

Assessing the Efficacy of Bisdipyrrins as Ligands for *in Vivo* Applications in Disease

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*This work is dedicated to my Family and Friends,
For you have all shaped me into the man I am today.*

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ABSTRACT

Porphyrins are tetrapyrrolic macrocycles which form highly stable metal complexes crucial to many biological processes. These complexes are characterised by a high kinetic barrier to decomplexation, which is a desirable quality in the design of new radiopharmaceuticals. Positron Emission Tomography (PET) imaging makes use of radionuclides such as copper-64 ($t_{1/2}$ 12.7 hours) to aid in the diagnosis of disease. Current reports of porphyrin ligands radiolabelled with copper-64 are limited, due to the typically forcing conditions required to achieve sufficient radiolabelling for application.

2,2'-Bisdipyrins are acyclic tetrapyrroles that form charge-neutral square-planar complexes with divalent metal cations. They are structurally analogous to porphyrins, although they are suggested to offer superior complexation kinetics under milder conditions. Accordingly, they hold promise as an alternative to porphyrins as potential radiotherapeutics. To date, there have been no reported attempts to translate these ligands into biological applications.

This thesis presents the synthesis and characterisation of new (2,2'-bisdipyrinato) metal(II) complexes and attempts to assess their efficacy for applications in the diagnostic imaging of disease.

A number of (2,2'-bisdipyrinato) copper(II) complexes have been synthesised and shown to be charge-neutral and approximately square-planar in structure based on crystallography. Cyclic voltammetry has elucidated a one-electron transfer process at ca. -1.1 V in all copper(II) complexes, and retention of this process with slight variations in potential for the corresponding Pd(II) and Ni(II) complexes evidences that this process is largely ligand-based in character. Slight shifts between the free base

ligand and complexes are associated with structural variations following metal ion coordination.

A (2,2'-bisdipyrinato) copper(II) complex was determined to have a K_D of $6 \pm 1 \times 10^{-15}$ M at pH 7.4 and 25°C, and is resistant to copper(II) removal following administration of a high affinity copper(II) chelator at up to 80°C with 50 equivalents of competitor. Interactions of the model ligand with bovine serum albumin (BSA) were demonstrated using fluorescence spectroscopy, and the protective effects of BSA following administration of a reducing agent on the complex supports the formation of BSA-complex interactions *in situ*. Preliminary work has also shown the aforementioned ligand based electrochemistry is potentially sufficient to mediate some decomplexation.

Cell studies involving four different cell lines have shown that membrane permeability is significantly affected by the peripheral substitution of the 2,2'-bisdipyrin. Oligo(ethylene glycol) substituents and negative charges limit the membrane permeability, while charge neutral or positively charged derivatives with comparatively small substituents were shown to cross membranes with the highest efficacy.

Preliminary radiolabelling of the 2,2'-bisdipyrins is efficient under mild conditions, although the presence of the oligo(ethylene glycol) substituents inhibits this. Biodistribution of a representative complex labelled with copper-64 in mice is distinct from the biodistribution of the unchelated [^{64}Cu]Cu(OAc)₂, indicating *in vivo* stability following administration.

The stability studies in conjunction with the membrane permeability and biodistribution of selected (2,2'-bisdipyrinato) copper(II) complexes suggest this new family of complexes are superior to porphyrins for applications in diagnostic imaging.

DECLARATION

This is to certify that:

- I. This thesis comprises only my original work towards the Degree of Doctor of Philosophy except where indicated.
- II. Due acknowledgement has been made to all other material used within this text.
- III. This thesis is less than 100,000 words in length, exclusive of tables, figures, schemes and appendices.

Jacob Rowan

10/04/2020

PREFACE

This thesis comprises my original work, alongside work completed by collaborators in various other laboratories. All the work presented herein is unpublished material not yet submitted for publication. The work presented in section 2.2.1 involving the synthesis and crystallography of CuL^2 was conducted by myself during an Honour's project. Crystallographic analysis of this complex discussed in subsequent sections was conducted as part of this research and not reported in my honours thesis. Electrochemistry experiments in section 2.8 on H_2L^2 , NiL^2 and CuL^2 were all obtained during the PhD project. Previous reports of the electrochemistry in my honours thesis was detailed using a different electrolyte.

Work conducted in section 4.2 using HEK293 cells and sections 4.3 and 4.4 using SKOV-3 cells was conducted by Dr. Karla Acevedo and Assoc. Prof. Tony White in the Department of Pathology, University of Melbourne, Parkville, Victoria, Australia. The results pertaining to HEK293 cell uptake has been previously reported in the candidate's Honours thesis and reproduced here for completion. Any analysis made herein is unique to this work, given the enhanced understanding of the target compounds. Cell studies on the ReN neural stem cells in sections 4.5 and 4.6 were conducted by Dr. Lotta Oikari, Dr. Hazel Quek and Assoc. Prof. Tony White at QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia.

All preliminary radiolabelling experiments were conducted by the candidate. Biodistribution experiments, PET imaging and image processing in section 5.4 were conducted by Dr. Lachlan McInnes, Ms Jessica Van Zuylekon and Dr. Carlene Cullinane at the Victorian Comprehensive Cancer Centre (VCCC) in Melbourne, Australia.

X-ray diffractometry experiments and structure solving was performed by Prof. Paul Donnelly, Prof. Jonathon White, or Dr. Asif Noor at School of Chemistry, Bio21 Molecular Science and Biotechnology Institute, Parkville, Victoria, Australia. All subsequent analysis was performed by the candidate.

Aspects of this research were financially supported by research grants awarded to Prof. Paul Donnelly by the NHMRC and ARC. My candidature was supported by funding from the Research Training Scholarship Program (formerly Australian Postgraduate Award).

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My research would not have been possible without the assistance of collaborators I had the pleasure of working alongside, or who I may not have had the chance to meet, but who had a large hand in the work presented in this thesis. Thank you for all your time and effort. Specifically, I would like to thank Assoc. Prof. Tony White, Dr. Lotta Oikari and Dr. Karla Acevedo, for all the work you did in the preliminary cell studies and letting me be a part of the process throughout. And to collaborators at the Victorian Comprehensive Cancer Centre, specifically Dr. Peter Roselt, Dr. Mohammad Haskali, Prof. Rodney Hicks, Dr. Carleen Cullinane and Ms. Jessica Van Zuylekom for your overwhelming help with my radiolabelling and animal studies.

I would like to acknowledge funding provided by the Australian Research Council and the National Health and Medical Research Council, which has supported the research presented in this thesis. I would also like to thank Mr William Schuster, Ms Norma Schuster, and The University of Melbourne for their generous donations in the form of scholarships.

To my PhD review panel, Assoc. Prof. Colette Boskovic and Prof. Craig Hutton, your support throughout my candidature has proven invaluable in my completion. I would also like to mention Prof. Jonathon White, who, with the assistance of Dr. Asif Noor and Prof. Paul Donnelly, acquired all the crystallographic data presented in this

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To my friends and family, I cannot begin to thank you for everything you have done for me over these past four years. Mum and dad, you have supported me and motivated me to make sure I kept going. Kayla, Beth and Bel, you were all my rocks during the hardest times. And you were my cheer squad during the best. Beth and Bel, you made coming to work every day unbelievably easy, and I will forever cherish the late nights and Uber eats we shared together.

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ABBREVIATIONS

3,2,3-tet	<i>N,N'</i> -bis(3-aminopropyl)-1,3-ethylenediamine
3,3,3-tet	<i>N,N'</i> -bis(3-aminopropyl)-1,3-propylenediamine
ACN	Acetonitrile
Atox1	Human antioxidant protein 1
β^+	Positron
BBB	Blood brain barrier
Bca	Bicinchoninic acid
BSA	Bovine serum albumin
Bu	Butyl
COSY	Correlation spectroscopy
CT	Computed tomography
CuATSM	Diacetylbis(N(4)-methylthiosemicarbazonato) copper(II)
CuGTSM	Glyoxalbis(N(4)-methylthiosemicarbazonato) copper(II)
Cys.OMe	Cysteine methyl ester
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DFT	Density-functional theory
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMF	<i>N,N</i> -Dimethylformamide
DMSO	Dimethyl sulfoxide
EDC·HCl	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	Ethylenediamine tetraacetic acid tetra anion
ΔE	Peak potential separation [$E_{ox} - E_{red}$]
E_m	Midpoint potential [$(E_{ox} + E_{red})/2$]
E_p	Peak potential

ESI-HRMS	Electrospray ionisation high resolution mass spectrometry
EtOAc	Ethyl acetate
FA	Fatty acid
Fe⁺	Ferricinium cation
Fc	Ferrocene
FDA	Fluorescein diacetate
FDG	2-fluorodeoxyglucose
GSH	Glutathione
GSSG	Reduced glutathione
HEK293	Human embryonic kidney 293
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
His.OMe	Histidine methyl ester
HMBC	Heteronuclear multiple bond correlation
HO-1	Heme monooxygenase-1
HOMO	Highest occupied molecular orbital
HPLC	High performance liquid chromatography
HSA	Human serum albumin
HSQC	Heteronuclear single quantum coherence
ICP-MS	Inductively coupled plasma mass spectrometry
IFE	Inner filter effects
ID	Injected dose
Ip_a	Anodic peak current
Ip_c	Cathodic peak current
LNCaP	Human prostate adenocarcinoma cells
LUMO	Lowest unoccupied molecular orbital

Me	Methyl
mpi	Minutes post-injection
MRI	Magnetic resonance imaging
NMR	Nuclear magnetic resonance
OAc	Acetate
PBS	Phosphate buffered saline
PET	Positron emission tomography
Pgp	P-glycoprotein
PI	Propidium iodide
PiB	Pittsburgh compound B
PMB	<i>p</i> -Methoxybenzyl
PMB-OH	<i>p</i> -Methoxybenzyl alcohol
ppb	Parts per billion
ppm	Parts per million
ReN	Human neural progenitor cells
R_f	Retention factor
ROS	Reactive oxygen species
SKOV-3	Human ovarian cancer cells
SOD1	Superoxide dismutase 1
SUV_{max}	Standard uptake value (maximum)
t_{1/2}	Half-life
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TSPP	<i>meso</i> -tetra(<i>p</i> -sulfonatophenyl)porphyrin

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1 INTRODUCTION

1.1 NATURAL TETRAPYRROLES

Macrocyclic tetrapyrroles such as porphyrins and corroles (Figure 1.1) are highly conserved biological ligands, coordinating metal ions including cobalt, magnesium and iron. Each ligand incorporates four pyrrole rings connected by either four methylene bridges or three methylene bridges and a direct pyrrole-pyrrole bond, for porphyrin and corrole respectively. Despite the ring contraction in corroles, both macrocycles are aromatic, with a conjugated 18 π -electron system and an overall planar structure.

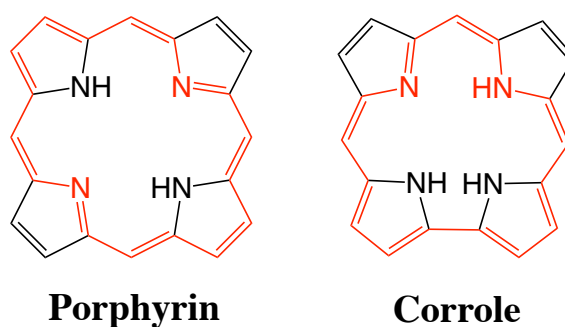


Figure 1.1. Macrocyclic core structure of porphyrin and corrole. 18 π -electron systems are indicated in red.

The highly conjugated nature of the above macrocyclic tetrapyrroles, and more specifically porphyrins, results in characteristic absorption spectra in UV-visible spectroscopy. Two distinct regions are observed in the spectra of free-base porphyrins. The Q-bands, which are a set of weak absorption bands in the region of 500 to 750 nm, are the result of a set of near-total parity-forbidden $S_0 \rightarrow S_1$ transitions. The Soret band, also known as the B-band, is a parity-allowed $S_0 \rightarrow S_2$ transition, and as such occurs at higher energy with markedly higher intensity.¹ An example spectrum for *meso*-tetraphenyl porphyrin is shown below (Figure 1.2).

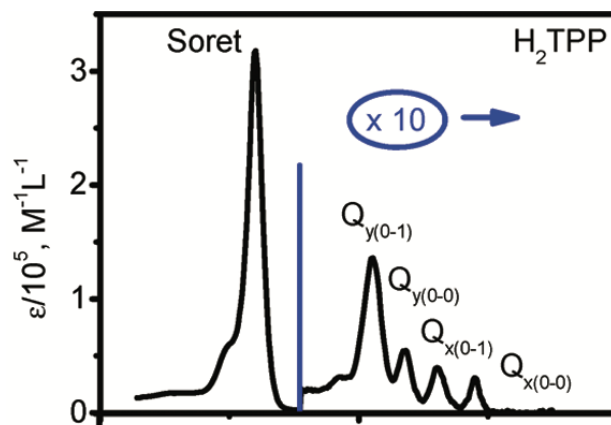


Figure 1.2. Experimental absorption spectrum of free base meso-tetraphenyl porphyrin. Adapted with permission from Nemykin, V. N., et. al., J. Phys. Chem. A **2010**, 114 (45), 12062-12066.² Copyright 2010 American Chemical Society.

Hemes are iron-containing porphyrins found in a diverse range of proteins including haemoglobin and cytochromes.³ Heme B (**1**, Figure 1.3a) is the most abundant form of heme, incorporating the ligand protoporphyrin IX. It is bound to proteins either covalently through the porphyrin ligand or coordinatively through an axially coordinated amino-acid side chain (Figure 1.3b). The remaining vacant coordination site is then responsible for interacting with various substrates, such as dioxygen in haemoglobin, for transportation and metabolism.

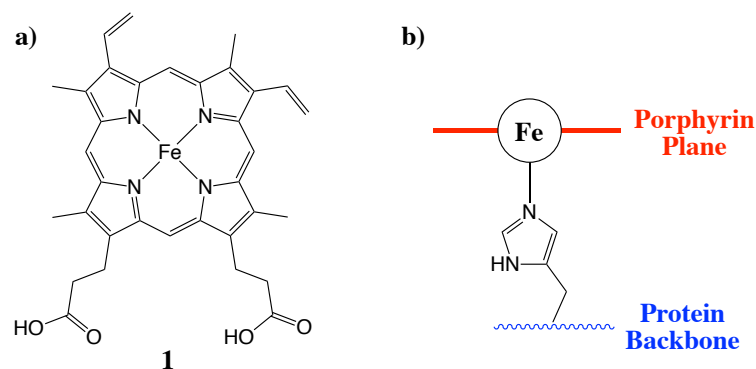
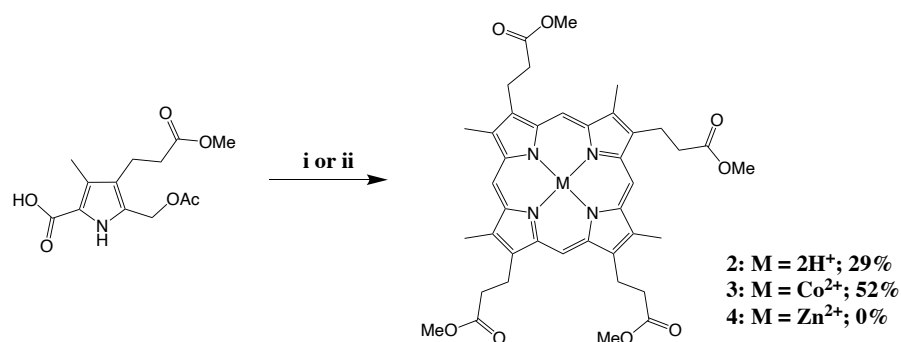


Figure 1.3. a) Structure of Heme B (**1**). b) Side-on schematic of the square-pyramidal Heme B-haemoglobin complex.

An extensive set of works conducted by Hans Fischer described the ubiquity of heme and its ligand, protoporphyrin IX, in nature.⁴⁻⁸ The structure and reactivity of

protoporphyrin IX and hemin, the Fe(III)–Cl counterpart of heme, was largely determined through isolation and spectroscopy of constituent pyrroles following oxidative degradation of the macrocycles. In the following 40 years, attempts to synthesise analogous structures in a synthetic laboratory utilised a number of different procedures to modulate substitution, macrocyclic ring size and metal incorporation.

Macrocyclic tetrapyrroles are synthesised using pyrrole building blocks, which undergo acid catalysed polymerisation followed by cyclisation. The macrocyclic products are typically isolated in low yields (~20-30%), a result of the intramolecular cyclisation of a linear tetrapyrrole being limited by the intermolecular polymerisation process.⁹ These yields are increased following addition of a metal ion to form the metal-tetrapyrrole complex, which promotes cyclisation through preorganisation of the ligand and the template effect. In the synthesis of the tetramethyl ester of coproporphyrin III, **2**, a precursor to protoporphyrin IX and heme, the yields are increased from 29% to 52% by the addition of cobalt(II) chloride,⁹ which results in the isolation of cobalt(II) coproporphyrin III tetramethyl ester, **3** (Scheme 1.1). Conversely, the addition of zinc acetate to the reaction inhibits cyclisation of the tetrapyrrole, instead favouring a non-templating Zn(II) complex.

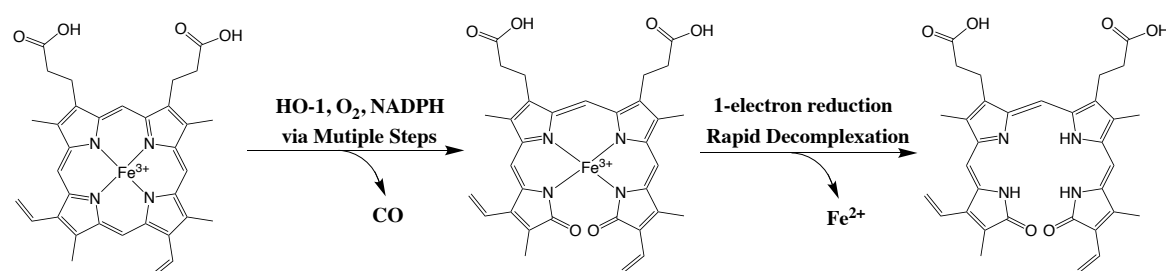


Scheme 1.1. Synthesis of coproporphyrin III tetramethyl ester. i) CH₃COOH, CH₃OH. ii) CoCl₂, CH₃COOH, CH₃OH.⁹

Unbound heme readily damages blood vessel walls through poor regulation of oxidation and the associated generation of reactive oxygen species (ROS).¹⁰ This is

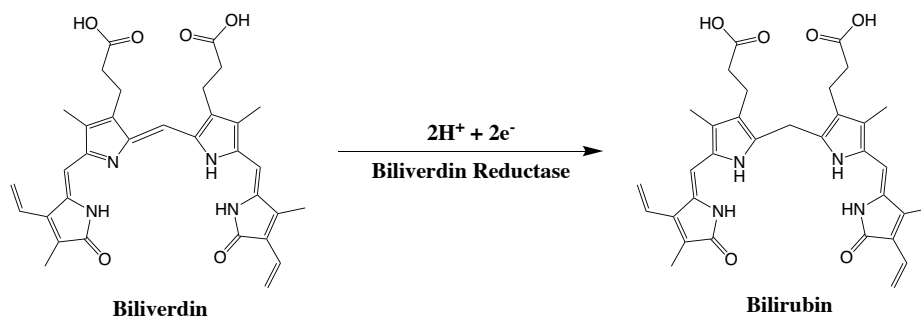
particularly prominent after haemolysis, the rupturing of red blood cells following tissue damage, in which free heme is released into the blood stream and surrounding tissues.

To mitigate the oxidative damage of free heme, it is rapidly metabolised by enzymes such as heme monooxygenase-1 (HO-1).¹¹ These enzymes catalyse oxidative ring opening of the protoporphyrin IX macrocycle, forming a highly unstable Fe(III) complex with the first of the major bile pigments, biliverdin (Scheme 1.2). The instability of the resulting complex leads to rapid and irreversible release of Fe(II) following a 1-electron reduction of the Fe(III)-biliverdinate complex.¹²⁻¹³ The free Fe(II) is then sequestered by iron transport proteins for proper metabolic processing (excretion or biological reincorporation).



Scheme 1.2. Heme monooxygenase-1 catalysed degradation of Heme B to liberate Fe(II) and carbon monoxide.¹¹

Biliverdin and its two-electron reduced counterpart, bilirubin, constitute the major bile pigments fated for excretion (Scheme 1.3). Their production through haemolysis following tissue damage is the cause of the characteristic bruising which often follows, attributed to their profound chromophoric nature (biliverdin – green [*Verdi*]; bilirubin – red [*ruber*]) prior to urinary excretion.



Scheme 1.3. Two-electron reduction of biliverdin to bilirubin by biliverdin reductase.

The flexibility of these tetrapyrroles facilitates the formation of monomeric and dimeric species when employed as ligands. The complexation of zinc(II) with octaethyl formylbiverdinate, for example, results in both a pentacoordinate metal complex with an axial aqua ligand, and the $\text{Zn}_2(\text{biliverdinate})_2$ dinuclear helical complex.¹⁴

The interesting metal-tetrapyrrole redox and photochemistry has led to the emergence of a field of work surrounding synthetic and naturally occurring metalloporphyrins. They have shown promise in applications in photodynamic therapy and catalysis,¹⁵ as well as ionophores for trafficking anions across biological membranes.¹⁶

The instability of the iron(III) biliverdinate complex is profound when contrasted with the stability of heme imposed by the macrocyclic effect. The macrocyclic nature of porphyrins consequently imposes a kinetic barrier to complexation, somewhat limiting specific applications. Acyclic tetrapyrroles have accordingly become intriguing in their own right. Initially, they generated interest in the design of stable acyclic tetrapyrrolic iron complexes but have since been shown to form stable metal complexes with a variety of metal cations.

1.2 SYNTHETIC TETRAPYRROLES: 2,2'-BISDIPYRRINS

Acyclic tetrapyrrole ligands including the 2,2'-bisdipyrrens (Figure 1.4) structurally resemble the tetrapyrrolic macrocycles. Like porphyrins and the related bile pigments, 2,2'-bisdipyrrens incorporate tetradentate N₄ metal binding sites, forming one 5-membered and two 6-membered chelate rings with a net charge of −2 following deprotonation.

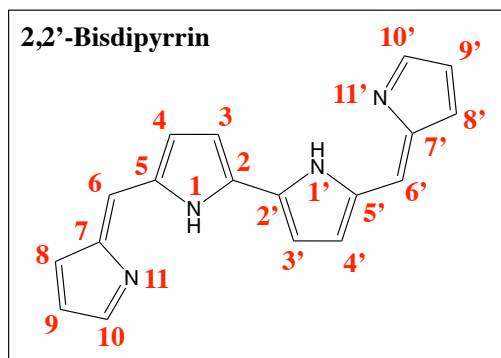
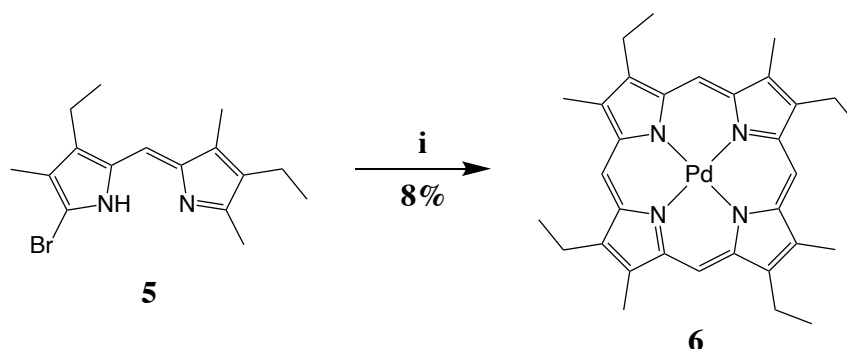


Figure 1.4. General structure and numbering of 2,2'-bisdipyrrens.

2,2'-Bisdipyrrens were first synthesised from their dipyrren analogues through metal-templated coupling in attempts to afford porphyrins and corroles. Much like the cobalt(II) coproporphyrin (**3**) discussed previously, a palladium(II) aetioporphyrin I complex, **6**, was afforded through palladium(II) template synthesis using the 2-bromodipyrren, **5**, according to Scheme 1.4.⁹



Scheme 1.4. Synthesis of palladium(II) aetioporphyrin I (**6**) from 1-bromo-3,8-diethyl-2,7,9-trimethyl-4,6-dipyrren (**5**, hydrobromide perbromide salt).

i) 3% Pd/SrCO₃, EtOH, Reflux, 20 hours.⁹

Ligand coupling reactions using dipyrin **5** and a 9-brominated analogue, **7**, result in a mixture of products. In conjunction with metalloporphyrin **6**, a palladium(II) metallocorrole, **8**, was obtained from a mixed ligand cyclisation, along with an acyclic dibrominated palladium(II) bisdipyrin, **9**, formed *via* the homoleptic palladium(II) complex with dipyrin **7** (Figure 1.5).

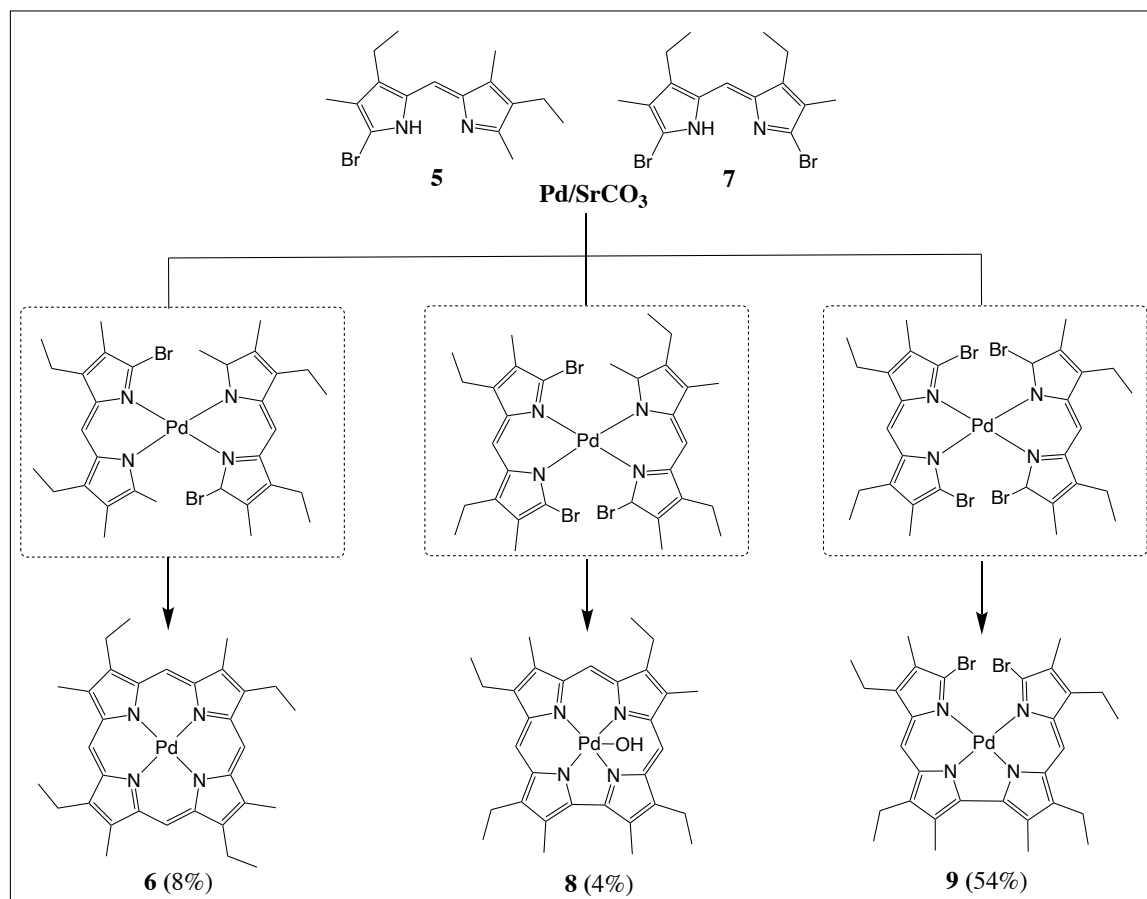


Figure 1.5. Mixed ligand metal-templated cross coupling reaction products and yields.^{9, 17}

While the porphyrin and corrole products **6** and **8** were obtained in low yields (<10%), the corresponding (2,2'-bisdipyrinato) palladium(II) was produced in 54% yield. Addition of hydrobromic acid to a solution of **9** affords the free ligand as the hydrobromide salt, from which the Cu(II) and Co(II) complexes were synthesised.¹⁷

The 2,2'-bisdipyrinato metal complexes can adopt mononuclear or dinuclear structures, dependant on ligand substitution and the selected metal ion. Bisdipyrin **10**, for example, is able to form mononuclear complexes with both Ni(II) and Cu(II), affording complexes **11** and **12**, as well as a dinuclear 2:2 helicate with Cu(II) (**13**, Figure 1.6) analogous to the structure of $\text{Zn}_2(\text{biliverdinate})_2$.¹⁸

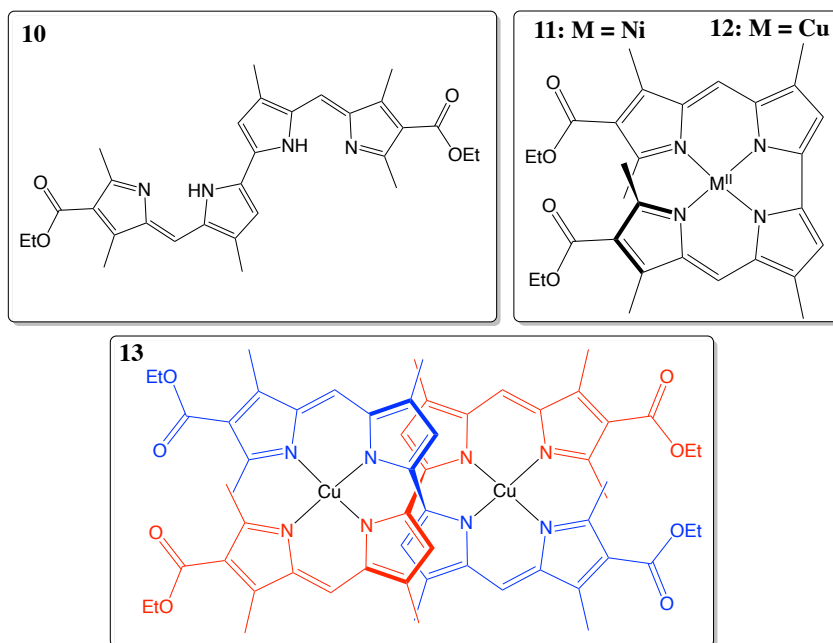


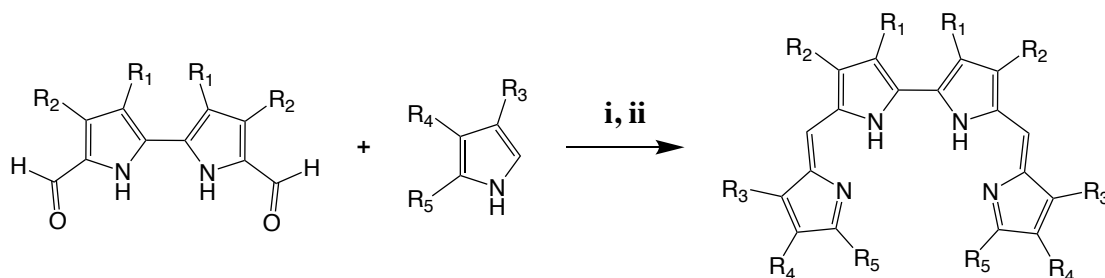
*Figure 1.6. Structure of bisdipyrin **10**, corresponding mononuclear Ni(II) and Cu(II) complexes **11** and **12**, and dimeric Cu(II) complex **13**.*

1.2.1 Synthesis of 2,2'-Bisdipyrins: From Bipyrroles

Work to improve the modest yields in the metal-templated synthesis of these tetrapyrroles led to the development of alternate synthetic approaches to these ligands. This has enabled synthesis of differentially substituted bisdipyrins, greatly improving ligand variability.

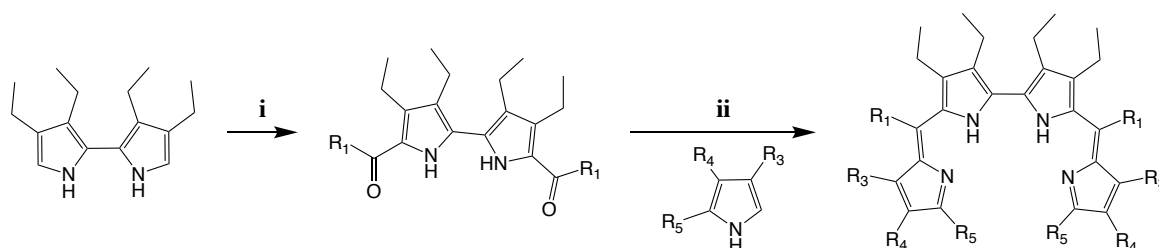
Direct synthesis of the 2,2'-bisdipyrins can be achieved through acid-catalysed condensation of a 2,2'-bipyrrole-5,5'-dicarbaldehyde with an α -unsubstituted pyrrole (Scheme 1.5).¹⁹ The use of a dicarbaldehyde leads to synthesis of the *meso*-unsubstituted

2,2'-bisdipyrin, which in many cases displayed thermal instability.¹⁹ The incorporation of β -pyrrole ring substitution (R_1 , R_2 , R_3 , R_4) was shown to reduce this instability, as does *meso*-substitution of the ligand. This reaction produces yields of 78 – 93%, depending on the starting materials.



*Scheme 1.5. Synthesis of meso-unsubstituted 2,2'-bisdipyrins. i) H^+ , CH_3OH ; ii) NEt_3 .*¹⁹

An analogous *meso*-(4-cyanophenyl) substituted ligand was synthesised through $AlCl_3$ catalysed α -substitution of a 2,2'-bipyrrole with 4-cyanobenzoyl chloride (Scheme 1.6), followed by subsequent reaction with the chosen pyrrole and phosphorus(V) oxytrichloride. Such reactions produce the 2,2'-bisdipyrins in 74 – 81% yields.¹⁹



*Scheme 1.6. General synthesis of meso-substituted 2,2'-bisdipyrins. i) 4-cyanobenzoyl chloride; ii) $POCl_3$.*¹⁹

1.2.2 Synthesis of 2,2'-Bisdipyrins: From Dipyrins

An alternative approach to the synthesis of 2,2'-bisdipyrins has been developed, which utilises *1H*-pyrrole as the primary building block. The conjugate acid of *1H*-Pyrrole has a pK_a of -3.8, attributed to the incorporation of the lone pair of electrons in the aromatic π -system.²⁰ As each of the ring atoms has an average electron density of 1.2

(6 electrons/5 atoms), it is considered electron rich, and as such can act as a donor of π -electrons in α - and β -electrophilic substitutions. Resonance structures (Figure 1.7) highlight an inversion of the dipole orientation with respect to other nitrogen heterocycles, such as pyridine and pyrrolidine. Electron density is removed from the nitrogen atom and placed on the ring carbons, creating uniquely nucleophilic carbon atoms.²¹

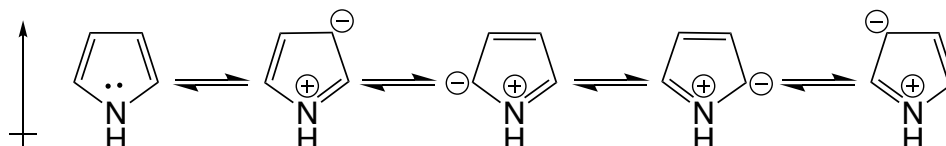
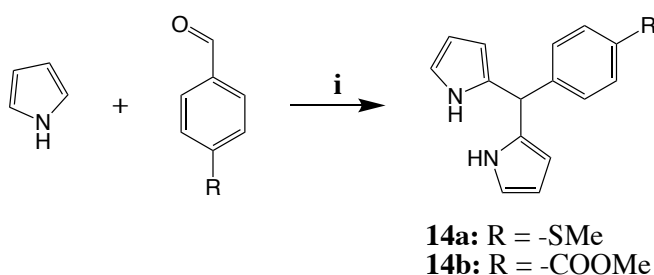


Figure 1.7. Resonance structures of 1H-pyrrole, highlighting the direction of the net dipole moment.

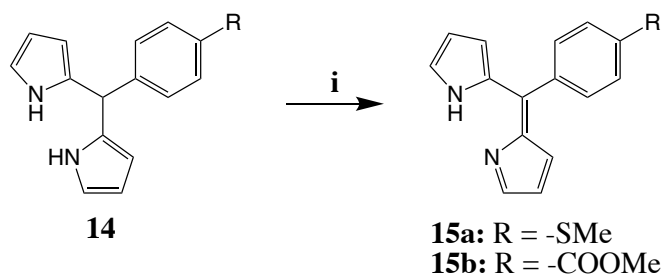
While both the α - and β -carbon atoms are nucleophilic, resonance stabilisation following reaction at the α -position leads to a favoured reaction product compared to the product of β -attack. Acid-catalysed condensation between pyrrole and aromatic aldehydes correspondingly affords the α -substituted aryl-dipyrromethane, **14** (Scheme 1.7) as the major product.



*Scheme 1.7. General synthesis of 5-aryl-4,6-dipyrromethanes, **14**. i) H^+ .*

A number of different Lewis acids have been investigated for catalysing the double condensation reaction.²²⁻²⁷ Optimisation of reaction stoichiometry, which classically involved a large excess of pyrrole to aldehyde (>20 equivalents), has led to the use of 0.18 M HCl with only three equivalents of pyrrole to afford the desired dipyrromethane in

60 – 97% yields.²⁸ Subsequent oxidation affords the desired *meso*-substituted 5-aryl-4,6-dipyrrens (Scheme 1.8).



Scheme 1.8. General synthesis of 5-aryl-4,6-dipyrrens, 15 from the corresponding dipyrromethanes. i) Oxidant.

Typically, benzoquinones such as *p*-chloranil and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) are used as the oxidant in these reactions (Figure 1.8).

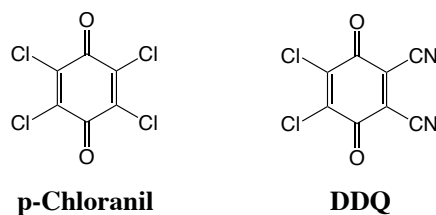
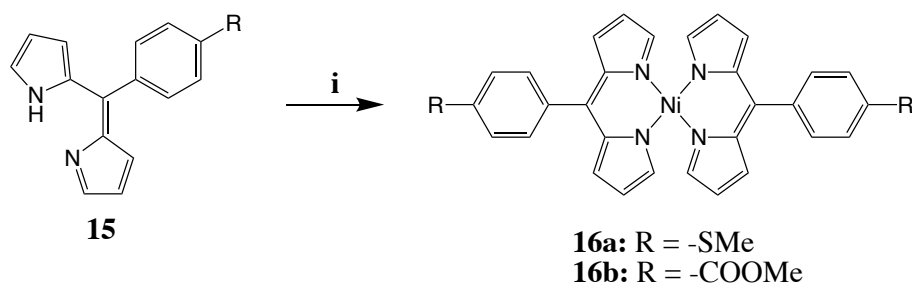


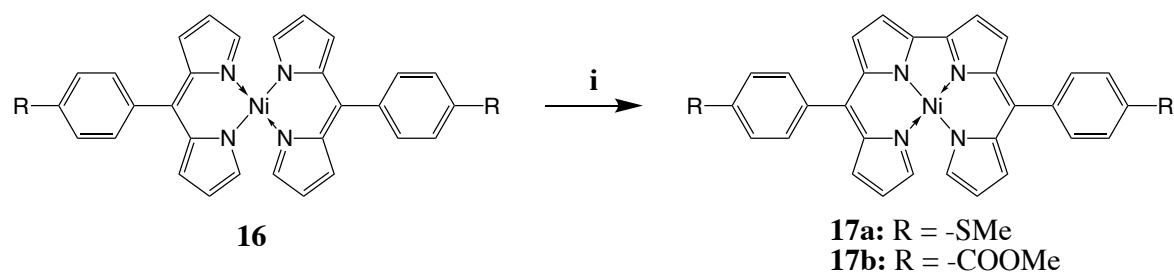
Figure 1.8. Structure of benzoquinone oxidants used in the synthesis of dipyrrens, p-chloranil and DDQ.

Meso-substituted 2,2'-bisdipyrrens ligands are then synthesised *via* by metal-templated oxidative-coupling of the corresponding dipyrren. The homoleptic Ni(II) complex is obtained through a one-pot oxidation of the dipyrromethane with DDQ followed by complexation with nickel(II).²⁹ Alternative procedures involve treating a methanolic solution of purified dipyrren with nickel(II) acetate tetrahydrate (Scheme 1.9).³⁰ The latter protocol is based on the synthesis of the copper(II) and zinc(II) analogues.³¹⁻³²



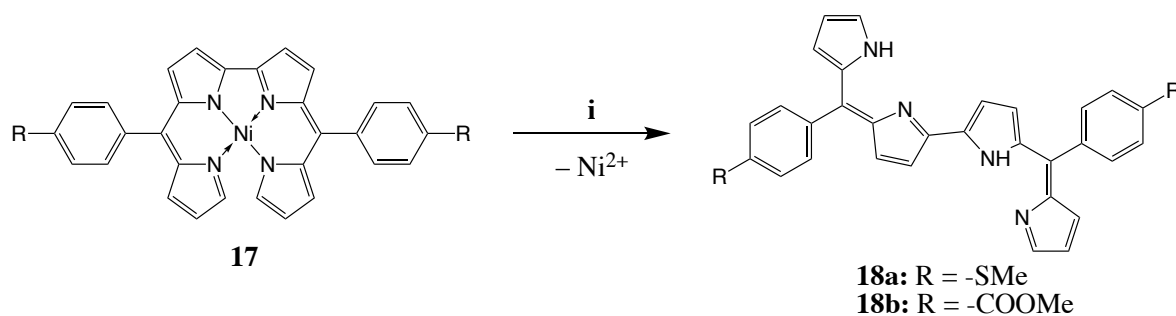
Scheme 1.9. Synthesis of the distorted tetrahedral bis(dipyrinato) nickel(II) complex, 16. i) Ni(OAc)₂·4H₂O, CH₃OH.

The oxidative coupling of the ligands with general structure **16** is carried out by DDQ in toluene at reflux in > 60% yields for various substituted phenyl derivatives (Scheme 1.10).²⁹⁻³⁰



Scheme 1.10. Nickel(II)-templated oxidative coupling of the dipyrinato ligands of 16 to give the (2,2'-bisdipyrinato) nickel(II) complex, 17. i) DDQ, toluene, reflux.

The resulting nickel(II) complexes are sensitive to acid-mediated decomplexation, as is the case for the first reported palladium(II) bisdipyrins. This allows rapid and high yielding removal of the metal ion to liberate the free base 2,2'-bisdipyrin following neutralisation (Scheme 1.11).²⁹



Scheme 1.11. Removal of the nickel(II) from 17 through acid-mediated decomplexation.

i) HCl (12 M), CHCl₃.

1.3 METAL-BISDIPYRRIN COMPLEXES

(2,2'-Bisdipyrinato) iron complexes were initially investigated as potential analogues for the aforementioned unstable iron bilins. Only one example of a quasi-stable Fe(III/IV) bilin complex with an axial chlorido ligand has been reported as a stable analogue of these natural heme metabolites (Figure 1.9a).³³ Reducing the sterically imposed distortion by using an octaethyl 2,2'-bisdipyrin (Figure 1.9b) results in increased stability of the pentacoordinate Fe(III) complexes for use as a models of the iron bilins.¹³

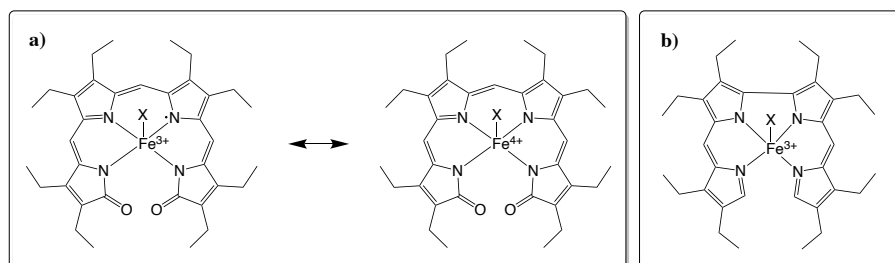
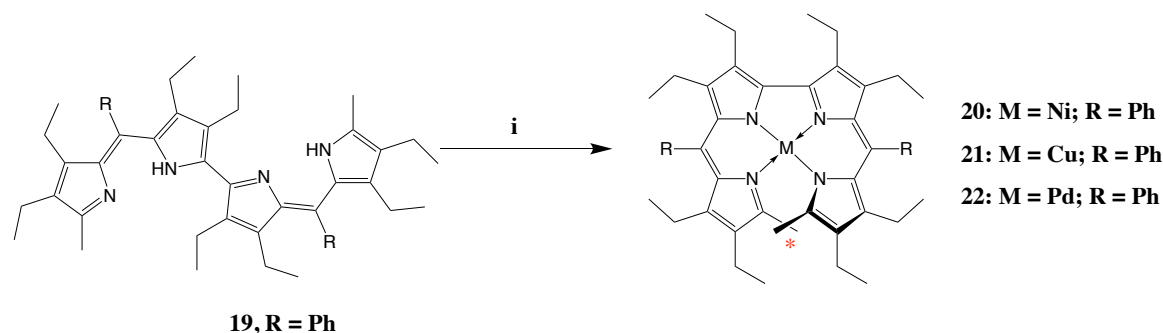


Figure 1.9. Stable iron-(octaethyl)tetrapyrrole complexes using a) an oxidised biliverdin scaffold (X = Cl),³⁴ and b) a 2,2'-bisdipyrin (X = F, Cl, Br, I).¹³

On top of the previously discussed metal complexes, which form an integral part to the synthesis of the ligands, various *meso*-substituted M(II) complexes with a dimethyloctaethyl-2,2'-bisdipyrin, **19**, have also been prepared (Scheme 1.12).³⁵⁻³⁶ Crystallography shows the formation of M- and P- helices due to steric clash of methyl

(Me) substituents at the 10 and 10' positions (*; Scheme 1.12), resulting in distorted square planar complexes that could be resolved *via* chiral chromatography.



*Scheme 1.12. Synthesis of Ni(II), Cu(II) and Pd(II) complexes of **19** using methanolic solutions of the metal diacetate with the ligand. i) $M(\text{OAc})_2$, CH_3OH .³⁵⁻³⁶ * Indicates C10-CH₃ and C10'-CH₃ steric clash.*

Complexes **20** to **22** are electrochemically active, as are the *meso*-unsubstituted analogues ($\text{R} = \text{H}$). Two reversible one-electron transfer processes are observed for each complex with E_m values between -0.2 and $+0.5$ V (vs the ferricinium/ferrocene couple, $[\text{Fc}^+/\text{Fc}]$) in the cyclic voltammograms. Two additional one electron transfer processes are evident at approximately -1.7 V and -2.3 V for each of the Ni(II) and Pd(II) complexes **20** and **22**, although only a single process is observed for the Cu(II) complex **21** at ca. -1.45 V. The authors suggest this less negative potential is attributed to a strain-dependant release of the metal ion and is likely not due to metal ion reduction.³⁶ Despite variations in the midpoint potential, E_m , values of the processes [where $E_m = (E_{\text{ox}} + E_{\text{red}})/2$], ligand **19** produces a similar cyclic voltammogram, indicating these processes are all largely ligand based in character. Metal-imposed structural variations such as ligand bite angle are established to be the major cause of variation in E_m values in each of the metal complexes, with loss of the second one-electron process at ca. -2.3 V for **21** proposed to result from geometric changes in the complex geometry following the first reduction step.

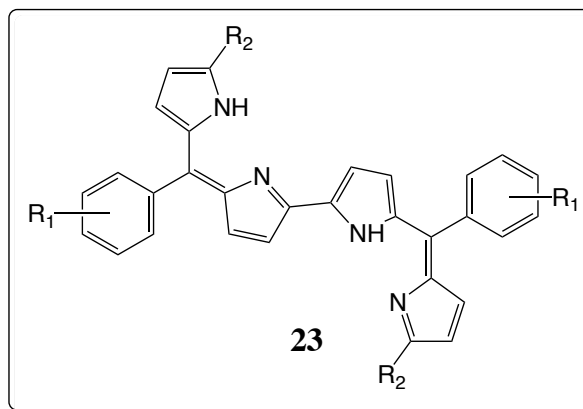


Figure 1.10. General structure of *meso*-aryl-2,2'-bisdipyrin, **23**.

Meso-aryl-2,2'-bisdipyrins of general structure **23** (Figure 1.10) have been used in the synthesis of metal(II)-based helicates. The effect of α -substitution and metal ion selection has shown that the formation of supramolecular helicates is largely driven by steric effects and metal-geometric biases.

Distorted square planar coordination geometries are obtained for complexes of the *meso*-phenyl-2,2'-bisdipyrin **23a** ($R_1 = R_2 = H$) with nickel(II), copper(II), cobalt(II), palladium(II) and platinum(II). Unlike these, the zinc(II) complexes are isolated as the 2:2 Zn_2L_2 helicate, due to the lack of metal-based geometric preferences.³⁷

Higher order polynuclear complexes of zinc(II) with analogous (2,2'-bisdipyrinato) ligands (**23**, R_1 [*para*] = -SMe or -COOMe, $R_2 = H$) have also been isolated, incorporating 2:2, 3:3 and 4:4 helical frameworks (Figure 1.11).³⁰

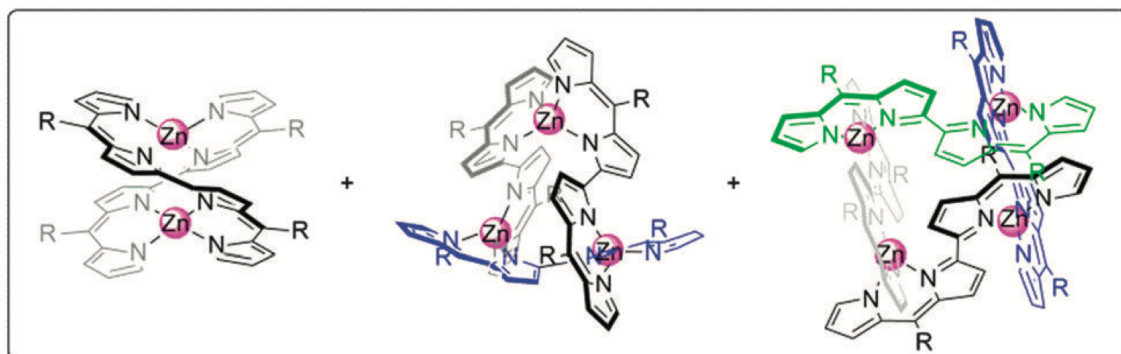


Figure 1.11. Structure of helicates generated in the complexation of bisdipyrrins with Zn(II) (Reproduced with permission from Baudron et al, *ChemComm* 2015, **51**, 5906-5909).³⁰

Incorporation of an α -phenyl substituent (**23b**, $R_1 = \text{H}$, $R_2 = \text{Ph}$) to impose steric strain on the complex is able to switch the geometry of the cobalt(II) complex from distorted square-planar to the Co_2L_2 helicate analogue of zinc. The divalent nickel, copper, palladium and platinum complexes of **23b** do not undergo the same shift. Rather, the complexes merely show enhanced distortion of the square planar complex seen for **23a**.³⁷

1.4 TETRAPYRROLES AS THERAPEUTICS AND RADIOPHARMACEUTICALS

Copper-64 (^{64}Cu) is a positron (β^+) emitting isotope of copper useful in Positron Emission Tomography (PET) imaging. Incorporation of this metal radionuclide into metal complexes through chelation is typically advantageous compared to low molecular weight radionuclides, including ^{11}C , ^{13}N , ^{15}O and ^{18}F . Most notably, the complexation kinetics of incorporating this radionuclide into ligands is, in many cases, rapid compared to the covalent incorporation required for the non-metal radionuclides. Considering this in addition to their shorter half-lives ($t_{1/2}$) (Table 1.1), designing new stable ligands for metal radionuclide chelation is an attractive alternative to current diagnostic and therapeutic tools.

*Table 1.1. Nuclear properties and applications of selected positron emitting isotopes.*³⁸⁻⁴³

	$t_{1/2}$ (min)	% β^+ decay	Application
^{11}C	20	99.6	Amyloid β imaging – Pittsburgh Compound B (PiB). Dopamine receptor imaging – [^{11}C]Raclopride.
^{13}N	10	99.8	Myocardial perfusion imaging– [^{13}N]NH ₃ .
^{15}O	2	100	Myocardial perfusion imaging– [^{15}O]H ₂ O.
^{18}F	110	96.7	Metabolic profiling in malignancies – [^{18}F]FDG ([^{18}F] 2-fluoro-2-deoxyglucose).
^{64}Cu	762	18	Hypoxia imaging – [^{64}Cu]CuATSM.

FDG: 2-fluorodeoxyglucose; ATSM: Diacetylbis(N(4)-methylthiosemicarbazone)

Although a smaller proportion of copper-64 undergoes β^+ decay (positron emission), with electron capture and β^- decay as dominating decay modes, its longer half-life ($t_{1/2}$ 12.7 hours) improves access for remote diagnostic centres and handling time for sample preparation.

Current reports of porphyrin ligands radiolabelled with copper-64 are limited. Despite offering highly stable metal complexes, associated poor complexation kinetics of porphyrins typically requires labelling to be conducted at elevated temperatures for

extended durations. Mild conditions have been reported for the labelling of porphyrin **24** (Figure 1.12) with copper-64 using ascorbic acid in acetate buffer (pH 5.6) at 37°C for 60 minutes. The ascorbic acid was selected as an additive to promote complexation by reducing the $^{64}\text{Cu(II)}$ to $^{64}\text{Cu(I)}$. Radiochemical purity of up to 96% was achieved after reverse-phase chromatographic purification, producing a specific activity on the order of 600 GBq per mmol of ligand.⁴⁴

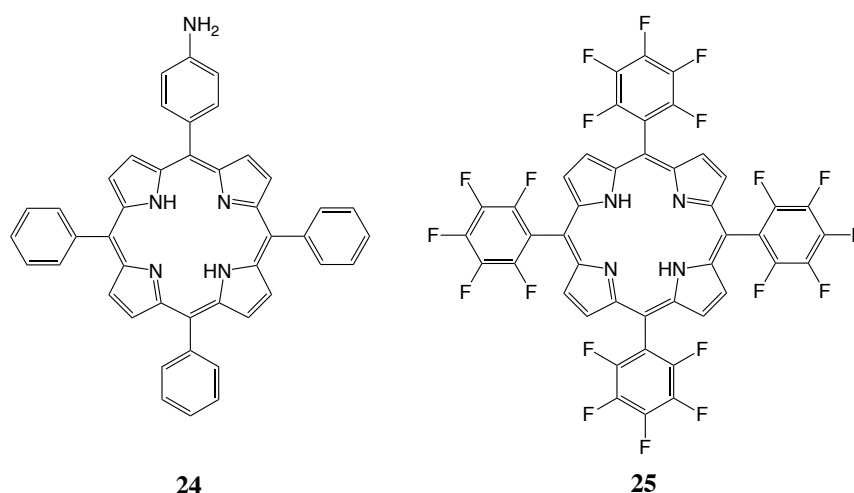


Figure 1.12. Structure of porphyrin ligands used to coordinate copper-64.

A similar approach to labelling porphyrin **25** (Figure 1.12) yielded up to 14 GBq per mmol of ligand following reflux in acetate buffer for 60 minutes.⁴⁵ Few other reports regarding the complexation of porphyrins to copper-64 have been published, and a review into the use of porphyrins as radiopharmaceutical ligands describes the sensitivity of various functional and biological groups to these typically harsh complexation conditions.⁴⁶ In addition, the previously described metabolism of porphyrins may result in biological instability following administration, leading to metabolic release of the radionuclide.

2,2'-Bisdipyrins are promising candidates for the development of radiopharmaceuticals. They are structurally similar to porphyrins and corroles, although with a lower barrier to complexation due to their acyclic structure. The corresponding copper(II)

complexes can be formed under mild complexation conditions in organic solvents rapidly at room temperature, adopting charge-neutral square planar structures.³⁶

Aside from the ability of **23a** and **23b** to form distorted square-planar metal complexes and supramolecular helicates, there has only been a single investigation of the electrochemical behaviour of these ligands and their complexes published at the time of writing.⁴⁷ Similarly, spectroscopic investigations on the α -unsubstituted derivatives of **23** remain limited in current literature. There have also been no studies on the biological activity of (2,2'-bisdipyrinato) copper(II) complexes. Other than the aforementioned acid sensitivity first seen in the decomplexation of the (2,2'-bisdipyrinato) palladium(II) complex **9**, no indication of their stability in aqueous solutions and in the presence of high-affinity copper(II) chelators has been reported.

1.5 AIMS OF THIS THESIS

This thesis will explore the applicability of a new family of 2,2'-bisdipyrrens and their corresponding copper(II) complexes as *in vivo* imaging agents using the copper-64 radionuclide. The work presented will address the synthesis and characterisation of new 2,2'-bisdipyrren M(II) complexes, with a particular interest in synthesis and characterisation of the copper(II) complexes.

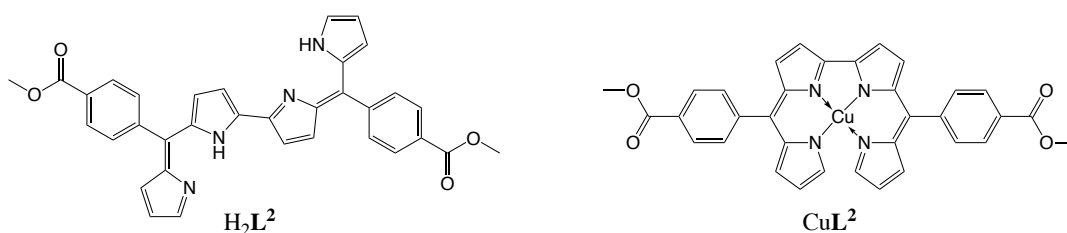


Figure 1.13. Structure of representative ligand, H_2L^2 , its corresponding copper(II) complex, CuL^2 .

The methyl-ester appended 2,2'-bisdipyrren, **18b** (hereafter H_2L^2 ; Figure 1.13) was selected as a means of facilitating bioconjugation to peptides and proteins for targeted application. The kinetic and redox stability will be discussed using CuL^2 as a stable charge-neutral model complex to determine the likelihood of decomplexation *in vivo*. Preliminary toxicity and cell uptake studies using various cell lines will be used to relate membrane permeability with lipophilicity and examine the biological fate of copper following complex cell uptake. In conjunction with radiolabelling experiments using copper-64, these results will be used to assess the potential for these ligands and complexes to be employed as PET imaging agents for imaging disease.

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2 SYNTHESIS AND CHARACTERISATION OF NEW BISDIPYRRIN M(II) COMPLEXES

2.1 BACKGROUND

Works conducted by Bröring *et al.* have highlighted the formation of distorted square planar metal complexes with Cu^{2+} , Ni^{2+} and Pd^{2+} with a 10,10'-dimethyl-2,2'-bisdipyrin (see Section 1.3). The distortion away from square planar was approximated by the distance between the terminal methyl carbon atoms.¹ In bilins, the presence of 10 and 10' substituents is highlighted to destabilise the resulting metal complexes.²⁻³

A previously synthesised 2,2'-bisdipyrin with methyl ester functionality, H_2L^2 was synthesised from the corresponding dipyrin, HL^1 (Figure 2.1), according to literature procedures discussed in Section 1.2.2.⁴⁻⁷ This ligand was selected as a representative compound to assess the coordination geometry and stability of a newly synthesised copper(II) complex. X-ray crystallography shows the ability of this ligand to form square-planar metal complexes with Ni(II), and as such this ligand is suggested to form analogous lipophilic complexes following coordination to copper(II).

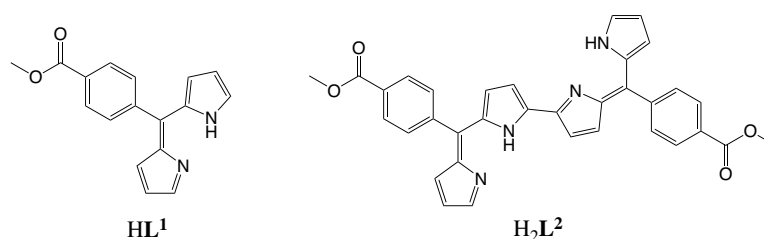


Figure 2.1. Structure of ligands, HL^1 and H_2L^2 .

The methyl ester groups were selected as a means of enabling derivatisation. The absence of any 10,10'-substitution on the ligand H_2L^2 is hoped to minimise the deviation observed in the complexes, and therefore increase stability and planarity of the resulting metal complexes.

2.1.1 Chapter Aims

The work presented in this chapter will explore the synthesis and characterisation of the copper(II) and palladium(II) complexes of L^2 . Hydrolysis of H_2L^2 to produce the diacid, H_2L^3 , and subsequent complexation attempts will be discussed, and the crystallographic differences and coordination geometries explored.

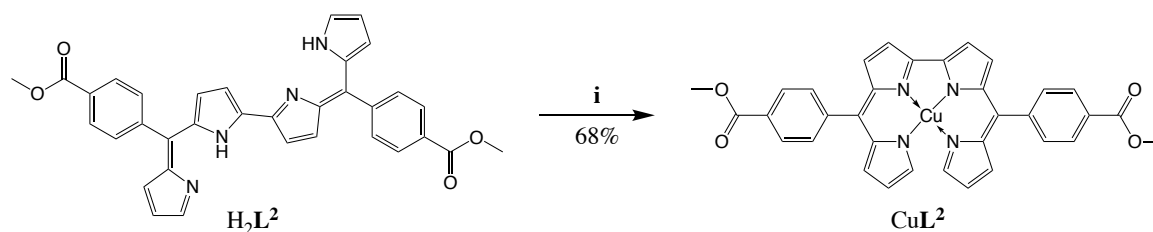
As a means of investigating the differences in electrochemistry and spectroscopic features due to the methyl ester groups, a set of new differently substituted 2,2'-bisdipyrrins and their copper(II) complexes will be synthesised and characterised. This will exploit the use of H_2L^3 to synthesise a number of N-substituted benzamidyl-2,2'-bisdipyrrins and their copper(II) complexes. Resulting variations in solubility will also be addressed.

2.2 SYNTHESIS AND CHARACTERISATION OF M(II) COMPLEXES OF H_2L^2

2.2.1 Copper(II) Complex

The work conducted in section 2.2.1 was conducted by the candidate during and honours research project.

The copper complex was synthesised from H_2L^2 using $Cu(OAc)_2 \cdot H_2O$ in a $CHCl_3/CH_3OH$ solution (Scheme 2.1).



Scheme 2.1. Synthesis of CuL^2 . i) $Cu(OAc)_2 \cdot H_2O$, $CH_3OH/CHCl_3$, 4 hours, room temp.

The compound was collected by vacuum filtration and crystals suitable for X-ray diffractometry were grown by slow evaporation of a toluene solution of CuL^2 (Figure 2.2).

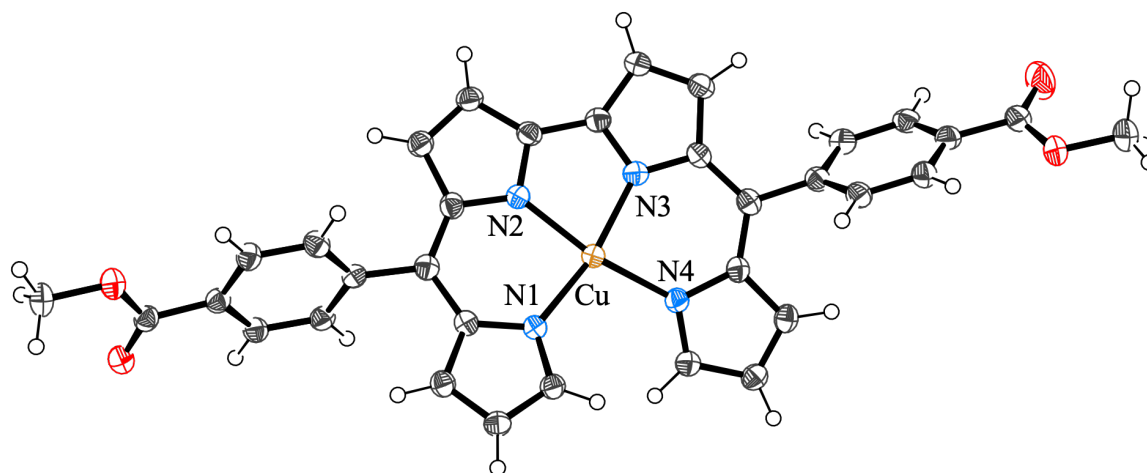
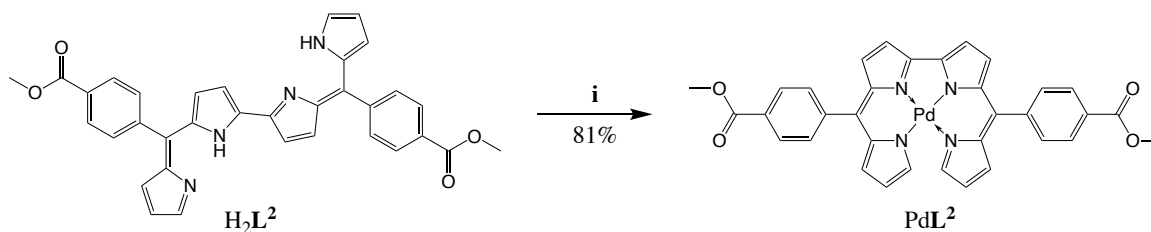


Figure 2.2. Ortep-3 representation of the structure of CuL^2 , as determined by X-ray crystallography. Thermal ellipsoids of non-hydrogen atoms shown at 50% probability level.

A similar distorted square-planar geometry to the 10,10'-substituted analogues synthesised by Bröring *et al.* is found in the above structure. The crystals were obtained as a racemate, consisting of the P- and M- helices. Electron paramagnetic resonance indicates the retention of axial symmetry in the complex, with a $g_{\parallel}$ value of 2.12, and the corresponding $g_{\perp}$ value of 2.04 (data not shown).

2.2.2 Palladium(II) Complex

The analogous palladium(II) complex was prepared by reacting H_2L^2 with $\text{Pd}(\text{OAc})_2$ in 81% yield (Scheme 2.2). This complex was synthesised as a diamagnetic and electrochemically inert analogue of the square planar nickel(II) and copper(II) complexes



Scheme 2.2. Synthesis of PdL^2 . i) $\text{Pd}(\text{OAc})_2$, $\text{CH}_3\text{OH}/\text{CHCl}_3$, 4 hours, room temp.

2. New Bisdipyrrin Metal(II) Complexes

Crystals of suitable quality for X-ray diffraction were grown from the slow diffusion of hexanes into a CH_2Cl_2 solution containing PdL^2 . The resulting structure is shown in Figure 2.3.

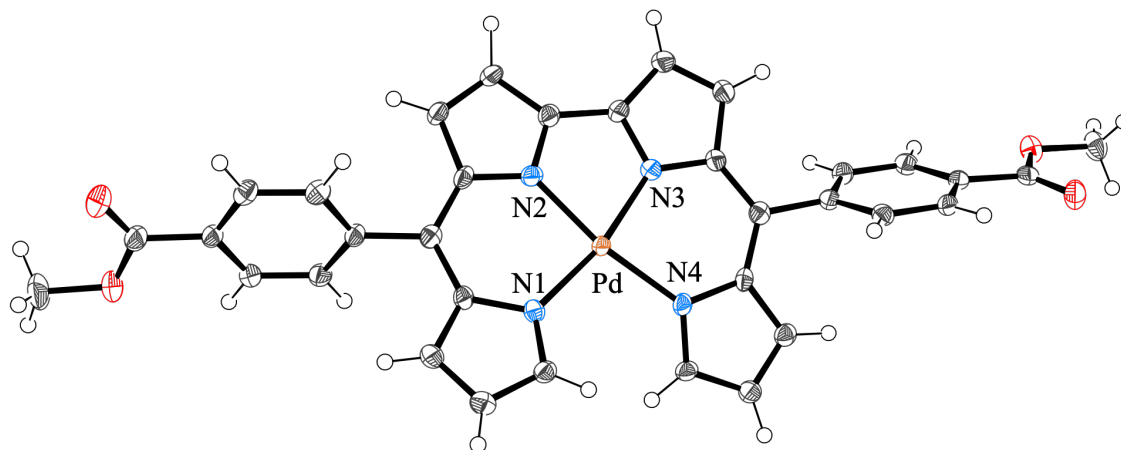


Figure 2.3. Ortep-3 representation of the structure of PdL^2 , as determined by X-ray crystallography. Thermal ellipsoids of non-hydrogen atoms shown at 50% probability level.

The X-ray crystal structure again shows a distorted square-planar geometry created around the central Pd(II) ion through an N_4 -coordination mode (Figure 2.4). This will be discussed in more detail in Section 2.4.

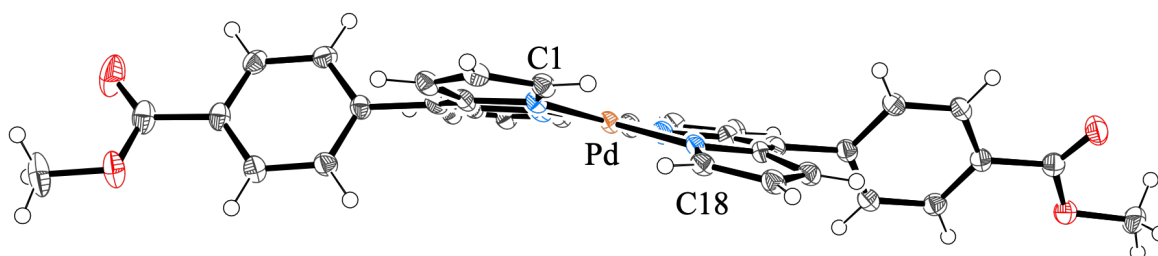


Figure 2.4. Side-on Ortep-3 representation of the structure of PdL^2 , as determined by X-ray crystallography. Thermal ellipsoids of non-hydrogen atoms shown at 50% probability level.

2. New Bisdipyrrin Metal(II) Complexes

The diamagnetic palladium(II) complex was characterised by nuclear magnetic resonance (NMR) spectroscopy. Comparing the hydrogen environments to H_2L^2 and NiL^2 shows a number of distinctive changes (Figure 2.5).

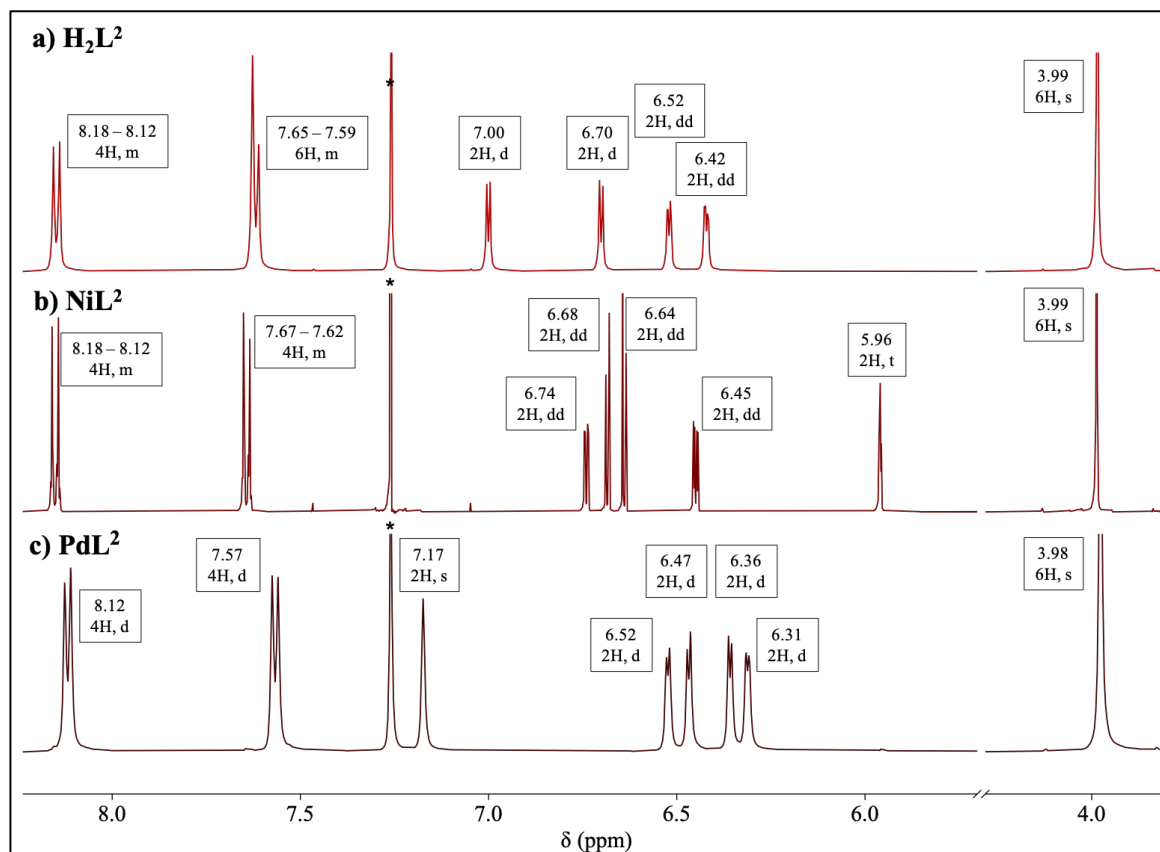


Figure 2.5. Comparison of the ^1H NMR spectra for a) H_2L^2 , b) NiL^2 and c) PdL^2 in $CDCl_3$ (500 MHz, * residual solvent peak).

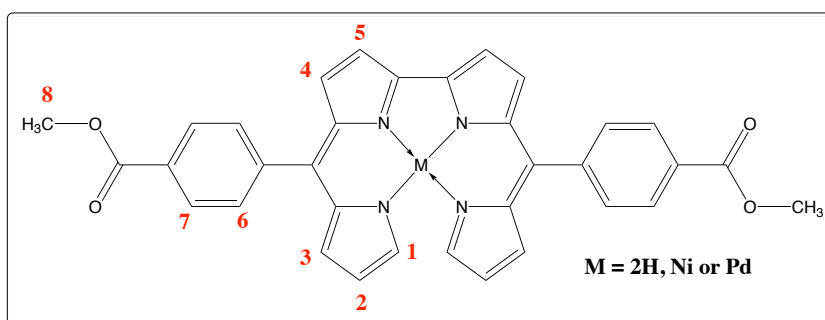


Figure 2.6. Hydrogen environment numbering for NMR characterisation.

The $-\text{CH}_3$ ($H8$) signal at δ 3.99 ppm is conserved across all three structures, as is the aromatic $-\text{CH}$ at approximately δ 8.15 ppm ($H7$), while a slight change is observed for $H6$ in the palladium complex ^1H NMR spectrum.

The pyrrolic $-\text{CH}$ signals ($H1-5$) show the largest variation between the complexes and free ligand. Correlation spectroscopy (COSY) NMR was used to assign the 3H ($H1-3$) and 2H ($H4/5$) spin systems (Figure 2.7).

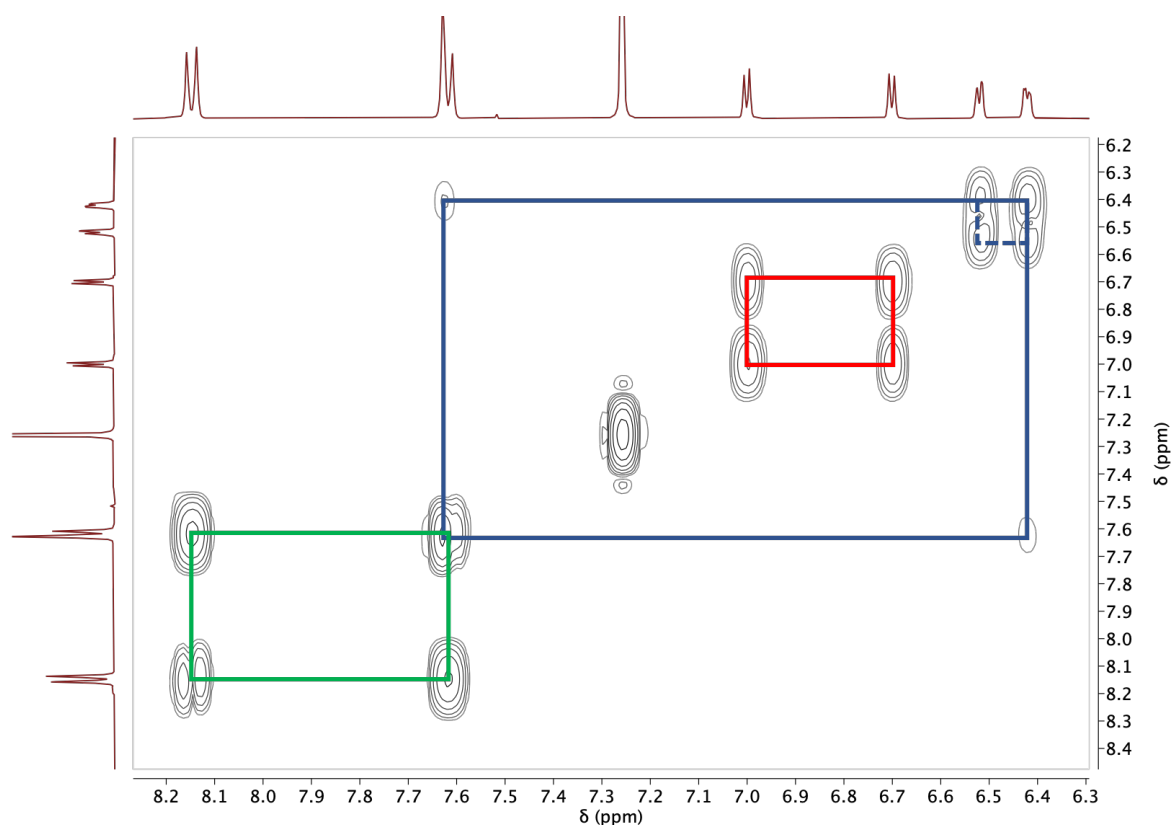


Figure 2.7. COSY NMR spectrum of H_2L^2 in CDCl_3 (500 MHz).

The signals corresponding to the 3H spin system are shown by the dark blue line. Overlap of one of these environments with that of a phenyl $-\text{CH}$ signal at δ 7.62 ppm ($6H$) is removed following coordination to either Pd(II) or Ni(II). This pyrrolic $-\text{CH}$ is shielded by complexation, and moves to δ 5.96 and δ 7.17 ppm for NiL^2 and PdL^2 , respectively (Figure 2.8 and Figure 2.9).

2. New Bisdipyrrin Metal(II) Complexes

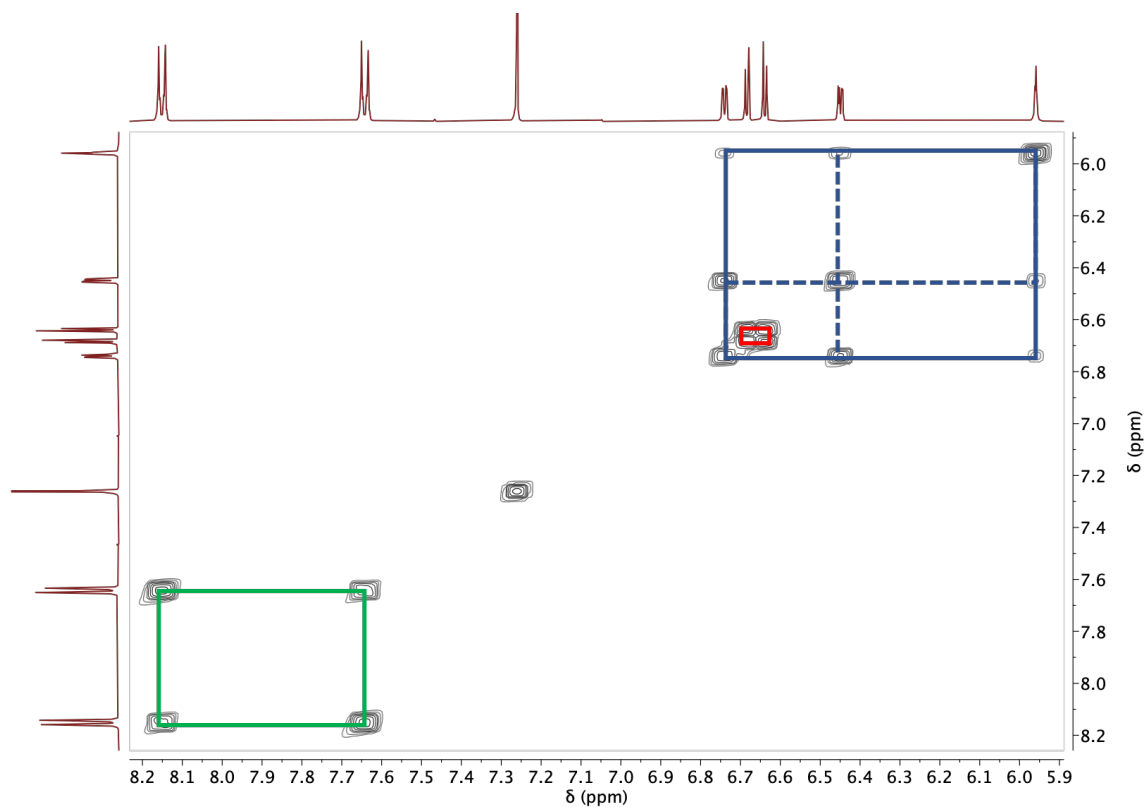


Figure 2.8. COSY NMR spectrum of NiL^2 in CDCl_3 (500 MHz).

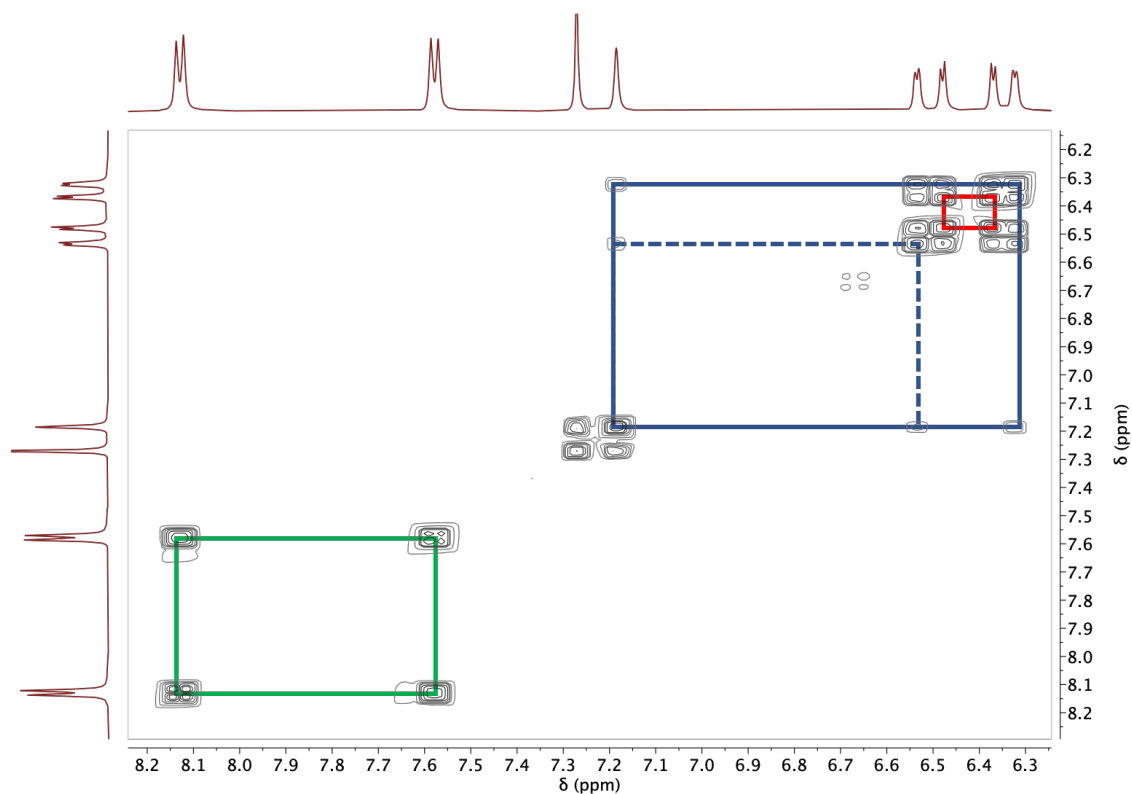


Figure 2.9. COSY NMR spectrum of PdL^2 in CDCl_3 (500 MHz).

2. New Bisdipyrrin Metal(II) Complexes

Based on these COSY NMR spectra, the proton environments for each of H_2L^2 , NiL^2 and PdL^2 were assigned (Table 2.1). The exact proton environments corresponding to each of the pyrrolic spin systems were, however, unable to be differentiated.

*Table 2.1. NMR Spectral assignment of H_2L^2 and the corresponding Ni(II) and Pd(II) complexes ($CDCl_3$, 500 MHz) (dd = Doublet of doublets; t = triplet; d = doublet; s = singlet; br = broad signal; m = multiplet; * overlapping signals). Assignments made using labels given in Figure 2.6.*

Environment	Assignment	H_2L^2	NiL^2	PdL^2
(3H) Pyr-H	<i>H1/2/3</i>	6.52 (2H, dd)	6.74 (2H, dd)	6.52 (2H, dd)
		6.42 (2H, dd)	6.45 (2H, dd)	6.31 (2H, dd)
		7.62 (2H, m*)	5.96 (2H, t)	7.17 (2H, br. t)
(2H) Pyr-H	<i>H4/5</i>	7.00 (2H, d)	6.68 (2H, d)	6.47 (2H, d)
		6.70 (2H, d)	6.64 (2H, d)	6.36 (2H, d)
Ph-H	<i>H6</i>	7.65–7.59 (4H, m*)	7.67–7.62 (4H, m)	7.57 (4H, d)
Ph-H	<i>H7</i>	8.18–8.12 (4H, m)	8.18–8.12 (4H, m)	8.12 (4H, d)
O-CH ₃	<i>H8</i>	3.99 (6H, s)	3.99 (6H, s)	3.98 (6H, s)

Additional 2-dimensional NMR experiments were used herein to attempt assignment, including heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) spectroscopy (data not shown). Coupling of *H7* to a carbon signal at 166.8 ppm in HMBC experiments confirmed that these signals are closest to the ester carbonyl group. Building on this, assignment of the phenyl ring carbon and hydrogen environments was confirmed. The presence of five quaternary carbons in the bisdipyrrin backbone, however, left the exact nature of the highly variable H-environment (Table 2.1, red) unknown. To best approximation, these signals correlate to the terminal protons (*H1*), experiencing the highest degree of change following complexation with Ni(II) or Pd(II).

One explanation for this change is the change in charge density, Z/r , on moving from Ni(II) to Pd(II), as well as the different shielding/deshielding effects of the 3d and 4d orbitals. Structural changes in the complex geometry evidenced by crystallography, which may also result in changes to the chemical environment of *HI*, will be discussed in Section 2.4.

2.3 SYNTHESIS OF A CARBOXYL-APPENDED BISDIPYRRIN

We have prepared a dicarboxylate-appended 2,2'-bisdipyrin through both direct saponification and transesterification, which will be employed in the derivation of new bisdipyrin ligands and complexes. Previous reports of the synthesis of NiL^3 and H_2L^3 (Figure 2.10), published since the following works were conducted, use a tetrahydrofuran (THF) solution containing either NaOH or LiOH in H_2O , respectively.⁸⁻⁹

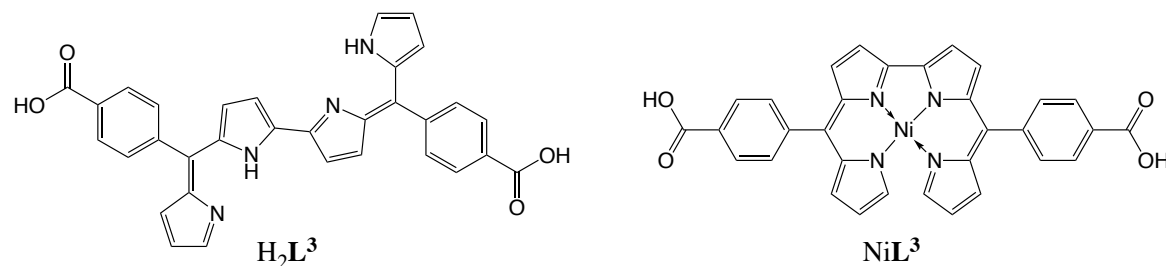
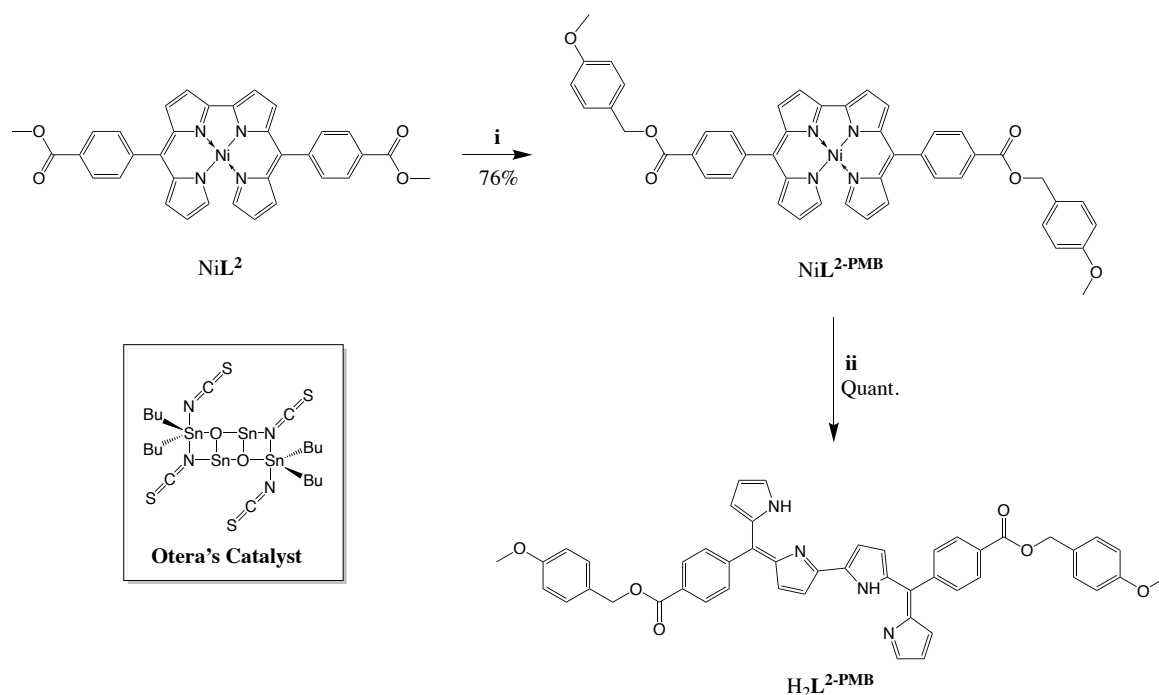


Figure 2.10. Structures of H_2L^3 and NiL^3 .

Purification involved precipitation using HCl to neutralise the excess base and resulted in yields of 73 and 88% for NiL^3 and H_2L^3 , respectively. In the following work, two alternate approaches to the production of these compounds were investigated, and one was found to be superior in terms of yield to all other reports.

2.3.1 Transesterification with Otera's Catalyst

The poor solubility of H_2L^2 in aqueous solvent systems and low molecular weight alcohols led to difficulty in hydrolysing the methyl esters to give the desired carboxylates. Initial transesterification followed by a subsequent organic phase cleavage to yield the desired product was carried out to mitigate these complications (Scheme 2.3).



*Scheme 2.3. Transesterification of NiL^2 to $\text{NiL}^{2\text{-PMB}}$ and subsequent nickel extrusion to give $\text{H}_2\text{L}^{2\text{-PMB}}$. i) *p*-methoxybenzyl alcohol, Otera's Catalyst, toluene, reflux, 48 hours. ii) Conc. HCl, CH_2Cl_2 . Inset: Structure of Otera's catalyst, first synthesised in 1993.¹⁰*

Transesterification of the complex NiL^2 to an acid-sensitive *p*-methoxybenzyl (PMB) ester was achieved in 76% yield through application of Otera's catalyst in toluene at reflux (Scheme 2.3). Otera's catalyst (Scheme 2.3 *inset*) has been identified as an efficient means of catalysing transesterification reactions in organic solvents and was able to be isolated from the reaction mixture during the chromatographic purification of the complex. Total consumption of the starting material was crucial here, due to the similarities in retention factor (R_f) of the methyl ester (0.72) and PMB ester product (0.68), which was monitored via thin-layer chromatography (TLC) (SiO_2 , CH_2Cl_2).

Crystals suitable for X-ray diffraction were grown from slow evaporation of a CHCl_3 solution of the complex, $\text{NiL}^{2\text{-PMB}}$ (Figure 2.11).

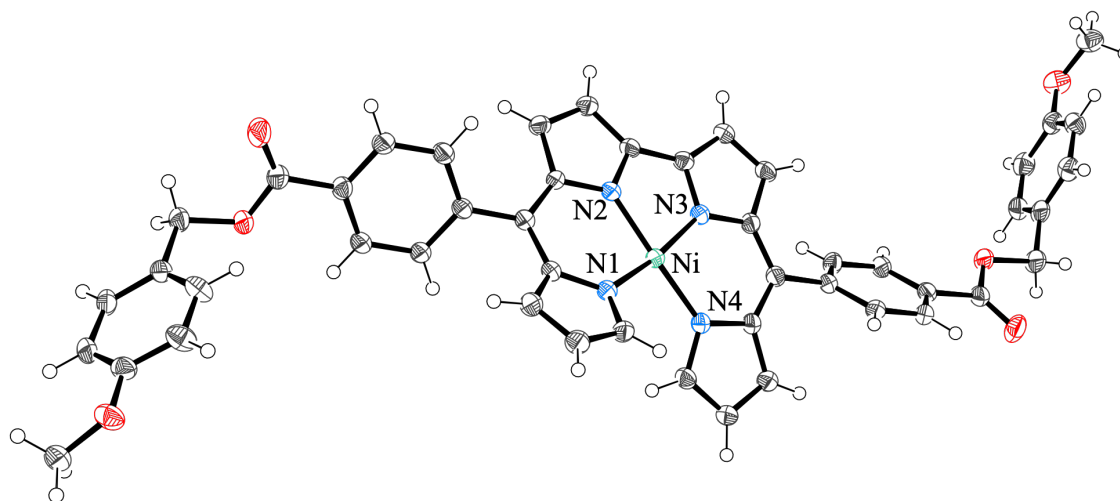


Figure 2.11. Ortep-3 representation of the structure of $\text{NiL}^{2\text{-PMB}}$, as determined by X-ray crystallography. Thermal ellipsoids of non-hydrogen atoms shown at 50% probability level.

The *p*-methoxybenzyl ester is stable to the 12 M HCl used in the proteolytic dissociation of the metal complex, giving ligand $\text{H}_2\text{L}^{2\text{-PMB}}$, as indicated by both mass spectrometry and NMR spectroscopy.

Electrospray ionisation–high resolution mass spectrometry (ESI-HRMS) shows a change in mass from m/z 822.1995 to m/z 767.2854, associated with both the loss of the coordinated Ni^{2+} ion and addition of three H^+ to give the ligand its required positive charge (Figure 2.12). No peak corresponding to PMB ester cleavage was observed.

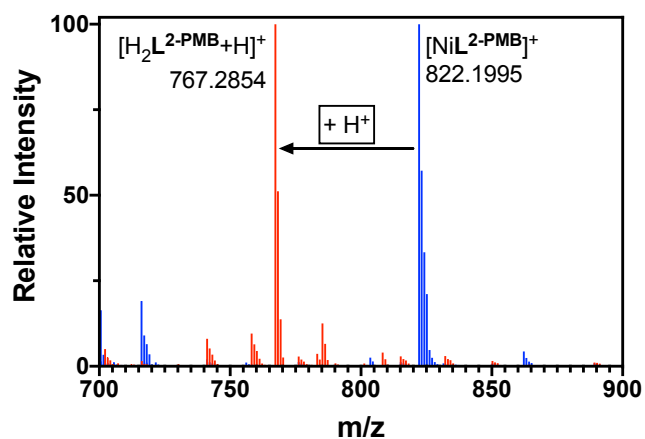


Figure 2.12. Mass spectra of $\text{NiL}^{2\text{-PMB}}$ (Blue trace) and $\text{H}_2\text{L}^{2\text{-PMB}}$ (red trace) following proteolytic dissociation of the metal complex.

The ^1H NMR spectrum highlights changes similar to that seen in previous analysis of H_2L^2 and its associated Ni(II) and Pd(II) complexes (Figure 2.13).

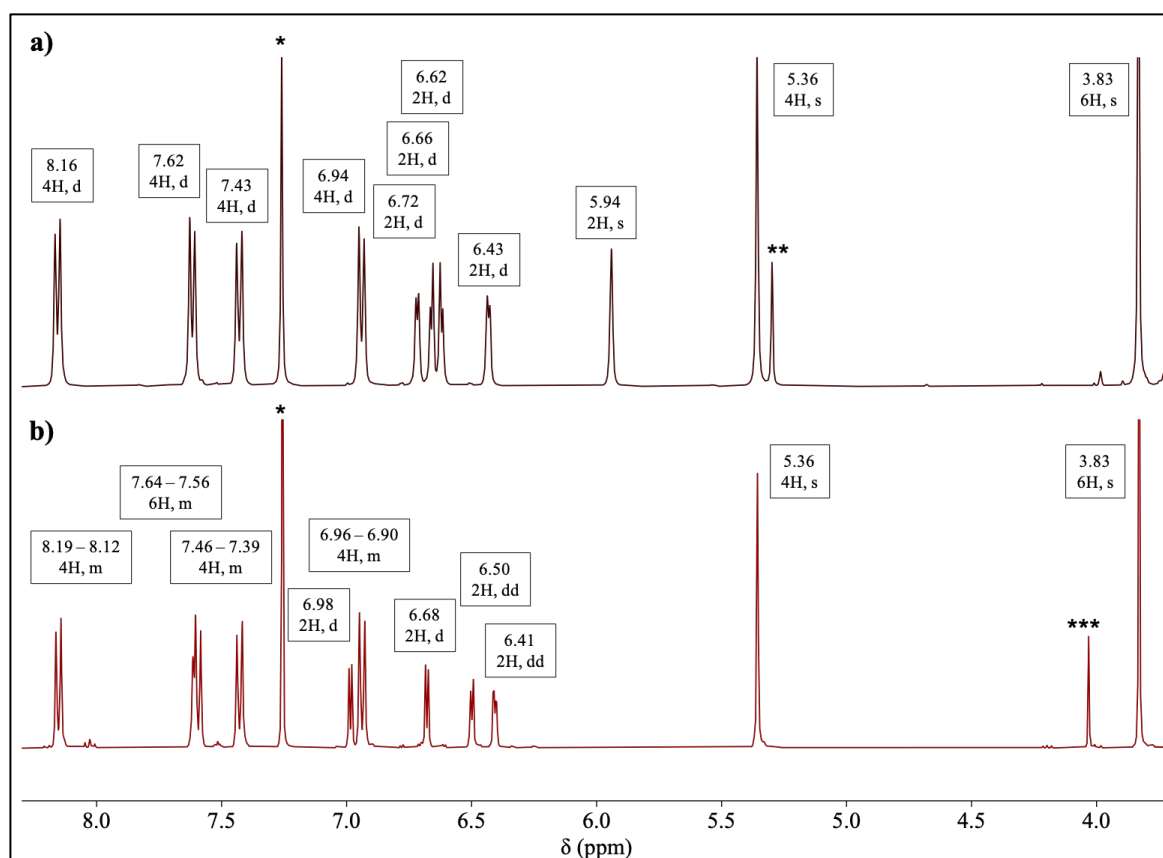


Figure 2.13. ^1H NMR spectra of a) $\text{NiL}^{2\text{-PMB}}$ and b) $\text{H}_2\text{L}^{2\text{-PMB}}$ in CDCl_3 (400 MHz).

*Residual Solvent Peak. ** CH_2Cl_2 peak. *** Unidentified impurity.

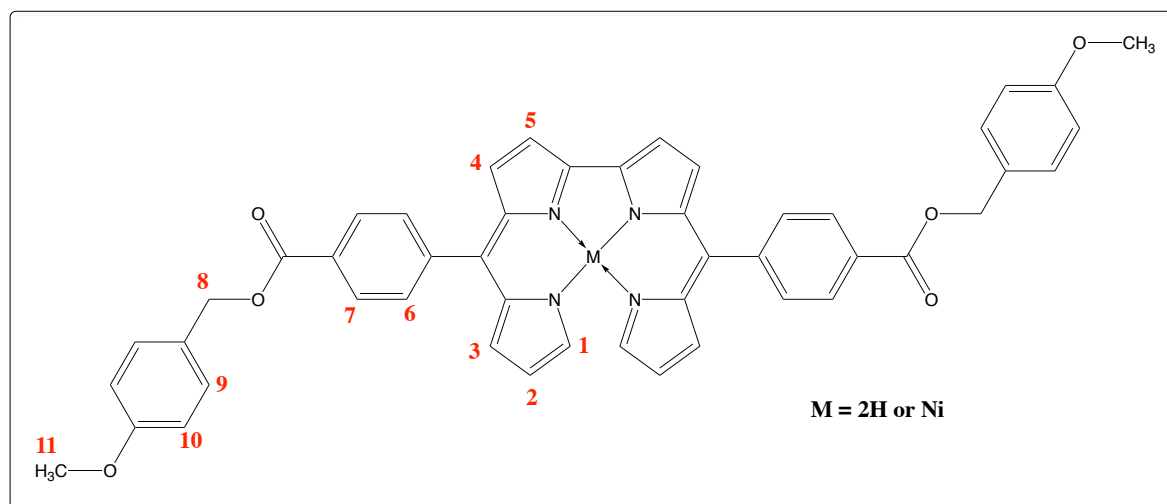


Figure 2.14. NMR proton environment numbering for H_2L^{2-PMB} and the Ni(II) complex.

Again, the pyrrolic proton environments corresponding to $H1-5$ (Figure 2.14) shift by varying degrees following decomplexation. The signals corresponding to $H8$ ($-\text{CH}_2-$, δ 5.36 ppm) and $H11$ ($-\text{OCH}_3$, δ 3.83 ppm) are unaltered following decomplexation, and phenyl proton signals $H6$, $H7$, $H9$ and $H10$ experience minimal change.

As was the case for L^2 , decomplexation leads to overlap of two signals at δ 7.64 – 7.56 ppm, producing a total integration of 6H. Comparison of the COSY NMR (Figure 2.15 and Figure 2.16) shows that this signal is a product of the marked shift of a single pyrrolic $-\text{CH}$ environment following decomplexation. The ^1H NMR assignments are given in Table 2.2.

2. New Bisdipyrrin Metal(II) Complexes

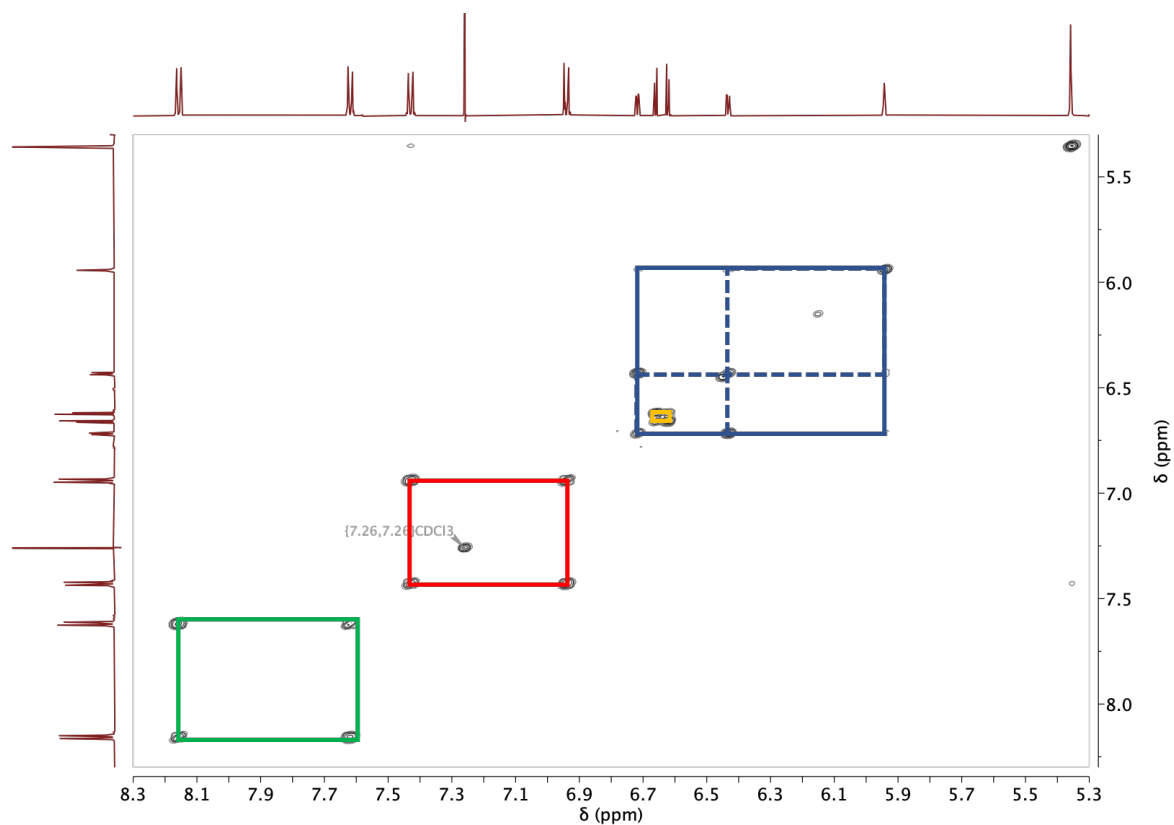


Figure 2.15. COSY NMR of H_2L^{2-PMB} in $CDCl_3$ (600 MHz).

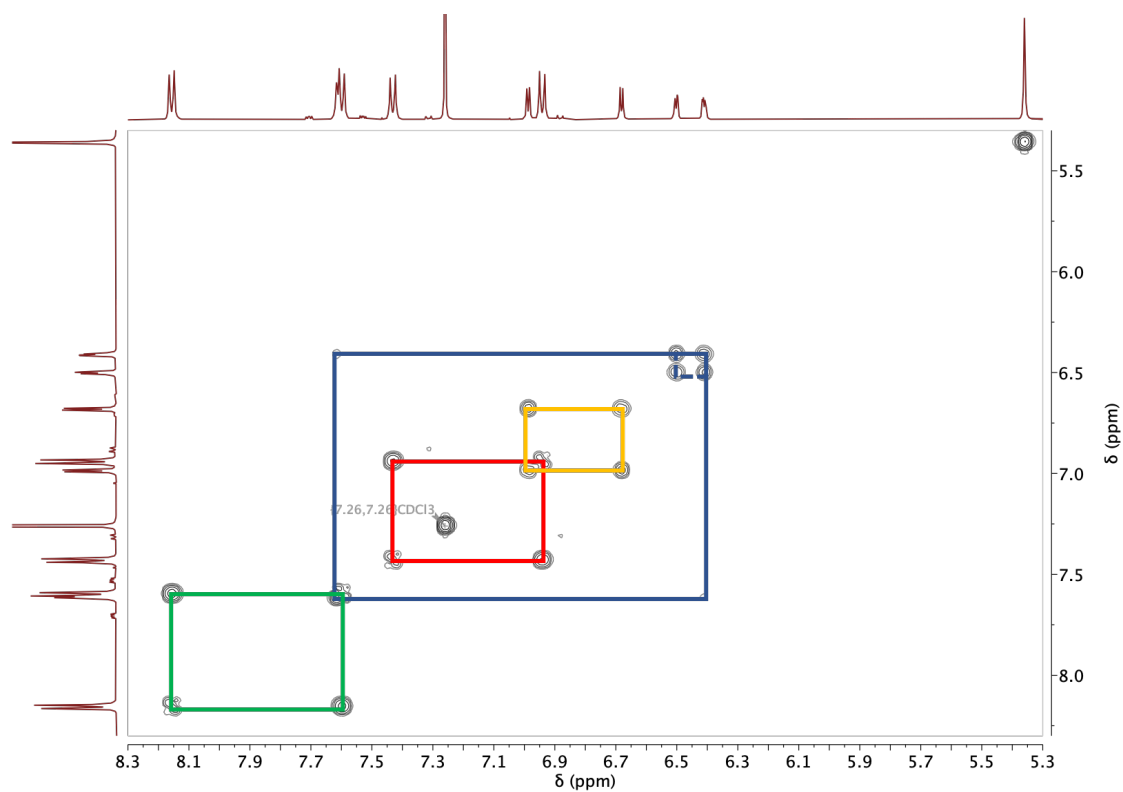


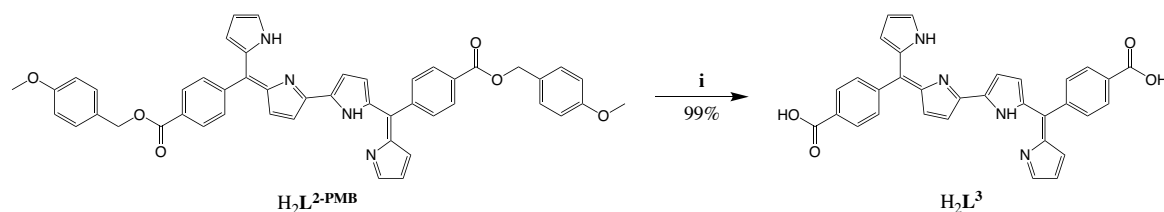
Figure 2.16. COSY NMR of NiL^{2-PMB} in $CDCl_3$ (600 MHz).

2. New Bisdipyrrin Metal(II) Complexes

Table 2.2. NMR Spectral assignment of H_2L^{2-PMB} and NiL^{2-PMB} complex ($CDCl_3$, 600 MHz) (*dd* = Doublet of doublets; *t* = triplet; *d* = doublet; *s* = singlet; *br* = broad signal; *m* = multiplet; * overlapping signals).

Environment	Assignment	H_2L^{2-PMB}	NiL^{2-PMB}
(3H) Pyr-H	$H1/2/3$	6.41 (2H, dd)	6.43 (2H, dd)
		6.50 (2H, dd)	6.72 (2H, dd)
		7.61 (2H, m)*	5.94 (2H, s)
(2H) Pyr-H	$H4/5$	6.68 (2H, d)	6.62 (2H, d)
		6.99 (2H, d)	6.68 (2H, d)
Ph-H	$H6$	7.64 – 7.56 (4H, m)	7.62 (4H, d)
Ph-H	$H7$	8.19 – 8.12 (4H, m)	8.16 (4H, d)
O-CH ₂	$H8$	5.36 (4H, s)	5.36 (4H, s)
Ph-H	$H9$	6.96 – 6.90 (4H, m)	6.94 (4H, d)
Ph-H	$H10$	7.46 – 7.39 (4H, m)	7.43 (4H, d)
O-CH ₃	$H11$	3.83 (6H, s)	3.83 (6H, s)

Following purification, the ester was hydrolysed by trifluoroacetic acid (TFA) (10% in CH_2Cl_2) to liberate the free acid bisdipyrrin H_2L^3 , which was purified by extraction into 1 M aqueous NaOH to remove *p*-methoxybenzyl alcohol (PMB-OH). This was followed by reacidification using HCl to extract the product back into ethyl acetate (Scheme 2.4). NMR and ESI-HRMS confirm loss of the PMB ester group.



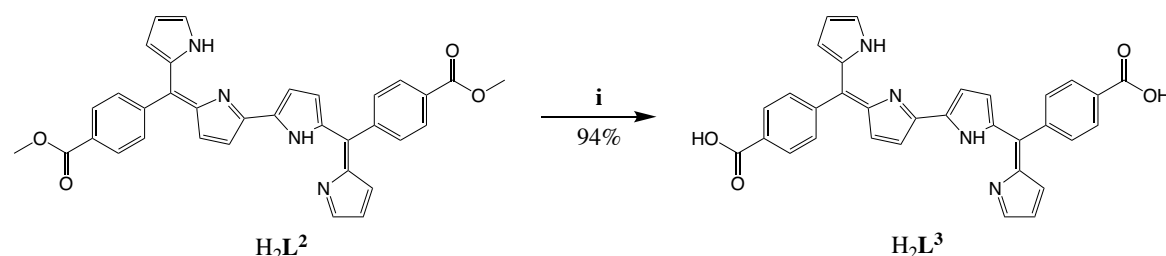
Scheme 2.4. Proteolytic cleavage of the acid-sensitive PMB-esters in H_2L^{2-PMB} to give the free diacid H_2L^3 . i) 10% TFA in CH_2Cl_2 .

2. New Bisdipyrrin Metal(II) Complexes

The products of the transesterification, proteolytic dissociation and ester cleavage steps were obtained in 76, 100 and 99% yield, respectively, leading to an overall efficiency of 75% over three steps.

2.3.2 Direct Saponification in Organic Solvents

A direct organic-phase ester hydrolysis protocol was also investigated, which has been used for a number of lipophilic esters.¹¹ This involved treating a solution of the ester in CH_2Cl_2 with a 3 N NaOH slurry in methanol at room temperature until hydrolysis was complete (Scheme 2.5).

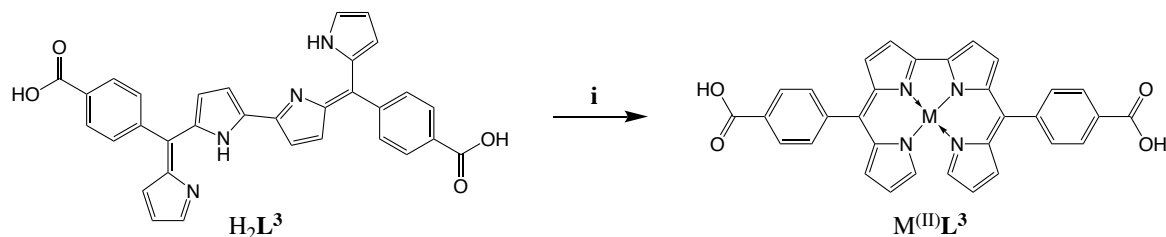


Scheme 2.5. Direct saponification of H_2L^2 to give the diacid H_2L^3 . i) 3 N NaOH in CH_3OH , CH_2Cl_2 , 48 hours.

Following purification, the dicarboxylate bisdipyrrin, H_2L^3 , was obtained in 94% yield, making it superior to the transesterification protocol and previously published synthetic routes.

Unlike in previous cases, where the synthesis of the Ni(II) complex has been a fundamental part of the synthesis of the bisdipyrrin ligand, H_2L^3 was achieved in the first instance after the nickel was removed. As such, we hoped to form the nickel(II) and copper(II) complexes with this ligand to examine any solubility changes (Scheme 2.6).

2. New Bisdipyrrin Metal(II) Complexes

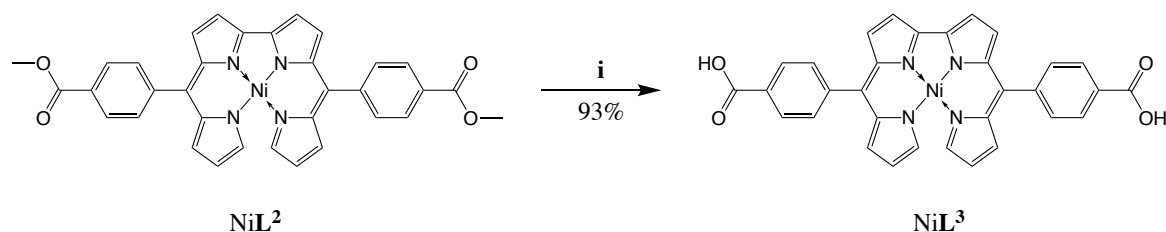


Scheme 2.6. Synthesis of ML^3 complexes ($M = Ni^{2+}$ or Cu^{2+}). i) $M(OAc)_2$, $CHCl_3/CH_3OH$.

2.3.3 Nickel (II) Complex

A precipitate rapidly forms following addition of the nickel(II) acetate to a solution of H_2L^3 . After isolating by filtration, this solid proved insoluble in all common organic solvents and aqueous solutions, suggesting that a coordination polymer had formed *via* coordination of the nickel(II) centre with the deprotonated carboxylates, rather than the desired discrete complex.

A second route to NiL^3 complex using the previously described organic saponification with CH_3OH was investigated (Scheme 2.7) as an alternative to the THF/NaOH procedure reported.⁸



Scheme 2.7. Synthesis of NiL^3 from NiL^2 . i) 3 N NaOH in CH_3OH , CH_2Cl_2 .

Following hydrolysis, extraction of the deprotonated diacid complex, $Na_2[NiL^3]$ into H_2O was efficient, leaving only unreacted starting material, if any, in the organic phase (CH_2Cl_2). The addition of one equivalent of HCl to the aqueous phase precipitates a fine solid, which was filtered and washed with H_2O to give the desired neutral complex in the absence of excess base.

2. New Bisdipyrrin Metal(II) Complexes

Comparison of this complex to the free ligand by NMR spectroscopy in deuterated dimethyl sulfoxide (DMSO) highlights similar shifts observed following decomplexation of NiL^2 , namely a shift in pyrrolic C-H chemical shifts. The removal of the self-protonation observed in H_2L^3 by effectively protecting the pyrrolic nitrogens allowed for substantially cleaner spectra (Figure 2.17). DMSO was used herein due to insolubility of H_2L^3 in CDCl_3 .

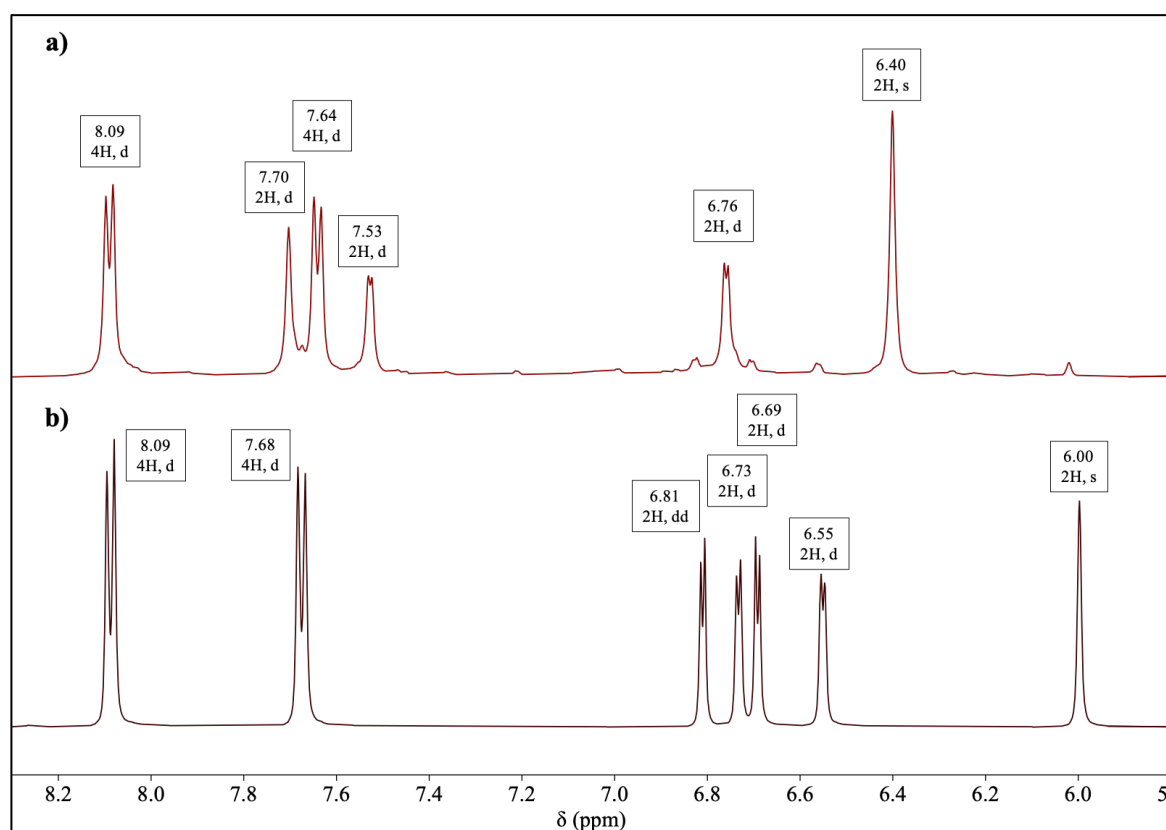


Figure 2.17. ^1H NMR spectra for a) H_2L^3 and b) NiL^3 (d_6 -DMSO, 500 MHz).

Crystals of suitable quality for X-ray diffraction were grown from slow evaporation of a N,N -dimethylformamide (DMF) solution of NiL^3 (Figure 2.18), in which the complex is interacting with two solvent molecules through H-bonding of the terminal carboxylates.

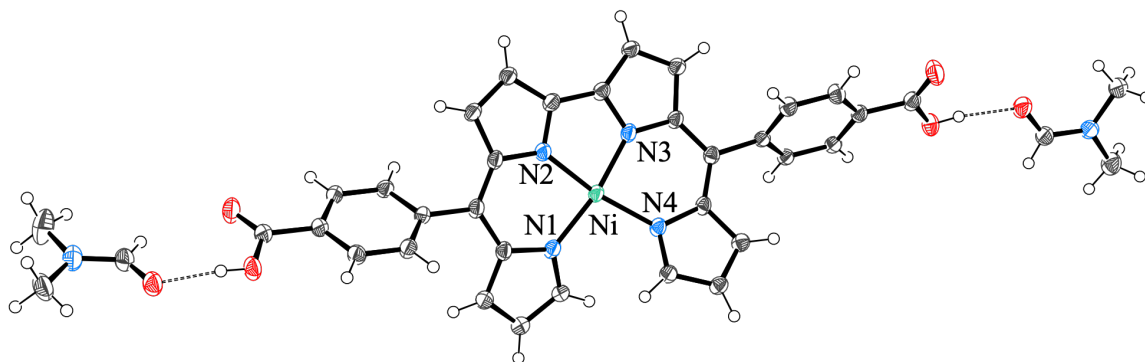
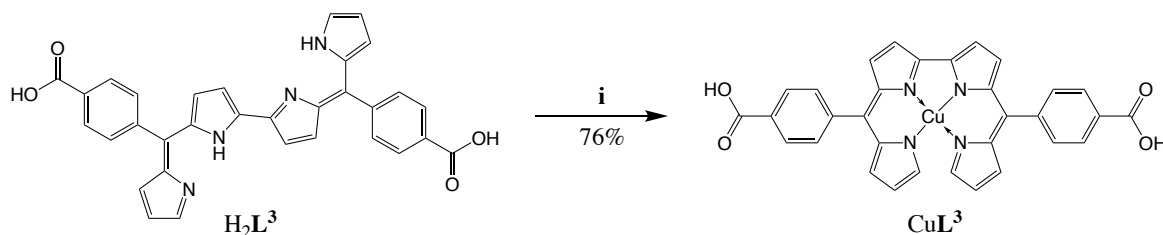


Figure 2.18. Ortep-3 representation of the structure of $\text{NiL}^3 \cdot 2\text{DMF}$, as determined by X-ray crystallography. Thermal ellipsoids of non-hydrogen atoms shown at 50% probability level.

2.3.4 Copper(II) Complex

The copper(II) complex was synthesised by the same method as for the methyl ester ligand, H_2L^2 (Scheme 2.8).



Scheme 2.8. Synthesis of CuL^3 . i) $\text{Cu}(\text{OAc})_2 \cdot \text{H}_2\text{O}$, $\text{CH}_3\text{OH}/\text{CHCl}_3$, 4 hours, room temp.

Following work-up, this product proved highly insoluble in aqueous solutions, with the exception of NaOH solutions, whereby the corresponding disodium salt of the complex was highly water soluble.

Crystals of suitable quality for X-ray diffraction were grown from slow evaporation of a DMF solution of CuL^3 (Figure 2.19), again exhibiting H-bonding to two molecules of solvent through the carboxylates.

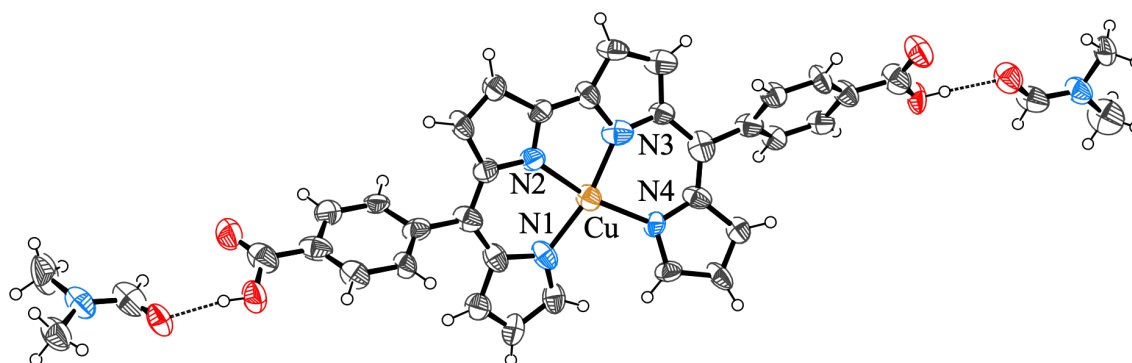


Figure 2.19. Ortep-3 representation of the structure of $\text{CuL}^3 \cdot 2\text{DMF}$, as determined by X-ray crystallography. Thermal ellipsoids of non-hydrogen atoms shown at 50% probability level.

Unfortunately, poor solubility at pH 7.4 was evident, and so while the complex will be an interesting comparison electrochemically and crystallographically, translation to cell and radiolabelling studies will be limited.

2.4 CRYSTALLOGRAPHY OF $M(\text{II})$ BISDIPYRRIN COMPLEXES

NMR spectroscopy of NiL^2 and PdL^2 highlights a dramatic shift in one pyrrolic $-\text{CH}$ environment within the $3H$ spin system. This has been tentatively assigned the terminal protons, located on the 10/10' carbon atoms. Crystallography of NiL^2 shows that these hydrogens lie above and below the plane approximated by the metal- N_4 chelate due to steric clash. The influence of the metal ion on this distortion will be discussed herein. For reference, the crystallographic tables for each complex is given below (Table 2.3 and Table 2.4).

Table 2.3. Crystallographic Table for (2,2'-bisdipyrrinato) complexes, CuL² and PdL².

	CuL²	PdL²
Empirical Formula	C ₃₄ H ₂₄ N ₄ O ₄ Cu	C ₃₄ H ₂₄ N ₄ O ₄ Pd
Formula Weight	616.11	659.01
Crystal System	Triclinic	Triclinic
Space Group	P $\bar{1}$	P $\bar{1}$
<i>a</i> /Å	9.6052(8)	9.4548(4)
<i>b</i> /Å	10.7834(7)	11.2390(5)
<i>c</i> /Å	14.8147(8)	14.4934(7)
α /(°)	95.554(5)	95.568(4)
β /(°)	105.650(6)	104.199(4)
γ /(°)	111.499(7)	111.966(4)
<i>V</i> /Å ³	1341.59(17)	1353.80(12)
<i>Z</i>	2	2
Temperature/K	130.0(1)	130.0(1)
λ /Å	1.54184	1.54184
Reflections Collected	9490	9629
Independent Reflections	5408	5369
R-Factor/%	6.62	4.15

Table 2.4. Crystallographic Table for (2,2'-bisdipyrrinato) metal(II) complexes.

	NiL³•2DMF	CuL³•2DMF	NiL²-PMB
Empirical Formula	C ₃₈ H ₃₄ N ₆ O ₆ Ni	C ₃₈ H ₃₄ N ₆ O ₆ Cu	C ₄₈ H ₃₆ N ₄ O ₆ Ni
Formula Weight	729.42	734.27	823.53
Crystal System	Triclinic	Triclinic	Triclinic
Space Group	P $\bar{1}$	P $\bar{1}$	P $\bar{1}$
<i>a</i> /Å	10.1047(3)	10.1230(19)	9.6093(4)
<i>b</i> /Å	10.3254(4)	10.477(3)	10.8850(4)
<i>c</i> /Å	17.3991(5)	17.110(2)	19.0071(8)
α /(°)	86.048(3)	86.142(16)	89.072(3)
β /(°)	76.812(3)	75.945(16)	87.830(4)
γ /(°)	68.800(3)	69.18(2)	69.923(4)
<i>V</i> /Å ³	1647.66(10)	1645.1(6)	1865.93(14)
<i>Z</i>	2	2	2
Temperature/K	99.97(18)	100.01(10)	130.01(10)
λ /Å	1.54184	1.54184	1.54184
Reflections Collected	21389	10324	13544
Independent Reflections	6773	6591	7672
R-Factor/%	6.68	13.02	6.14

2.4.1 PdL^2 , CuL^2 and NiL^2 Crystallographic analysis.

A list of bond lengths and angles of particular interest are given in Table 2.5. Atom labels are shown in Figure 2.20, using PdL^2 as the representative example. NiL^2 data obtained from the supplementary information from Baudron *et al.*⁷ All transition metal ionic radii discussed herein refer to the M(II) 4-coordinate (square planar) Shannon radii previously reported.¹²

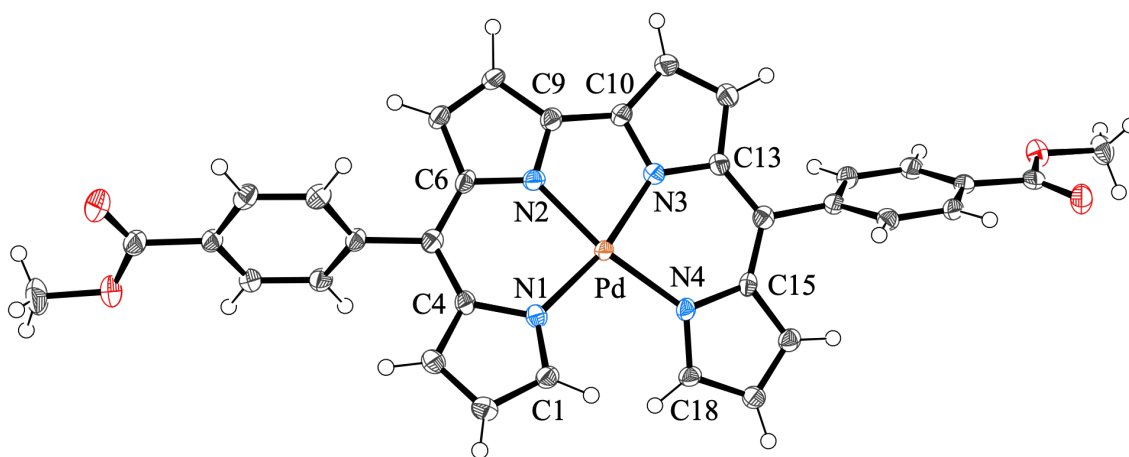


Figure 2.20. Atom labels for PdL^2 for crystallographic analysis.

Table 2.5. Selected bond lengths (Å) and bond angles (°) in NiL², CuL² and PdL². Effective metal ionic radius corresponds to +2 oxidation state in square planar geometries.¹²

		NiL ²	CuL ²	PdL ²
	M(II) ionic radii	0.49	0.57	0.64
Bond Lengths	C9–C10	1.446(3)	1.448(4)	1.452(4)
	C1–C18	3.093	3.286	3.372
	M–N1	1.8940(13)	1.986(2)	2.042(2)
	M–N2	1.8496(14)	1.944(2)	1.971(2)
	M–N3	1.8496(14)	1.959(2)	1.965(2)
	M–N4	1.8940(13)	1.983(2)	2.040(2)
Bond Angles	N1–M–N2	89.72(6)	88.38(10)	88.44(10)
	N2–M–N3	82.15(8)	79.72(10)	79.56(10)
	N3–M–N4	89.72(6)	88.35(10)	88.70(10)
	N1–M–N4	99.61(8)	104.25(9)	103.51(10)
	N1–M–N3	167.95(6)	166.21(9)	167.13(11)
	N2–M–N4	167.95(6)	166.18(10)	167.84(11)
	C4–C5–C6	122.31(13)	123.8(3)	125.5(3)
	C13–C14–C15	122.31(13)	123.6(3)	124.8(3)

Complexes PdL² and CuL² both exhibit M–N bond lengths typical of metal-pyrrole complexes, increasing with ionic radius of the metal ion.^{2, 13-17} Moreover, the M–N bond lengths for each of the complexes are within the typical values for M(II) porphyrins and M(II) corroles.¹⁸⁻²³

The C9–C10 bond length of approximately 1.45 Å for each complex corresponds to partial double bond character (bond order of 1.5),²⁴ indicating the extension of conjugation of each dipyrin subunit. The ligand expands with increasing the size of the coordinated metal ion, based on bond angles C4–C5–C6 and C13–C14–C15. This is in agreement with the previously reported analysis of the (3,3',4,4',8,8',9,9'-octaethyl-10,10'-dimethyl-2,2'-bisdipyrin) palladium(II) and copper(II) complexes.¹

2. New Bisdipyrrin Metal(II) Complexes

To quantify the deviation from square-planar geometry, the τ_4 -parameter, adapted from τ_5 for describing the geometry of 5-coordinate metal complexes, was calculated using bond angles N1–M–N3 and N2–M–N4 as α and β , respectively, according to Equation 2.1,²⁵

$$\text{Equation 2.1} \quad \tau_4 = \frac{360 - \alpha - \beta}{360 - 2\theta}$$

where $\theta = \cos^{-1}(-1/3)$, or $\sim 109.5^\circ$. A τ_4 value of 1 indicates formal tetrahedral geometry, while a value of 0 corresponds to a formal square-planar complex. The τ_4 values of NiL^2 , CuL^2 and PdL^2 are 0.171, 0.196 and 0.177, respectively, confirming a distorted square planar geometry for each.

The ligand-based distortions of these complexes have also been described through a set of dihedral angles centred on the C2–C2' (crystallographically C9–C10) bond (Table 2.6).

Table 2.6. Dihedral angles (°) for NiL^2 , CuL^2 and PdL^2 centred around the C9–C10 bond.

	DIHEDRAL ANGLES (°)		
	C1–C9–C10–C18	N1–C9–C10–N4	N2–C9–C10–N3
NiL²	21.2	12.6	4.31
CuL²	14.3	9.00	5.14
PdL²	11.7	6.25	5.79

The N2–C9–C10–N3 dihedral angle represents the torsion in the 5-membered chelate ring for each metal ion. This angle increases with metal ionic radius, indicating the introduction of ring-strain to chelate a larger metal ion. The C1–C9–C10–C18 and N1–C9–C10–N4 dihedral angles represent the twisting in the dipyrrolyl-subunits following metal coordination. Increasing the ionic radius of the metal causes an overall reduction in this torsion, which may result from a reduction in the steric clash of the two terminal

α -hydrogen atoms. This reduction is associated with an increase in the M–N bond distances from Ni(II) to Pd(II) and corresponds to increasing planarity.

The τ_4 values calculated for NiL², CuL² and PdL² contradict the geometry observed in the crystal structures (Figure 2.21) and these dihedral angles. To correct for this, an alternate approach to calculating the α and β angles was adopted.

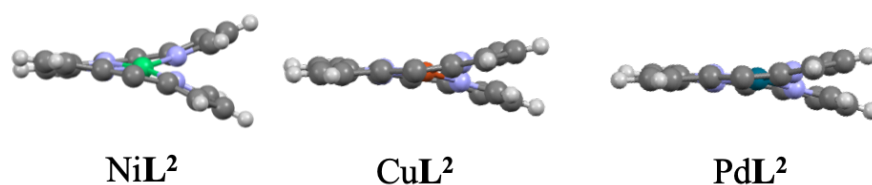


Figure 2.21. Side-on representations of the crystal structures of each of NiL², CuL² and PdL², highlighting the distortion of planarity. Meso-substituents omitted for clarity.

2.4.2 Refining the τ_4 Parameter

To better approximate the structural variation, a modified approach to the τ_4 calculation was used, in which the angles α and β in Equation 2.1 were calculated using a plane defined by N2–M–N3. This plane is inherently perfect with respect to the least-squares regression analysis, allowing for the calculation of bond angles using simple trigonometry. It requires the plane to be defined using two adjacent ligand donor atoms and measures the distance between this plane and the remaining two donor atoms. A comparison of the three complexes of L² is given in Table 2.7.

Table 2.7. Refined τ_4 parameter calculations.

Complex	NiL ²		CuL ²		PdL ²	
Classic τ_4	0.171		0.196		0.177	
Defined Plane	N2–M–N3		N2–M–N3		N2–M–N3	
N Label	N1	N4	N1	N4	N1	N4
M–N bond Length	1.894	1.894	1.986	1.983	2.042	2.040
N-Plane Distance	0.294	0.294	0.244	0.239	0.166	0.113
Sin(θ)	0.16	0.16	0.12	0.12	0.08	0.06
θ (°)	8.93	8.93	7.06	6.92	4.66	3.18
180 – θ (α/β)	171.07	171.07	172.94	173.08	175.34	176.82
Refined τ_4	0.127		0.099		0.056	

This approach forces the parameter to reference an intrinsic plane for the complex and ensures the measured bond angles are perpendicular to this plane, rather than the smallest crystallographic angle. This is particularly important for polydentate ligands, which are unable to coordinate independently of the other donor atoms. This approach was validated against the original publication in which the ligands were not spatially restricted relative to each other. These values were also compared to the $\tau_{\text{tet-sq}}$ values obtained using an alternative method developed for bidentate ligands,²⁶ which gave values of 0.130, 0.99 and 0.57 for NiL², CuL² and PdL² respectively.

These τ_4 values indicate an increase in square planar character with increasing metal ion radius. To extend on this, NiL^{2-PMB}, NiL³ and CuL³ were also analysed.

2.4.3 NiL^{2-PMB}, NiL³ and CuL³ Crystallography

Each of the three derivative complexes, NiL^{2-PMB}, NiL³ and CuL³ display N–M–N bond angles typical of Ni(II) and Cu(II) pyrrole bonds. A list of important bond lengths and angles is given in Table 2.8.

Table 2.8. Selected bond lengths ($\text{\AA}$), bond angles ($^\circ$) and dihedral angles ($^\circ$) in $\text{NiL}^{2\text{-PMB}}$, NiL^3 and CuL^3 .

		$\text{NiL}^{2\text{-PMB}}$	$\text{NiL}^3 \cdot 2\text{DMF}$	$\text{CuL}^3 \cdot 2\text{DMF}$
	M(II) ionic radii	0.49	0.49	0.57
Bond Lengths	C9–C10	1.438(3)	1.432(3)	1.418(14)
	C1–C18	3.069	3.050	3.285
	M–N1	1.8984(19)	1.910(2)	1.965(8)
	M–N2	1.8637(19)	1.863(2)	1.944(8)
	M–N3	1.8559(18)	1.859(2)	1.936(8)
	M–N4	1.9000(19)	1.910(2)	1.990(8)
Bond Angles	N1–M–N2	89.37(8)	89.84(9)	87.4(3)
	N2–M–N3	82.25(8)	81.63(9)	80.0(3)
	N3–M–N4	89.85(8)	89.69(9)	88.5(3)
	N1–M–N4	99.43(8)	99.71(9)	104.9(3)
	N1–M–N3	168.41	168.19	165.0(4)
	N2–M–N4	169.13	168.82	166.5(4)
Dihedral Angles	C1–C9–C10–C18	18.90	16.79	15.84
	N1–C9–C10–N4	9.70	9.09	9.83
	N2–C9–C10–N3	5.75	4.31	5.59
	τ_4 values	0.110	0.108	0.108

These structures produce refined τ_4 values of 0.110, 0.108 and 0.108, indicating a similar coordination environment for both Cu(II) and Ni(II) complexes. While C9–C10 bond lengths again indicate partial double bond character, this bond in CuL^3 is significantly reduced, resulting in the observed increase in dihedral angles N1–C9–C10–N4, N2–C9–C10–N3 and C1–C9–C10–C18 compared to CuL^2 . This contraction also manifests as an increase in twisting observed in the ligand, and correlates to a slight increase in the τ_4 value.

2.4.4 Interpreting the Crystallography

Assuming the solid-state structure of these complexes is retained in solution, the crystallographic data may aid in the understanding of the observed variation in ^1H NMR. By arranging the complexes in order of decreasing τ_4 , the resulting trend is shown to coincide with the *reverse* trend observed for the metal ionic radius:

$$\text{NiL}^2 > \text{NiL}^{2\text{-PMB}} > \text{NiL}^3 = \text{CuL}^3 > \text{CuL}^2 > \text{PdL}^2$$

$$\text{Ni}^{2+} (0.49 \text{ \AA}) < \text{Cu}^{2+} (0.57 \text{ \AA}) < \text{Pd}^{2+} (0.64 \text{ \AA})$$

This increase in deviation from planarity is expected to place the α -hydrogens located on C10 and C10' (crystallographically C1 and C18) in proximity of the aromatic π -electron cloud of the adjacent pyrrole ring. This would correspond to a net shielding effect of the relevant nuclei, and consequently account for the dramatic change in chemical shift of these protons. The increase in planarity of the palladium complex and overall ligand expansion would reduce this shielding compared to the nickel complex, shifting the signal downfield with respect to the other pyrrolic -CH environments.

Previous work relating the X-ray crystallographic analysis of (2,2'-bisdipyrrinato) metal(II) complexes describe the effect of subtle deviations in metal chelation on the electrochemical behaviour in cyclic voltammetry experiments.¹ Based on the above results, the differences in dihedral angles N2–C9–C10–N3 and τ_4 values are hypothesised to correspond to a variation in the cyclic voltammograms of the relevant complexes. The electrochemistry of NiL^2 , CuL^2 and PdL^2 will be discussed in Section 2.8.

2.5 SYNTHESIS OF NEW BISDIPIRRINS WITH DIFFERENT MESO FUNCTIONAL GROUPS

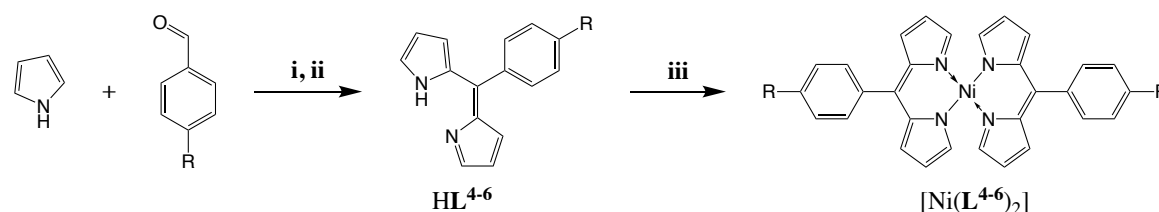
Following the synthesis for H_2L^2 , a series of analogous ligands with the same tetradentate binding functionality were synthesised. Four alternative benzaldehydes, 4-chlorobenzaldehyde, 4-nitrobenzaldehyde, and 4-cyanobenzaldehyde, were used to yield dipyrin and bisdipyrin ligands HL^{4-6} and H_2L^{7-9} (Table 2.9).

Table 2.9. Ligand substitution profile for dipyrins HL^{4-6} and bisdipyrins H_2L^{7-9} .

Dipyrin	Bisdipyrin	R_1
L^1	L^2	-COOMe
—	L^3	-COOH
L^4	L^7	-Cl
L^5	L^8	-CN
L^6	L^9	-NO ₂

2.5.1 Synthesis and Characterisation of $[NiL_2]$ Complexes

Dipyrins HL^{4-6} were synthesised according to the protocol described for HL^1 in 24%, 12% and 42% yield for the 4-chlorophenyl, 4-cyanophenyl and 4-nitrophenyl *meso*-substituted dipyrins, respectively (Scheme 2.9).



Scheme 2.9. General synthesis of $[NiL_2]$ complexes. (i) 0.18 M HCl, H_2O/CH_3OH , (ii) DDQ, $CHCl_3/CH_3OH$, (iii) $Ni(OAc)_2 \cdot 4H_2O$, CH_3OH .

Subsequent complexation to a nickel(II) ion was again achieved by treating the dipyrins HL^{4-6} in CH_3OH with $Ni(OAc)_2 \cdot 4H_2O$, producing complexes $[Ni(L^{4-6})_2]$ in 75%, 62% and 79% yields for the chloro-, cyano- and nitro-substituted complexes, respectively.

The proton NMR spectra for $[\text{Ni}(\text{L}^1)_2]$ and complexes $[\text{Ni}(\text{L}^{4-6})_2]$ are shown below (Figure 2.22). Previous work on the complex $[\text{Ni}(\text{L}^1)_2]$ highlighted the inability to elucidate the identity of the proton environment at δ 10.45 ppm due to the lack of cross-peaks in 2D COSY NMR experiments.

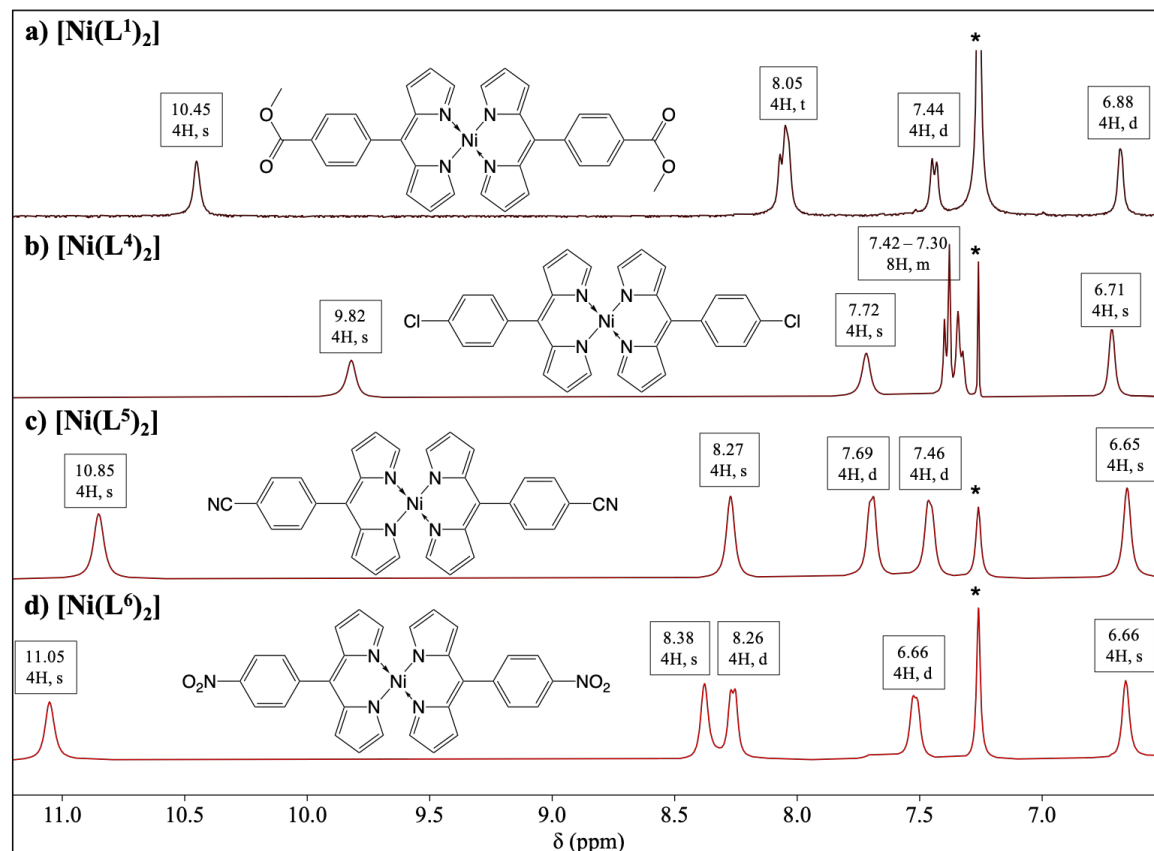


Figure 2.22. ^1H NMR spectra of the $[\text{Ni}(\text{L})_2]$ complexes in CDCl_3 (400 MHz).

* *Residual solvent peaks.*

Peak broadening of these spectra is indicative of fluxional processes in the pseudotetrahedral complexes about the nickel(II) centre, increasing the paramagnetic character of the complex. This removes peak multiplicity and in turn has made assignment of the signals difficult. Examination of the $[\text{Ni}(\text{L}^4)_2]$, $[\text{Ni}(\text{L}^5)_2]$, and $[\text{Ni}(\text{L}^6)_2]$ COSY spectra showed very weak coupling to the deshielded proton with two other signals. This confirms that the

deshielded environment in each complex (> 9 ppm) corresponds to a pyrrole proton. The representative COSY NMR for $[\text{Ni}(\text{L}^5)_2]$ is shown below (Figure 2.23).

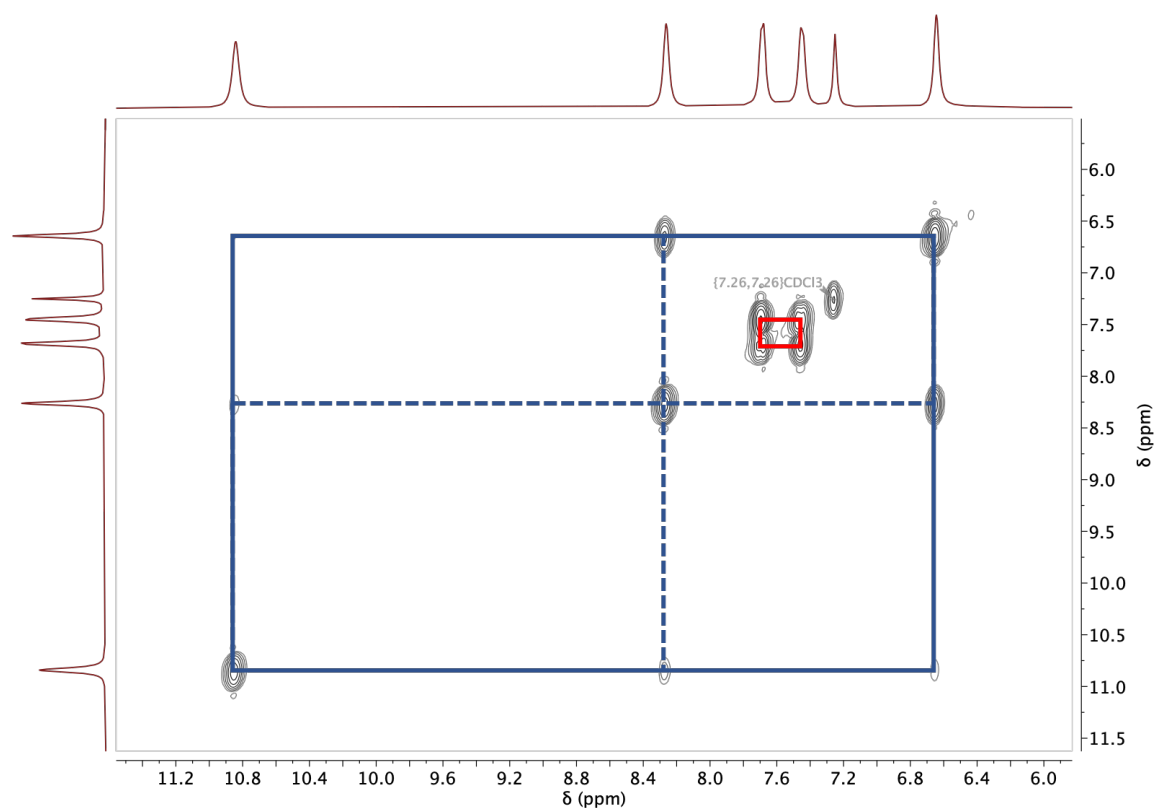


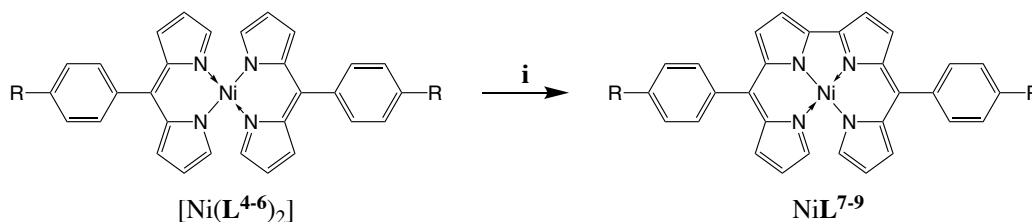
Figure 2.23. COSY NMR spectrum of $\text{Ni}(\text{L}^5)_2$ in CDCl_3 (400 MHz).

Each of the three dipyrin signals assigned by COSY NMR undergo downfield shifts following complexation to the Ni(II) ion. Their considerable deshielding follows from the crystallographic evidence discussed in Section 2.4.4, in which the α -hydrogens are expected to be spatially isolated from the π -electron cloud on the adjacent dipyrin ligand. This would limit the shielding effects near the nickel(II) ion, which would cause the observed downfield shift of this environment.

These complexes were then carried on to the next step in the synthesis of the (2,2'-bisdipyrinato) nickel(II) complexes.

2.5.2 Synthesis and Characterisation of NiL^{7-9} and H_2L^{7-9}

Oxidation of the $[\text{NiL}_2]$ complexes using DDQ and purification using flash chromatography (SiO_2 , CHCl_3 or CH_2Cl_2) yielded NiL^{7-9} in 40 to 60% yields as dark brown solids (Scheme 2.10).



Scheme 2.10. General synthesis of NiL^{7-9} . i) DDQ, toluene, reflux.

Chromatographic purification was typically efficient. In some cases, decomposition of the oxidant over time produced a by-product which shows a similar R_f to that of the complexes. Gravity chromatography was able to reduce the overlap between these bands and provided superior purity to flash chromatography in cases where this yellow decomposition product was observed.

The resulting proton NMR spectra of the purified complexes are given in Figure 2.24.

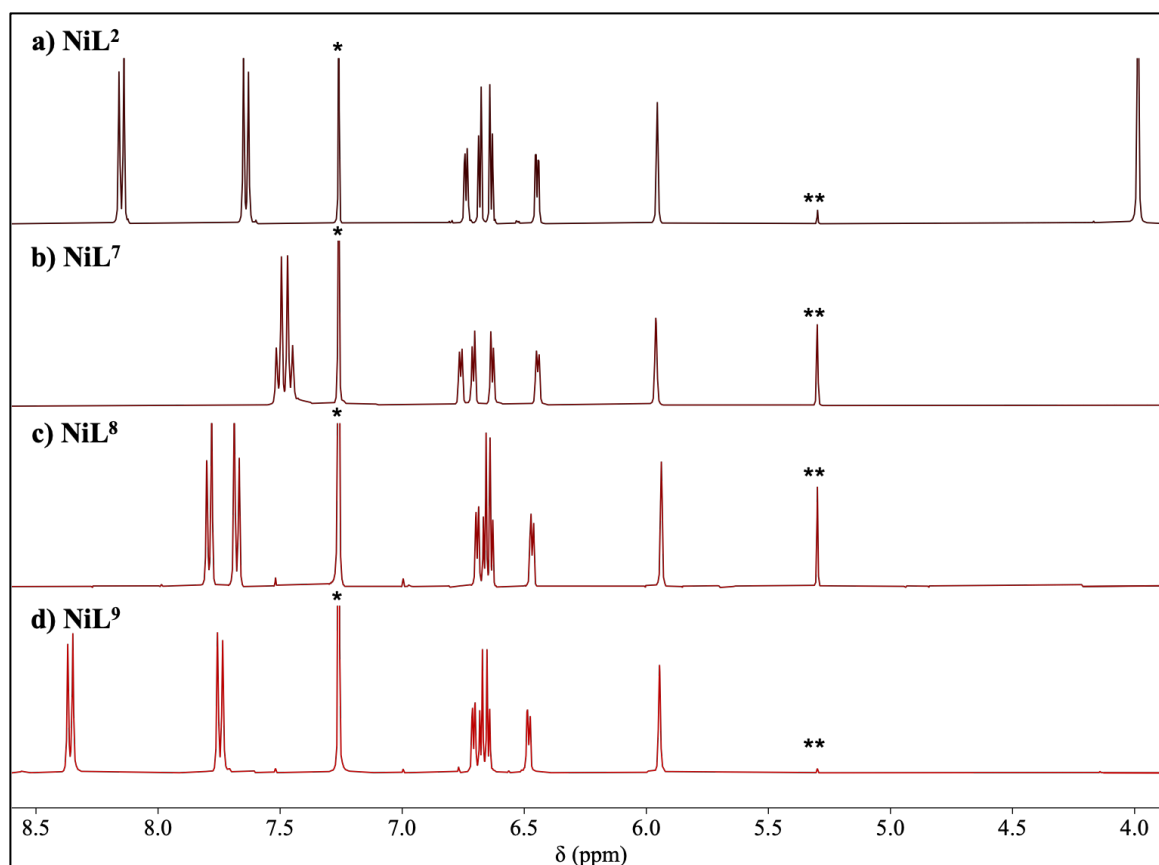
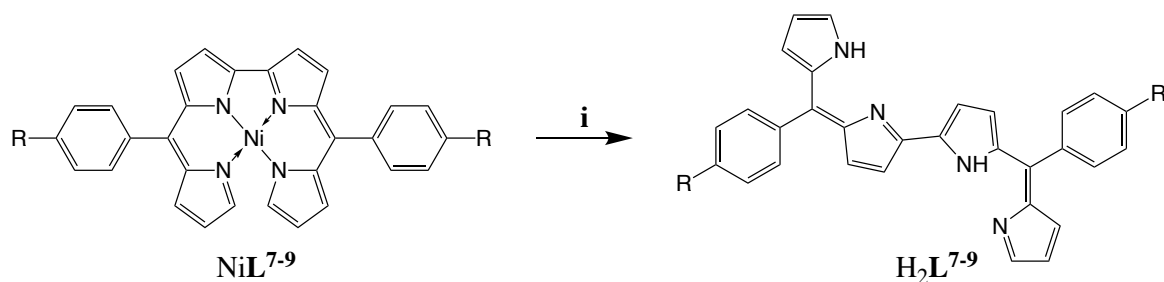


Figure 2.24. ^1H NMR spectra of the NiL complexes in CDCl_3 (400 MHz). a) NiL^2 ; b) NiL^7 ; c) NiL^8 ; d) NiL^9 . *Residual solvent signal. **Residual CH_2Cl_2 .

The peak broadening and deshielding effects observed for the bis(dipyrinato) nickel(II) complexes are no longer apparent. A total of 7 aromatic H-environments are present for each complex, consisting of five pyrrolic $-\text{CH}$ and two phenyl $-\text{CH}$ environments. The shielded pyrrolic $-\text{CH}$ is observed in all cases, showing some degree of structural ubiquity across these complexes. The two phenyl $-\text{CH}$ environments change significantly with the *para*-substituent, indicative of altered electronic inductive effects on the aromatic ring.



Scheme 2.11. General synthesis of H_2L^{7-9} . i) conc. HCl , CH_2Cl_2 .

Following acid-mediated decomplexation (Scheme 2.11), the same terminal pyrrolic proton environment is deshielded to exhibit chemical shifts of approximately δ 7.6 ppm (indicated by X, Figure 2.25). This is consistent with the removal of shielding effects following loss of the Ni(II) ion.

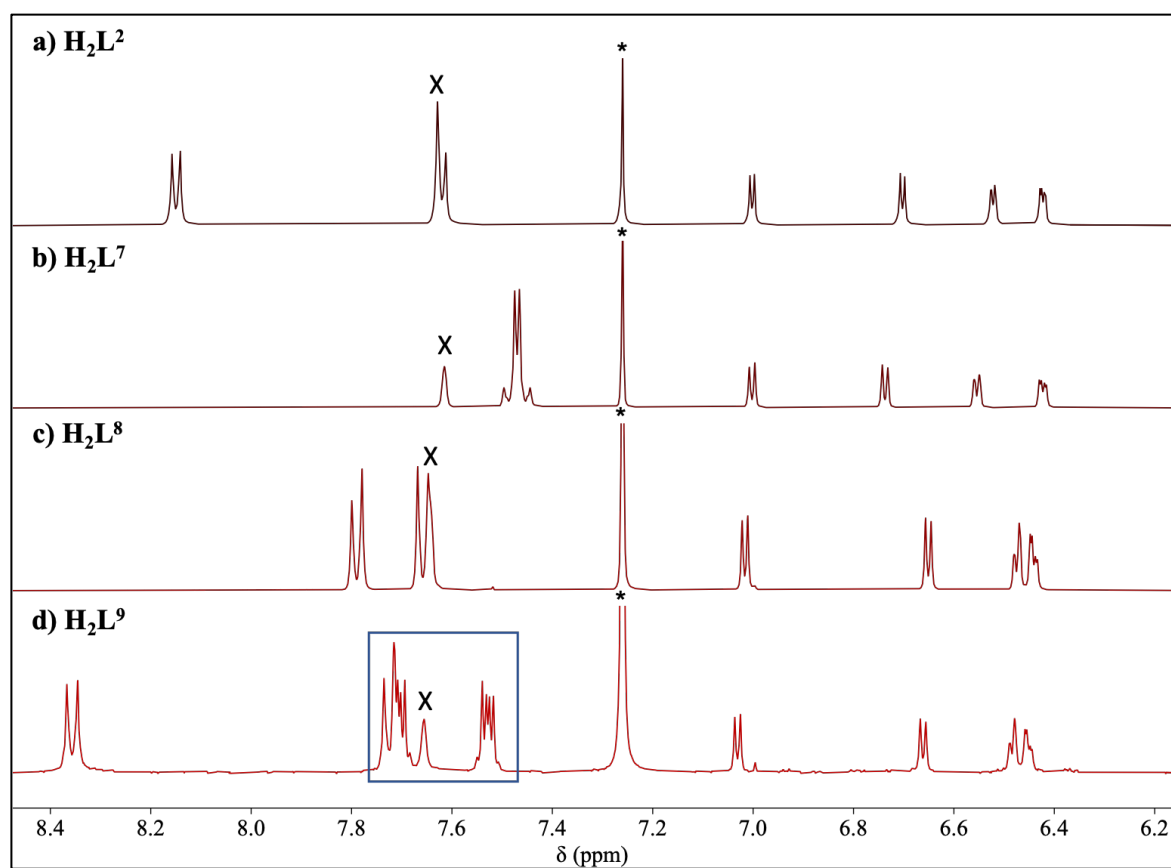


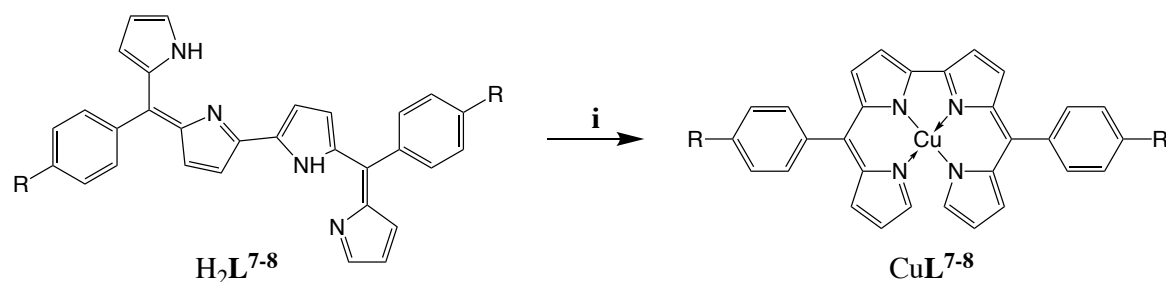
*Figure 2.25. ^1H NMR spectra of the H_2L ligands in CDCl_3 (400 MHz). a) H_2L^2 ; b) H_2L^7 ; c) H_2L^8 ; d) H_2L^9 . * Residual solvent signal.*

2. New Bisdipyrrin Metal(II) Complexes

Unexpectedly, the ligand H_2L^9 shows an impurity that overlaps with the ligand's resonances at δ 7.53 and δ 7.71 ppm. These peaks are shown to emerge during the acid-mediated decomplexation, despite using analytically pure NiL^9 . ESI-HRMS has provided no insight into the nature of this impurity, and no attempts at purification using column chromatography were successful. As such investigation of this ligand was discontinued.

2.5.3 The Copper Complexes, CuL^{7-8}

The copper complexes for each of the ligands were synthesised using the same methodology developed during the previous work with ligands H_2L^2 and H_2L^3 (Scheme 2.12).



Scheme 2.12. General synthesis of complexes CuL^{7-9} . (i) $\text{Cu}(\text{OAc})_2 \cdot \text{H}_2\text{O}$, $\text{CHCl}_3/\text{CH}_3\text{OH}$.

CuL^7 and CuL^8 were isolated as brown solids in 63 and 43% yield, respectively. Due to the paramagnetic nature of the copper(II) complexes, a combination of elemental analysis and ESI-HRMS were used to characterise the reaction products (see Section 7.2.2), which identified the presence of $[\text{CuL}]^+$ in the sample. Although some free ligand was observed, this is unsurprising given the use of acidic solvents in the instrumentation. To verify this, UV-Visible absorption spectroscopy was used, which enabled differentiation of the free ligand and intact complex. The spectroscopic and electrochemical properties of these complexes will be discussed in Sections 2.7 and 2.8.

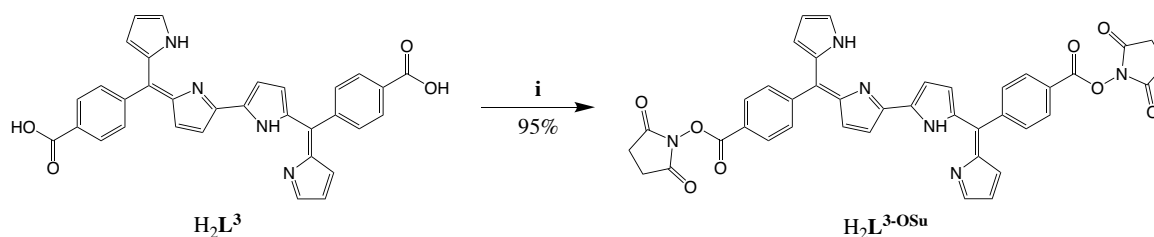
2.6 LIGAND DERIVATISATION THROUGH AMIDATION

The ability to expand on the family of bisdipyrins through amidation is a valuable tool in developing and optimising the development of radiopharmaceuticals. The coordination of the tetrapyrrole core to copper(II) in aqueous solution has been shown to occur rapidly and under mild conditions. Later work will highlight the results of translating this into incorporation of the copper-64 radioisotope.

CuL^2 and its derivatives are highly hydrophobic, accounting for poor solubility in low molecular weight alcohols and water. In the hopes of developing a means of modulating this solubility, short chain amino oligo(ethylene glycols) were selected as small solubilising groups to use in the amidation of H_2L^3 .

This project is focussed on the development of largely lipophilic copper(II) complexes. While increasing the aqueous solubility of these complexes seemingly deviates from this research goal, passage across biological membranes requires a balance in the lipophilicity and hydrophilicity of a complex to avoid membrane-trapping.

Conversion of the free acid groups to an activated succinamidyl ester was achieved using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) in DMF with *N,N*-diisopropylethylamine (DIPEA) and *N*-hydroxysuccinamide (Scheme 2.13).



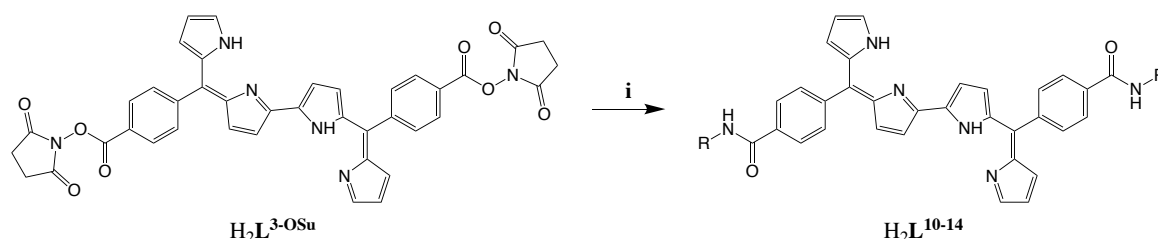
Scheme 2.13. Activation of the diacid ligand, H_2L^3 to the activated ester, $\text{H}_2\text{L}^{3\text{-OSu}}$.

i) N-hydroxysuccinamide, EDC·HCl, DIPEA, DMF.

Following extraction into CHCl_3 , this ligand was isolated in 95% yield, and subsequently used without further purification to react with the desired amine to give the corresponding amide.

2.6.1 Synthesis of amides H_2L^{10-14}

The selection process regarding which amines would be used was based on the trade-off between solubility and molecular weight. Lipophilic amines were not of interest as they are expected to exacerbate the poor water solubility seen for H_2L^2 . Instead, small hydrophilic amines ranging in molecular weight from 61.0 g mol⁻¹ to 149.0 g mol⁻¹ were selected, incorporating a number of hydrogen bond donors and acceptors, and reacted with H_2L^{3-OSu} according to Scheme 2.14.



Scheme 2.14. Amidation of the bisdipyrrin ligand through reaction of H_2L^{3-OSu} with various amines. (i) $R-NH_2$, K_2CO_3 , $CHCl_3$.

Three different oligo(ethylene glycol)amines were chosen to react with the ligand H_2L^{3-OSu} , along with two 2-substituted ethylamine derivatives as indicated in Table 2.10, producing H_2L^{10-14} .

Table 2.10. R-groups and ligand designations for amide-derivatives of H_2L^{3-OSu} .

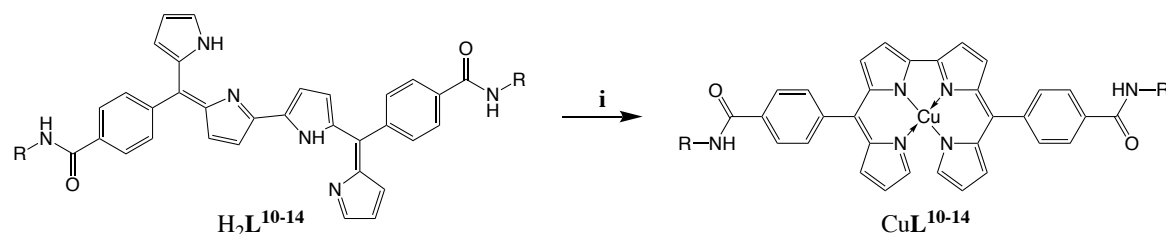
Ligand	R-group	Reaction Yield (%)
H_2L^{10}		63
H_2L^{11}		72
H_2L^{12}		68
H_2L^{13}		66
H_2L^{14}		61

2. New Bisdipyrrin Metal(II) Complexes

Purification of each ligand was achieved by neutralising a solution of the ligand in 1 M HCl with NaHCO₃ and extracting the free base into ethyl acetate (EtOAc). Ligands were synthesised in 61 to 72% yields. These ligands were then used in the synthesis of the corresponding copper(II) complexes.

2.6.2 The Copper Complexes, CuL¹⁰⁻¹⁴

Complexation was achieved in low to moderate yields by the reaction of the ligand in CH₃OH with Cu(OAc)₂·H₂O (Scheme 2.15). In all cases, organic-phase complexation to copper(II) caused precipitation of the desired complex for analysis.



Scheme 2.15 Synthesis of CuL¹²⁻¹⁶. (i) Cu(OAc)₂·H₂O, CHCl₃/CH₃OH.

These complexes were characterised by ESI-HRMS and elemental analysis, confirming isolation of the desired products as variably solvated complexes.

The solubility of CuL¹³ and CuL¹⁴ were noted to be dramatically increased in CH₃OH, while the solubility of CuL¹⁰ was markedly reduced in chlorinated and organic solvents, with no improvement in aqueous solubility. To examine the effects of the aromatic substituent on the solubility of these complexes, the corresponding logD values were determined.

2.6.3 *LogD Determination*

Partition coefficients are an excellent indicator of the ability for drugs and poisons to cross physical barriers in both the laboratory and in biology. A blood/gas partition coefficient, for example, assesses the ability for some gaseous compound, X, to be absorbed into the blood stream after exposure to the gas. This, for example, is particularly useful when considering the delivery of anaesthetics to a patient via inhalation.

Calculation of a partition coefficient relies on the ability to measure the concentration of a compound, X, in each of the two phases, A and B, of interest. Typically, a partition with 1-octanol/H₂O is used to determine the partition coefficient of non-ionisable compounds, which can be calculated according to Equation 2.2.

$$\text{Equation 2.2.} \quad \text{Log}P_{A/B} = \log_{10} \frac{[X]_A}{[X]_B}$$

An important distinction when considering compounds bearing ionisable groups should be made, whereby the pH of the aqueous phase becomes increasingly important. As such, many compounds are examined for their logD, or distribution coefficient, which uses a pH buffered aqueous phase, such as phosphate buffered saline (PBS) instead of water in the classic 1-octanol/H₂O partition experiment. The relevance of pH in biological media makes a logD calculation far more biologically useful when considering translation from chemical synthesis to biological application.

LogD values have been used previously as an estimate of membrane permeability.²⁷ They can be determined using any analytical technique from which a ratio of [X]_{1-octanol}/[X]_{PBS} phase can be ascertained. Such methods include high performance liquid chromatography (HPLC), radiotracer counting techniques and spectroscopic methods including UV-visible spectroscopy.

2. New Bisdipyrin Metal(II) Complexes

The radionuclide counting method incorporates a shake-flask method to partition a radiolabelled compound between the two phases. In the case of metal radionuclides, this protocol is relatively simple assuming no complications are encountered in the radiolabelling process. Many refinements here, including pre-saturation of the 1-octanol with PBS, and *vice versa*, as well as a pre-wash step to reduce the presence, if any, of impurities, improve the accuracy of this protocol.²⁸

Radioactive counting methods crucially rely on accessibility to a useful radionuclide, which is limited by proximity to a source, half-life, and cost. Instead, using the refinements described for the shake-flask method, inductively-coupled plasma mass spectrometry (ICP-MS) will be used herein to measure elemental copper levels in each layer to represent the partitioning of each complex.

ICP-MS involves atomisation and subsequent ionisation of an analyte into its constituent elemental parts using an argon-plasma, before being introduced to a mass spectrometer for detection and processing, providing part per billion, or $\mu\text{g/L}$ detection levels of analyte.

The following analysis involved partitioning each of the copper(II) complexes between 1-octanol and PBS (0.01 M, pH 7.4). Following HNO_3 digestions, the concentration of Cu^{2+} was determined by ICP-MS, and $[\text{Cu}^{2+}]$ in each layer was corrected by subtraction of a blank reference sample. The resulting ratio was converted to the corresponding logD values (Table 2.11).

Table 2.11. LogD ($\pm$ SD) values for various CuL complexes at pH 7.4.

Sample	R-Group	LogD pH 7.4
CuL²	-COOMe	1.64 $\pm$ 0.65
CuL³	-COOH	1.52 $\pm$ 0.16
CuL¹⁰	-CONH-EtOH	1.59 $\pm$ 0.17
CuL¹¹	-CONH-PEG2OH	1.81 $\pm$ 0.25
CuL¹²	-CONH-PEG3OH	2.03 $\pm$ 0.10
CuL¹³	-CONH-EtOMe	1.46 $\pm$ 0.18
CuL¹⁴	-CONH-EtNMe ₂	1.59 $\pm$ 0.25

The variations in logD for each of the complexes based on substituent selection, although small, supports that these complexes may be tailored for different applications. These results indicate a potential increase in lipophilicity for CuL¹² compared to CuL². It is also suggested that CuL¹³ and CuL¹⁴ represent the highest degree of hydrophilicity of these complexes, which is supported by the experimental evidence of improved solubility in CH₃OH. CuL³ and CuL¹⁰ also show improved hydrophilicity, however in practice they exhibit the least solubility in both aqueous and organic solvents.

2.7 ELECTRONIC SPECTROSCOPY

2.7.1 Absorption Spectroscopy

In solution, the ligands are intensely blue/purple, while the corresponding copper(II) complexes uniformly appear dark yellow/brown. Despite a weak solvatochromism effect, H_2L^2 and CuL^2 show absorption bands similar to the Soret (S) band and Q-bands of porphyrins and their complexes described in section 1.1. The UV-visible absorption spectra of H_2L^2 and CuL^2 in DMSO are shown below (Figure 2.26a and Figure 2.26b).

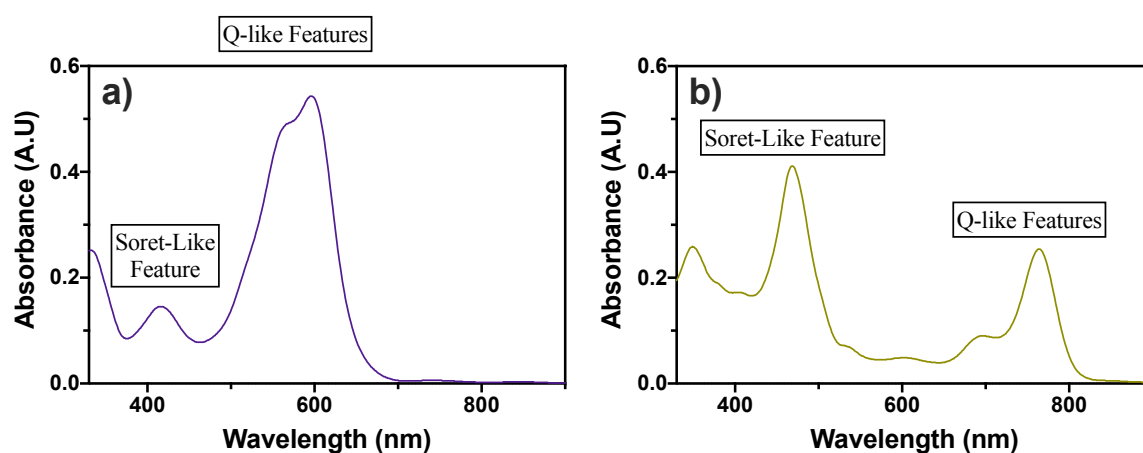


Figure 2.26. Absorption spectra of a) H_2L^2 and b) CuL^2 in DMSO (10 μM).

These porphyrinic features differ in intensity ($\epsilon[\text{Soret}] \gg \epsilon[\text{Q-band}]$) and number of constituent Q-bands, although respond in a similar manner to metal coordination. As such, the analogous bisdipyrrin absorption bands, which are noted to be largely ligand based, will be referred to as the S-like and Q-like features.

Addition of TFA to both H_2L^2 and CuL^2 causes a shift in the absorption spectra associated with protonation (Figure 2.27, green trace).

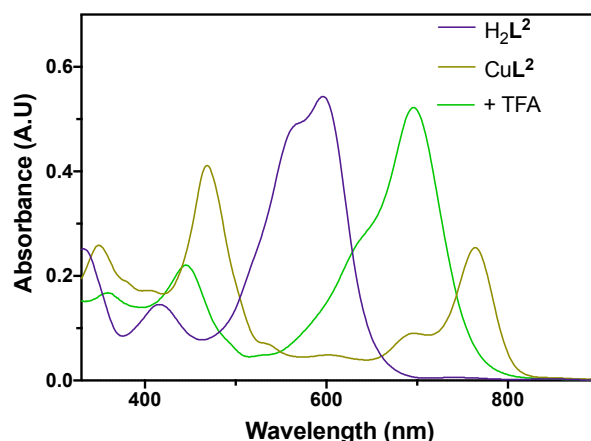


Figure 2.27. Absorption spectra for H_2L^2 and CuL^2 in DMSO ($10\ \mu M$) before and after treatment with TFA (0.5% v/v).

The differences in the Q-like features between ligand and complex enables efficient monitoring of the interchange between the three species in solution. Application to protein and biochemical assays requires the use of buffered aqueous media to maintain physiological pH (7.4), which is hoped to be sufficiently high in pH to prevent formation of the protonated species. This will be discussed in more detail in chapter 3.

Complexation of Cu^{2+} to H_2L^2 results in a bathochromic shift in both the S-like and Q-like features following complexation of the ligand to a copper(II) ion. Analysis of the other bisdipyrrins, H_2L^3 and H_2L^{7-8} , and their copper(II) complexes shows the same red shift following complexation (Figure 2.28). The values for λ_{max} and estimated values of the molar extinction coefficients ϵ ($M^{-1}\ cm^{-1}$) for each of the ligands and complexes are given in Table 2.12.

2. New Bisdipyrrin Metal(II) Complexes

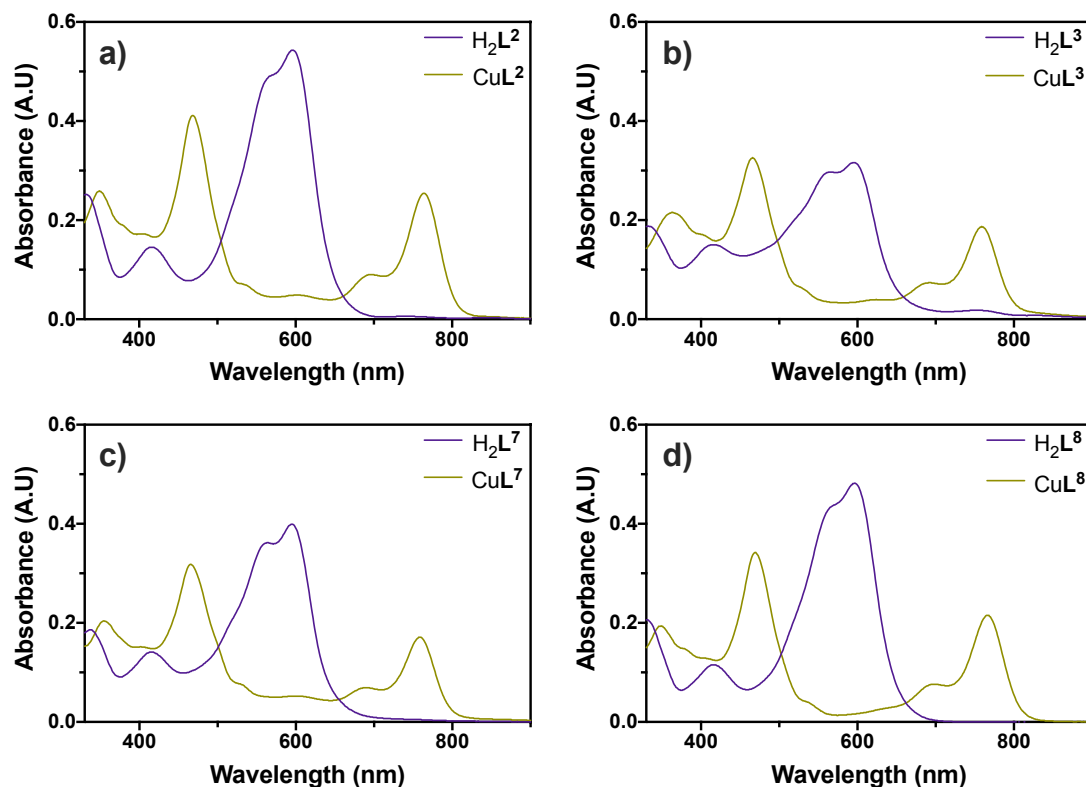


Figure 2.28. UV-Visible absorption spectra for various H_2L and CuL at $10\ \mu M$ in DMSO.
a) L^2 ; b) L^3 ; c) L^7 ; d) L^8 .

Table 2.12. Comparison of λ_{max} for CuL and H_2L for various ligands in DMSO at approximately $10\ \mu M$ concentrations.

		λ_{max} [Q-like Bands] (nm)			λ_{max} [S-like Bands] (nm)		
Ligand	R-Group	H ₂ L	CuL	$\Delta\lambda$	H ₂ L	CuL	$\Delta\lambda$
L ²	- COOMe	596	696	100	416	468	52
		567	764	197			
L ³	- COOH	595	692	97	416	466	50
		565	761	196			
L ⁷	- Cl	599	690	91	414	466	52
		564	759	195			
L ⁸	- CN	596	698	102	416	469	53
		576	766	190			
Approx. ϵ (x 10 ⁴ M ⁻¹ cm ⁻¹)		3.9–5.5	1.5–2.5	–	1.1–1.5	3.2–4.1	–

Analysis of complexes CuL^{10-14} also produced an absorption spectrum consistent with the previous complexes analysed (Figure 2.29).

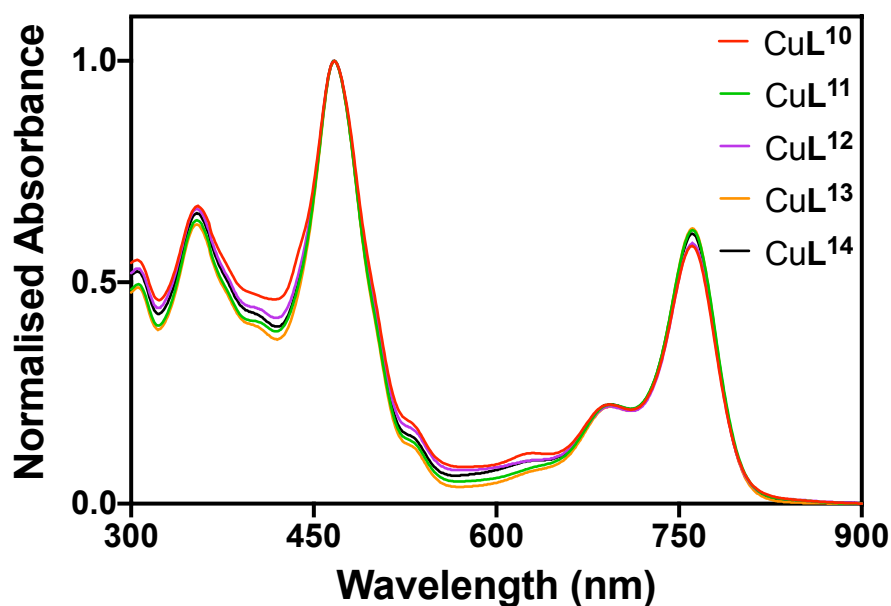


Figure 2.29. Normalised UV-Visible absorption spectra of CuL^{10-14} in DMSO.

2.7.2 Emission Spectroscopy

Previous work by the candidate has shown that H_2L^2 is emissive when excited at the Q-like features in CH_2Cl_2 . Although weak, further analysis of this emission is presented herein. The fluorescence spectroscopy was conducted in acetonitrile (ACN), in which the same previously described Q-like and S-like features are evident, with a slight variation in the λ_{max} for each peak. The emission spectra for H_2L^2 are shown in Figure 2.30. The excitation spectrum shown (purple line) corresponds to emission at 675 nm. The excitation spectrum for emission at 638 nm is coincident at the Q-like features (580 and 550 nm) with no corresponding peak at 406 nm.

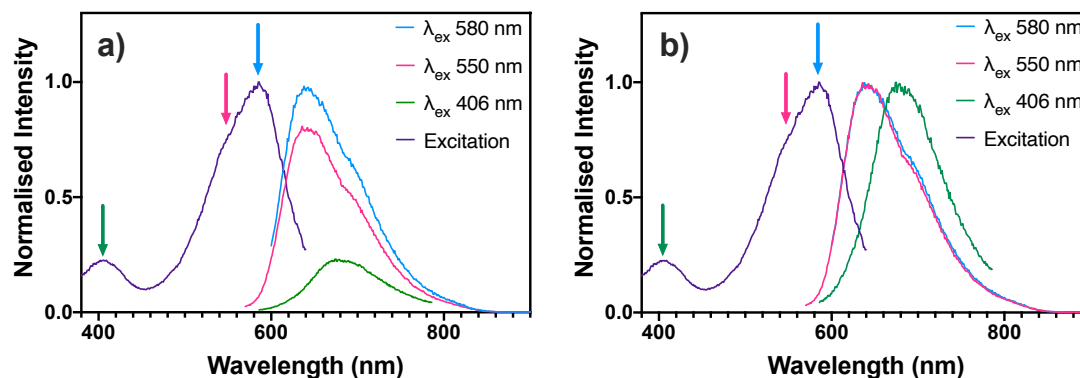


Figure 2.30. Emission (λ_{ex} 580 nm, 550 nm and 406 nm) and excitation (λ_{em} 675 nm) spectra for H_2L^2 in ACN. a) Spectra normalised to intensity of the excitation spectrum in each case to maintain relative intensity. b) Spectra normalised to intensity of the emission spectrum in each case to reflect spectroscopic differences.

Excitation of H_2L^2 at both Q-like bands (550 nm and 580 nm) yields fluorescence with a peak maximum at 638 nm, and a slight shoulder at 694 nm, suggesting non-radiative decay from a higher energy state following absorption at 550 nm to the same fluorescent excited state created following absorption of 580 nm light. Fluorescence is also observed following excitation at the Soret-like band (406 nm), although a corresponding decrease in fluorescent intensity is also evident. This emission exhibits a slight red-shift in the spectrum, with a λ_{max} at 675 nm, which becomes evident on normalisation of the data (Figure 2.30b).

Much like H_2L^2 , the other free base ligands all show fluorescence following excitation at the Q-like band of longest wavelength. The results of these experiments are summarised in Table 2.13. In all cases, the copper(II) complexes proved non-emissive, with any ligand-based fluorescent properties quenched following complexation.

2. New Bisdipyrrin Metal(II) Complexes

Table 2.13. Summary of emission properties of H_2L^2 , H_2L^7 and H_2L^8 , in ACN (sh.: peak is a weak shoulder).

Ligand	R-Group	λ_{ex} (nm)	λ_{em} (nm)	Stokes Shift (cm^{-1})
H_2L^2	- COOMe	580	638 694 (sh.)	1.57×10^3
		550	638 694 (sh.)	—
		406	675	(9.82×10^3)
H_2L^7	- Cl	580	628	1.32×10^3
H_2L^8	- CN	582	639	1.53×10^3

2.8 ELECTROCHEMISTRY

While the 4-coordinate tetradentate ligand is suggested to show improved stability compared to dipyrin and other bidentate analogues, the presence of intracellular reductants such as cysteine-rich metallothioneins have the capacity to lower the intracellular stability of these complexes. Previous works have demonstrated the importance of redox stability in copper(II) complexes for *in vivo* selectivity and stability.²⁹⁻³⁰ A reduction potential of -0.85 V for a $\text{Cu}^{2+}/\text{Cu}^+$ process versus the ferricenium/ferrocene couple (vs. Fc^+/Fc) can inhibit reductive liberation of copper into the intracellular environment.

The ability of Cu(II) in CuL^2 to resist reduction to Cu(I) is an important consideration in considering *in vivo* stability. Resisting the copper(I) scavenging properties of intracellular reductants requires the design of kinetically and thermodynamically stable, as well as electrochemically inert complexes to inhibit reductive decomplexation.

The analogous metal complexes discussed in Section 1.3 incorporating the dimethyloctaethyl-2,2'-bisdipyrrin (Figure 2.31) exhibit interesting electrochemistry, ascribed as ligand-based processes influenced by metal ion coordination.¹ The fully substituted bisdipyrrin complexes are capable of stabilising the formation of radicals, leading to a high degree of electrochemical reversibility.

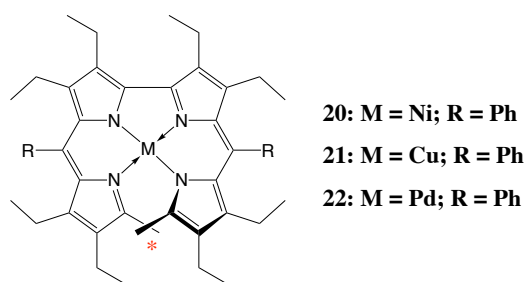


Figure 2.31. Structures of Ni(II), Cu(II) and Pd(II) complexes of the 10,10'-dimethyl-3,3',4,4',8,8',9,9'-octaethyl-6,6'-diphenyl-2,2'-bisdipyrrin ligand.^{1, 31} * Indicates C10-CH₃ and C10'-CH₃ steric clash.

The electrochemistry of (2,2'-bisdipyrrinato) copper(II) and palladium(II) with only *meso*-substituents have not been previously explored and were therefore investigated in this work using cyclic voltammetry. Reported peak potentials are given as midpoint potential (E_m) values, where $E_m = (E_{ox} + E_{red})/2$, or peak potentials, E_p , in the case of irreversible processes. Peak potential separation, ΔE , were calculated using $\Delta E = E_{ox} - E_{red}$. All potentials are reported versus the ferricenium/ferrocene couple ($Fc^+/Fc = 0.00$ V), which was used as an internal standard for all analyses.

2.8.1 Extending the Electrochemical Understanding of H_2L^2 and CuL^2

Analysis of H_2L^2 was conducted in dry DMF using tetrabutylammonium hexafluorophosphate, $[Bu_4N]PF_6$ as the electrolyte (Figure 2.32).

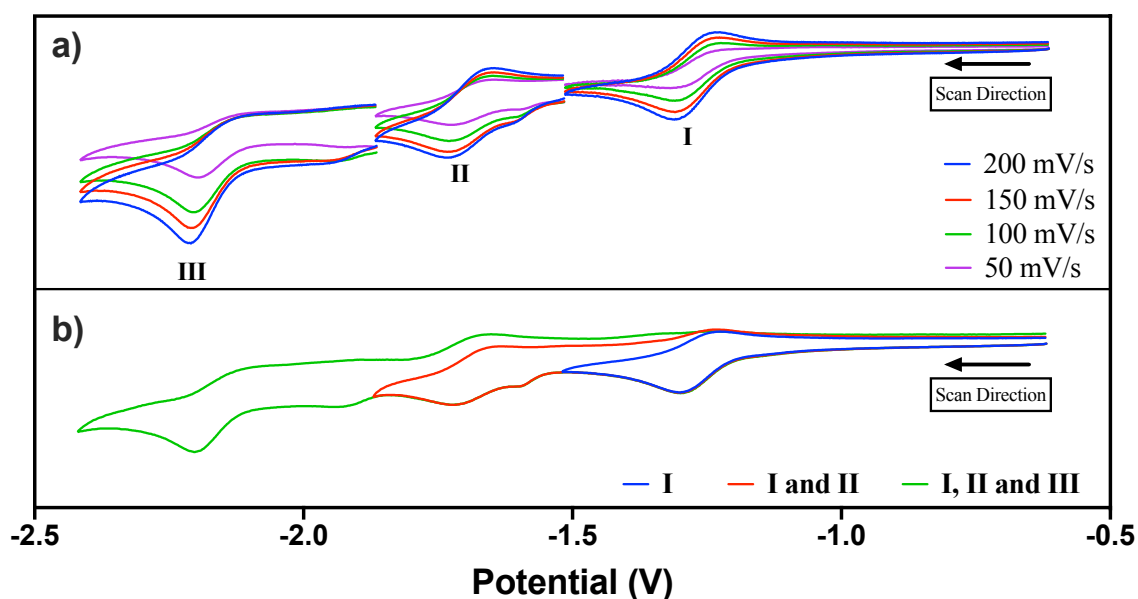


Figure 2.32. Cyclic voltammograms for H_2L^2 at approximately 1 mM, in dry deoxygenated DMF with 100 mM $[Bu_4N]PF_6$ (vs. Fc^+/Fc) a) Individual scans for processes I, II and III (as indicated) at various scan rates. b) Scans of Process I (blue), processes I and II (red) and processes I, II and III (green) at 100 mV/s.

Process III ($E_p = -2.20$ V) is tentatively assigned to the irreversible reduction of the methyl ester group. This process is strongly influential on the reversibility of processes I

($E_m = -1.27$ V) and II ($E_m = -1.69$ V), as seen in Figure 2.32b, in which there is an apparent loss of the return oxidation wave corresponding to process I.

The waves evident at oxidising potentials (> 0.2 V vs. Fc^+/Fc) are assigned to oxidation of the tetrapyrrole (Figure 2.33).

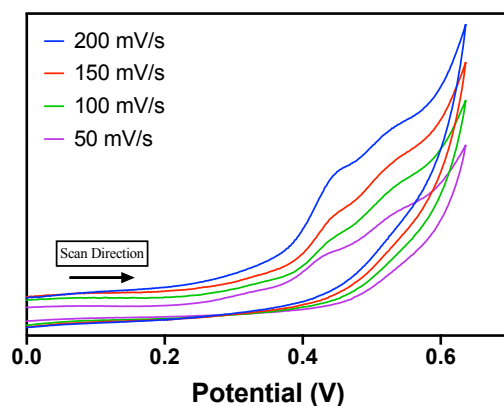


Figure 2.33. Cyclic voltammograms for the oxidation processes of H_2L^2 at various scan rates.

The cyclic voltammogram of CuL^2 reveals three quasi reversible electron-transfer processes at negative potentials vs. Fc^+/Fc (Figure 2.34) at $E_m -1.09$ V (process I), -1.83 V (process II) and -2.23 V (process III).

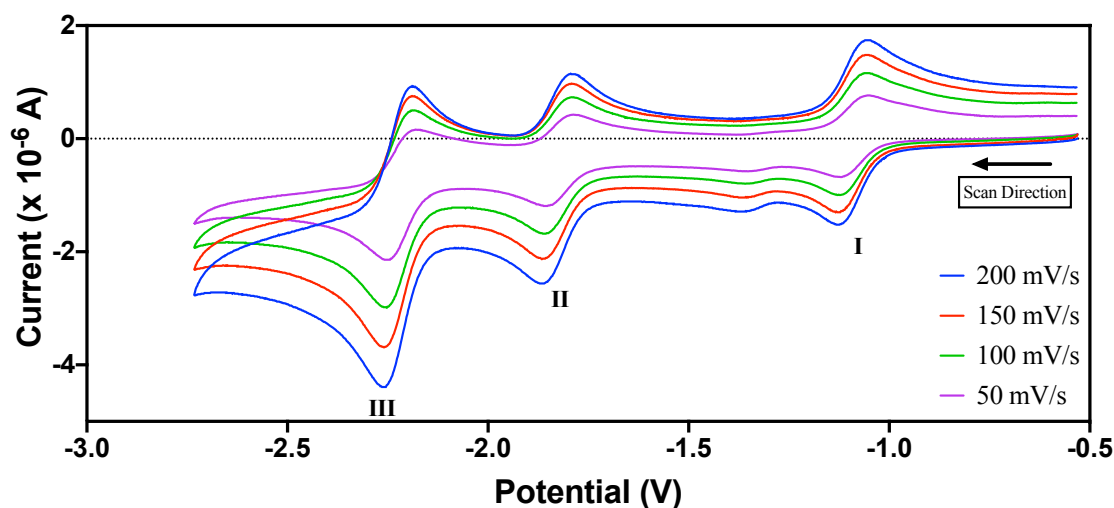


Figure 2.34. Cyclic voltammogram for CuL^2 at approximately 1 mM, in dry deoxygenated DMF with 100 mM $[\text{Bu}_4\text{N}]\text{PF}_6$.

Under the application of oxidising potentials, the copper(II) complex displays an irreversible process with an E_p of +0.278 V, tentatively assigned as a Cu(II)/Cu(III) process, although it is likely not independent of the analogous ligand process (Figure 2.35a).

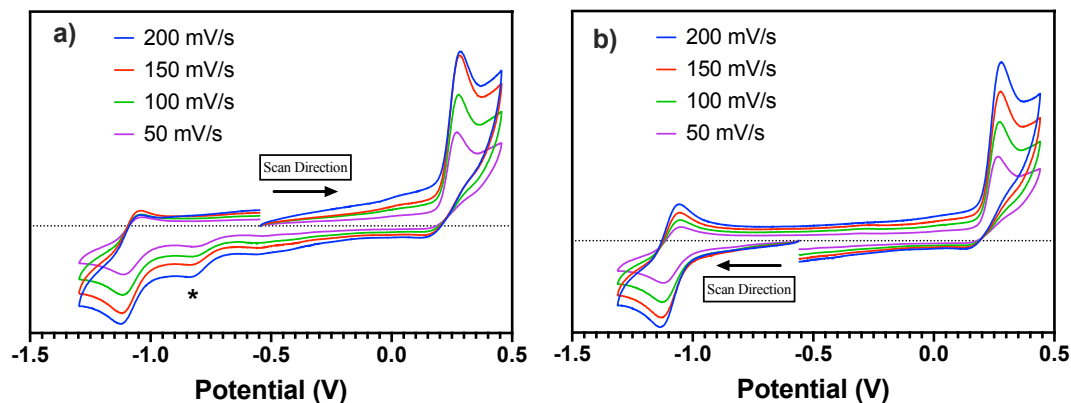


Figure 2.35. Cyclic voltammograms of CuL^2 incorporating process I (-1.90 V) and the oxidation process (0.278 V). a) Forward scan direction: left to right. * indicates the reduction process associated with the oxidation process. b) Forward scan direction: right to left.

The return reduction peak for the products of this electrochemical/chemical transformation can be observed at approximately -0.84 V (Figure 2.35a; *). Scanning at faster scan rates does not result in improved reversibility, suggesting that this is an electrochemically irreversible (E_i) process. In addition, the irreversibility of this process seemingly reduces the apparent reversibility of process I ($E_m -1.09$ V). This may be a result of adsorption of the oxidised species to the electrode, or else a chemical change of the species undergoing reduction. Reversing the scan direction (Figure 2.35b) removes any clear relationship between the two processes, suggesting an electrochemical-chemical electrochemical (ECE) mechanism dependent on the irreversible oxidation process of the complex, in which the chemical product of the first electron transfer process is electrochemically active.

2.8.2 Electrochemical Comparison of PdL^2 to H_2L^2 , NiL^2 and CuL^2

To gain better insight into this electrochemistry, the palladium complex was used as a redox-innocent metal analogue. It has already been shown that the PdL^2 complex shows a high degree of structural similarity to the copper(II) complex, with only a slight difference in the observed deviation from true square-planar coordination geometry.

While palladium(II) complexes are relatively well known, as are the analogous nickel(II) and platinum(II) complexes, the exploration of these metals in their monovalent oxidation states is still in their infancy. Palladium metal, or $\text{Pd}(0)$, is unusual in its electron configuration. The intrinsic stability of a full subshell over partially filled subshells results in a $[\text{Kr}]4\text{d}^{10}$ electronic configuration rather than the expected $[\text{Kr}]4\text{d}^85\text{s}^2$. As a d^{10} metal, $\text{Pd}(0)$ exhibits no crystal field stabilisation due to the equal and complete occupancy of the 4d orbitals, and therefore the coordination environment shows no metal-based preference and instead relies largely on the ligands.

Unlike copper(I), palladium(I) is postulated to form paramagnetic complexes due to the presence of a single unpaired electron in the 4d-subshell. This makes it analogous to the valence shell configuration of $\text{Cu}(\text{II})$, and as such shows a similar propensity to form square-planar coordination complexes due to *Jahn-Teller* distortions. A comprehensive review in the 1980's, however, highlighted the presence of a single known mononuclear palladium(I) complex with the 1,2-dicyanothiolene ligand (Figure 2.24).³²

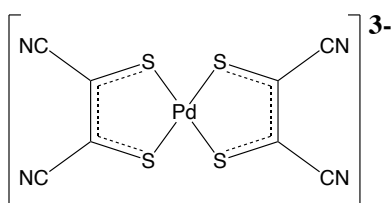


Figure 2.36. Structure of the palladium(I) bis(dithiolene) anion, stabilising an otherwise unstable palladium(I) ion.

2. New Bisdipyrrin Metal(II) Complexes

In every other case explored by this review, the palladium(I) complexes were either dinuclear or polynuclear in nature, and often with direct Pd–Pd bonds. This coincides with the observation that these complexes exhibit diamagnetic properties, which suggests the formation of a σ -bond between the two metal centres and a pairing of the electrons. Mononuclear monovalent palladium complexes have been theorised by Density functional theory (DFT) calculations, and evidenced to exist, at least transiently, through extensive stabilisation of a coordinatively saturated Pd(0) centre through the synthesis of a tetrakis(silyl)palladium(0) complex.³³ These complexes showed two distinguishable one-electron quasi-reversible couples at -0.77 and -0.62 V (in THF vs. Fc^+/Fc), suggesting a stepwise oxidation from Pd(0) to Pd(II) *via* Pd(I). Other works investigating the electrochemical oxidation of Pd(0) highlighted a correlation between scan rate and the accessibility of Pd(I) in what was defined as an endergonic, and therefore disfavoured, one-electron reduction process, followed rapidly by a geometric rearrangement before liberation of a Pd(II) species after a second one electron oxidation.³⁴

The limited ability for the PdL^2 complex to form Pd–Pd bonds and undergo structural rearrangement suggests that a single electron reduction process would therefore be disfavoured, again likely to be an endergonic process which would rapidly reduce further to the Pd(0) counterpart. With this in mind, the palladium bisdipyrrin complex was investigated for any electrochemical processes as a means of comparison to both the free ligand and copper(II) complex (Figure 2.37).

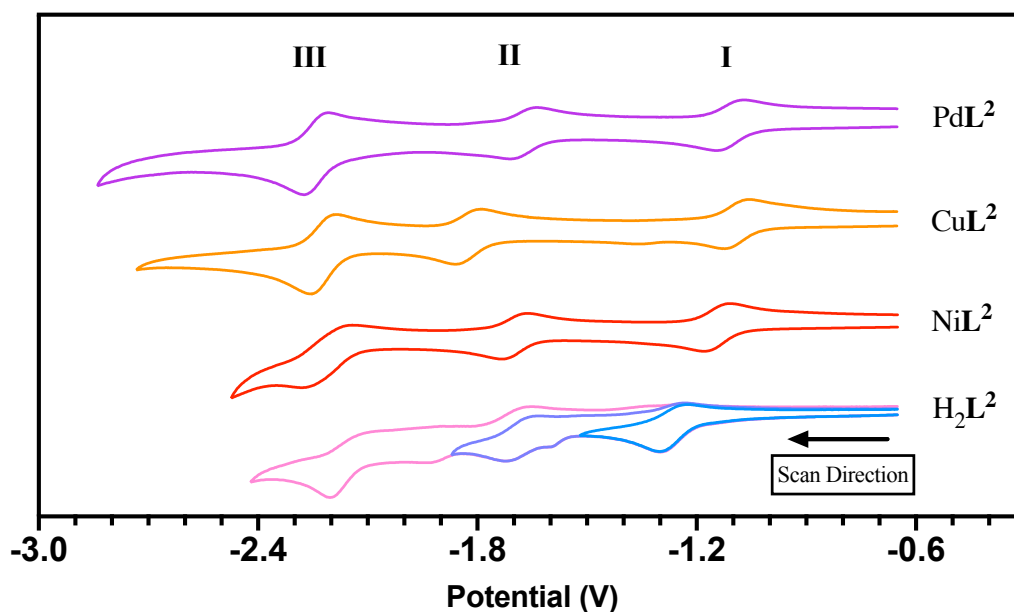


Figure 2.37. Cyclic voltammograms of H_2L^2 , NiL^2 , CuL^2 and PdL^2 at approximately 1 mM in dry, deoxygenated DMF using 100 mM $[\text{Bu}_4\text{N}]\text{PF}_6$ as co-analyte. Scans shown were conducted at 100 mV/s scan rates and referenced to $\text{Fc}^+/\text{Fc} = 0.00$ V (1 mM internal reference).

The lack of structural variation between the copper(II) and palladium(II) complex and high correlation between the cyclic voltammograms supports assignment of each process as largely ligand-based redox changes. This is further confirmed when considering the complex NiL^2 , in which the cyclic voltammogram is analogous to that of the palladium(II) complex (Figure 2.37). The individual process data is summarised in Table 2.14, along with the $\text{I}_{\text{pa}}/\text{I}_{\text{pc}}$ ratio from the corresponding plots of I_{pa} (anodic peak current) or I_{pc} (cathodic peak current) (μA) against $(\text{Scan Rate})^{1/2}$ ($\text{V}^{1/2}$).

Table 2.14. Summary of the electrochemical data for H_2L^2 , CuL^2 and PdL^2 . $|I_{pa}/I_{pc}|$ calculated from the ratio of gradients from the plot of $I_{pa/c}$ (μA) against $(\text{Scan Rate})^{1/2}$ ($V^{1/2}$).

Process	Compound	E_m (V)	ΔE (mV)	$ I_{pa}/I_{pc} $
	Ferrocene	0.00	64	1.05
Ox	H_2L^2	$> 0.40^*$	—	—
	NiL^2	0.387*	—	—
	CuL^2	0.278*	—	—
	PdL^2	0.386*	—	—
I	H_2L^2	−1.27	77	—
	NiL^2	−1.15	73	1.04
	CuL^2	−1.09	69	1.37
	PdL^2	−1.11	67	0.82
II	H_2L^2	−1.69	79	3.11
	NiL^2	−1.69	61	0.95
	CuL^2	−1.83	75	1.02
	PdL^2	−1.67	67	0.77
III	H_2L^2	−2.20*	—	—
	NiL^2	−2.21	150	—
	CuL^2	−2.23	74	0.77
	PdL^2	−2.24	79	1.15

*Value is a peak potential, E_p .

Process III is assigned to be a two-electron reduction process, based on the increased peak heights, involving the methyl ester group with an E_m of ca. −2.23 V in all cases. Reversibility is distinctly diminished for the nickel(II) complex and the free ligand, corresponding to changes in the EC (electrochemical-chemical) mechanism following change of the metal ion.

The two one-electron processes, I and II, are, as a result of the high correlation between the three complexes and the relative shift following complexation by the free-base ligand, ligand-based in character. Process I displays quasi-reversible characteristics, and is evidently independent of the metal ion present, with an E_m of −1.1 V in each of the

complexes. Its difference from the free base ligand (-1.27 V) is hypothesised to be a result of the conformational changes and the difference in highest occupied and lowest unoccupied molecular orbitals (HOMO and LUMO) energy levels before and after complexation. It is accordingly concluded that this one-electron transfer process is centred on the tetrapyrrolic backbone and presumed to be minimally affected by the aromatic ring substituents.

2.8.3 4-Phenyl Substituent Effects on Electrochemistry

To verify the effect of these distal substituents, the copper(II) complexes CuL^3 ($R = -\text{COOH}$), CuL^7 ($R = -\text{Cl}$) and CuL^8 ($R = -\text{CN}$) were also analysed. The cyclic voltammograms of CuL^2 , CuL^7 and CuL^8 for process I, II and III at 100 mV/s are shown in Figure 2.38. CuL^3 is not shown due to poor scan quality, a result of exceedingly poor solubility and the irreversibility of the processes.

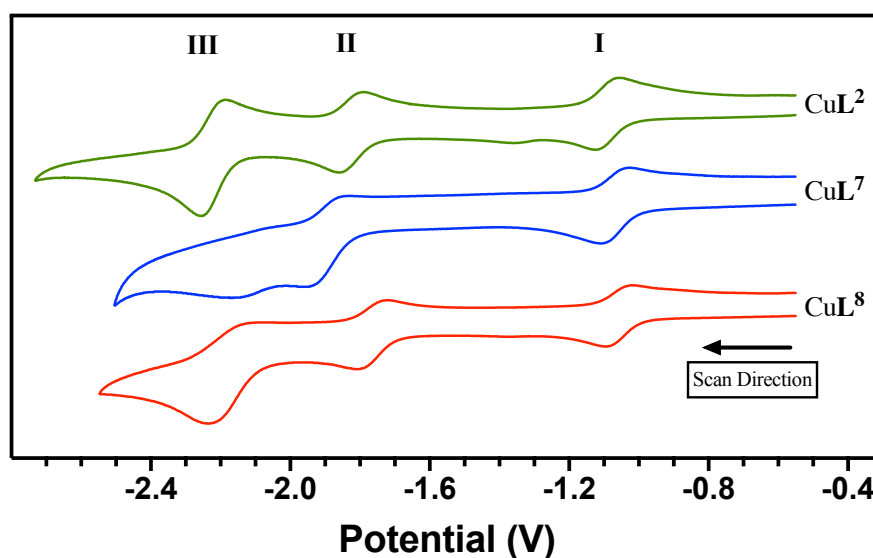


Figure 2.38. Cyclic voltammograms for the complexes CuL^2 , CuL^7 and CuL^8 at 100 mV/s.

The consistency of processes I and II in terms of reduction potential across the copper(II) complexes is evident, the values of which are summarised in Table 2.15, including the values for CuL^2 discussed previously.

Table 2.15. Summary of the electrochemical data for a variety of substituted (2,2'-bisdipyrrinato) Cu(II) complexes.

Compound	Substituent	PROCESS I		PROCESS II	
		E_m (V)	ΔE (mV)	E_m (V)	ΔE (mV)
CuL²	-COOMe	-1.09	69	-1.83	75
CuL³	-COOH	-1.15	> 80	-2.07	73
CuL⁷	-Cl	-1.11	71	-2.00	–
CuL⁸	-CN	-1.06	68	-1.77	65

From the corresponding Hammett plot using the E_m/E_p values for **CuL²**, **CuL⁷** and **CuL⁸**, and reported Hammett constants, σ ,³⁵⁻³⁶ a linear correlation exists for both process I and the irreversible oxidation process of each complex ($R^2 \sim 0.95$) (Figure 2.39). Despite a linear relationship, a diminishingly small gradient (< 0.05 V/[2 σ]) indicates a weak influence of the substituent's inductive effects on the measured E_m values. Process III is tentatively assigned a result of the redox non-innocence of the phenyl ring *para*-substituents. Results from the analysis of the Cu(II), Ni(II) and Pd(II) complexes of **L²** indicate that process II is heavily dependent on the metal ion and its interactions with the ligand, and supporting evidence from the Hammett plot for process II indicates an appreciable influence of the *para*-substituents on the measured E_m values (~ 0.27 V/[2 σ]).

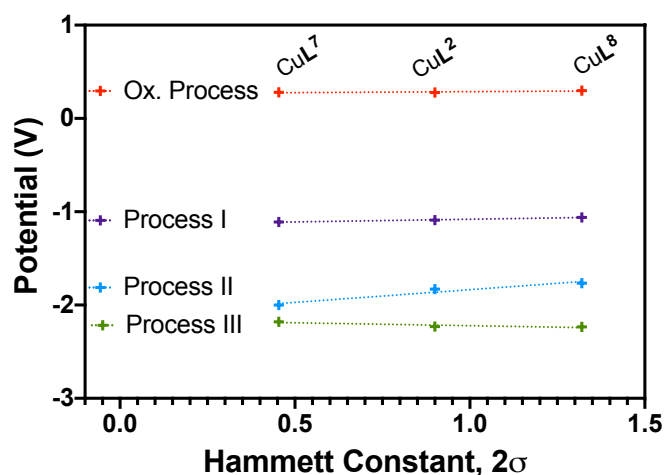


Figure 2.39. Changes in redox potential for processes I, II and III, and the oxidising process for **CuL²**, **CuL⁷** and **CuL⁸** with Hammett constants, 2σ .³⁵⁻³⁶

The diminishingly small gradients here reflect the structural data presented in section 2.4. The observed torsion between the phenyl ring and the dipyrin motif is indicative of the poor electronic distribution between the groups. The inductive effects of the *para*-substituent on the phenyl ring therefore exhibit the largest effect on the electrochemistry within the phenyl rings (Process II).

2.8.4 Electrochemistry of CuL^{10-14}

Expanding on the previous electrochemistry, the cyclic voltammograms of complexes CuL^{10-14} were also acquired (Figure 2.40).

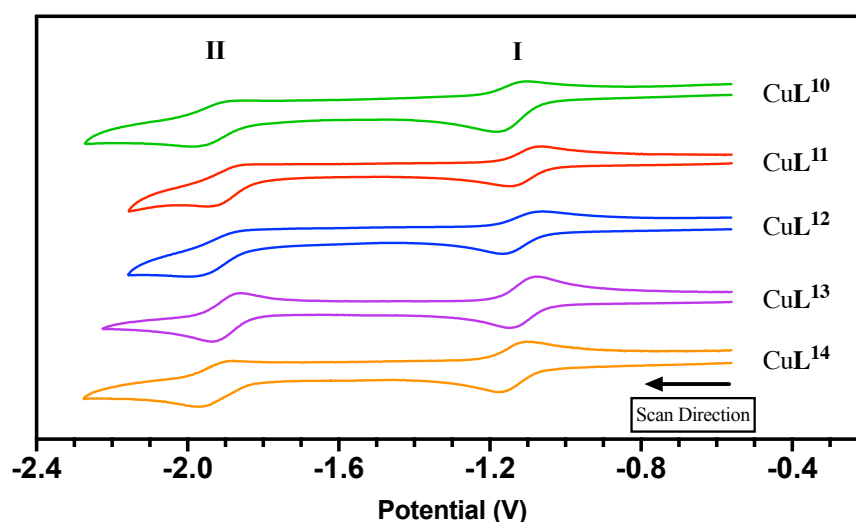


Figure 2.40. Normalised cyclic voltammograms for the complexes CuL^{10} , CuL^{11} , CuL^{12} , CuL^{13} and CuL^{14} at 100 mV/s at approximately 1 mM in dry, deoxygenated DMF using 100 mM $[\text{Bu}_4\text{N}]\text{PF}_6$ as co-analyte. Scans shown were conducted at 100 mV/s scan rates and referenced to $\text{Fc}^+/\text{Fc} = 0.00 \text{ V}$ (1 mM internal reference).

These complexes were expected to display similar electrochemistry due to the retention of the N-substituted benzamidyl group at the *meso*-position of the bisdipyrrin ligand. Indeed, processes I and II are highly conserved with respect to their E_m values (Table 2.16).

Table 2.16 Summary of the electrochemical data for a variety of substituted (2,2'-bisdipyrrinato) Cu(II) complexes.

Compound	PROCESS I		PROCESS II	
	E _m (V)	ΔE (mV)	E _m (V)	ΔE (mV)
CuL¹⁰	−1.14	65	−1.99*	–
CuL¹¹	−1.11	73	−1.95*	–
CuL¹²	−1.12	79	−2.00*	–
CuL¹³	−1.11	88	−1.90	80
CuL¹⁴	−1.14	69	−1.92	73

*Value is a peak potential, E_p.

Variability in the reversibility of the observed processes is evident. For CuL¹⁰⁻¹², process II shows diminishing peak current intensity of the return oxidation wave, while the same processes in CuL¹³⁻¹⁴ appear quasi-reversible.

Additional and increasingly complex processes are observed at potentials below −2.0 V. The added complexity on increasing oligo(ethylene glycol) length is assigned a result of the additional ether oxygens facilitating further electron-transfer processes.

Of the utmost importance herein is the consistency in the E_m value for process I. In all copper(II) complexes, this value is approximately −1.1 V vs. Fc⁺/Fc. The identity of the peripheral substituents on the phenyl rings exhibits minor effects on this process, regarded as a result of the distance from the site of this one-electron process. Based on previous reports, it is therefore expected that these complexes would be able to resist intracellular reduction.²⁹⁻³⁰

2.9 SUMMARY

The work presented in this chapter has described the synthesis and characterisation of a family of new copper(II) complexes incorporating 2,2'-bisdipyrrin ligands. A number of derivatives of H_2L^2 were characterised, demonstrating the minimal effects peripheral substitution has on the spectroscopic and electrochemical properties. Differences in lipophilicity, approximated by their respective logD values, and marked differences in solubility were achieved through incorporation of a variety of different *N*-substituted benzamidyl ligands and complexes. Despite changing peripheral substitution, the copper(II) complexes all show conservation of a one-electron transfer process at approximately -1.1 V, suggesting these complexes would be able to resist the intracellular reducing environment. The stability of these copper(II) complexes in the presence of high-affinity competitors and reducing agents will be explored in Chapter 3.

2.10 REFERENCES

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3 ASSESSING THE STABILITY OF CuL²

3.1 CHAPTER AIMS

The bisdipyrrin family of ligands synthesised herein form their corresponding copper(II) complexes under mild conditions in organic solvents. Electrochemistry measured using cyclic voltammetry suggests a degree of redox stability when translating these complexes into biological assays, and the variation in logD values based on substituent selection supports that these complexes may be tailored to cross biological membranes. Previous reports, however, have described the instability of complexes analogous to CuL² in the presence of acid,¹ which has since been exploited as a means of affording the free ligands synthetically.²⁻³

While rapid complexation kinetics are favourable for use in targeted PET imaging, stability in a biological system can affect the pharmacokinetics and pharmacodynamics of the metal complex and radionuclide. Until now, no evidence for the stability of these complexes in the presence of high affinity competitors for copper(II) has been reported. The following work examines the complexation and stability of the respective copper(II) complexes in aqueous solvent systems. The use of bovine serum albumin (BSA) as a solubilising agent will be investigated, and the impact of a large protein on the complex with respect to both the stability and reactivity of CuL² will be discussed.

3.2 STABILITY OF CuL^2 IN SOLUTION

3.2.1 Copper Titration and Extinction Coefficient Calculation

H_2L^2 and CuL^2 were analysed in HEPES buffer (10 mM pH 7.4) using 10% DMSO to maintain solubility [HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)]. The observed spectral features were similar to those observed in neat DMSO, with conservation of the complementarity of absorption. Buffers with a pH below 5 were found to effect decomplexation, demonstrating a working pH of > 5 is required to maintain stability.

A titration of Cu(II) into a solution of H_2L^2 (ca. 10 μM) in 10% DMSO in HEPES buffer (10 mM, pH 7.4) revealed four distinct isosbestic points, indicative of the direct conversion from one species to another, suggesting reaction of the ligand with Cu(II) leads to the formation of a 1:1 complex (Figure 3.1, Table 3.1). Spectra were acquired within 2 hours due to evident precipitation at longer time points, although spectra collected immediately post-addition highlight instantaneous complex formation.

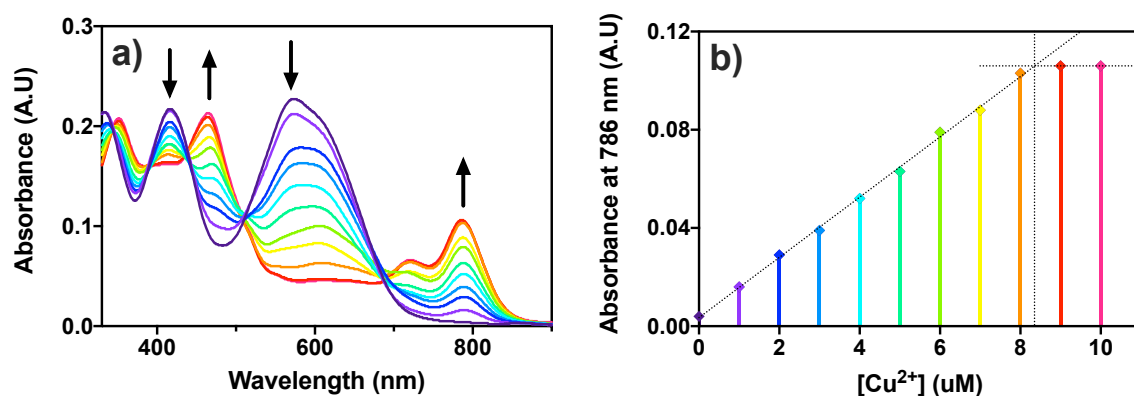


Figure 3.1. a) UV-Visible spectra for H_2L^2 (10% DMSO in 10 mM HEPES buffer, pH 7.4) following titration with Cu^{2+} (AAS Standard; HNO_3). b) Change in absorbance with increasing $[\text{Cu}^{2+}]$ at 786 nm.

*Table 3.1. Wavelengths ($\lambda_{\max}/\text{nm}$) and extinction coefficient ($\epsilon/M^{-1} \text{ cm}^{-1}$) values for H₂L² and CuL² (10% DMSO in 10 mM HEPES buffer pH 7.4). *Value for shoulder.*

	Near-UV		S-Band		Q-bands			
	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$
H₂L²	333	2.56 x 10 ⁴	416	2.60 x 10 ⁴	573	2.72 x 10 ⁴	616*	2.36 x 10 ⁴
CuL²	351	2.49 x 10 ⁴	464	2.55 x 10 ⁴	721	0.79 x 10 ⁴	786	1.27 x 10 ⁴

Introducing the same sample of CuL² into ammonium acetate buffers at both pH 5 and pH 8 indicates no decomplexation of the complex over 2 hours, revealing the complex is, at the very least, definitively stable within the pH range of 5 to 8 over short time intervals, which incorporates physiological pH.

3.2.2 Kinetic Challenges of CuL²

Application of metal complexes to cells and animals is challenged by the multitude of high-affinity metal sinks within the cell or organism of interest. In single cells, the intracellular environment hosts a number of high-affinity peptides and proteins with non-specific metal binding sites, as well as many small molecule chelators that may exploit redox functions to extract a metal from the exogenous complex being introduced.⁴

We have assessed the stability of CuL² in the presence of the tripeptide glutathione (GSH), cysteine methyl ester (Cys.OMe), histidine methyl ester (His.OMe) and ethylenediamine tetraacetic acid disodium salt (Na₂H₂EDTA, hereafter EDTA).

GSH is a tripeptide (Figure 3.2) present in cells at average concentrations of 2 mM. It is primarily a regulator of cell oxidative stress through its interaction with various enzymatic reductants and subsequent oxidation to reduced dimeric glutathione, GSSG.⁵

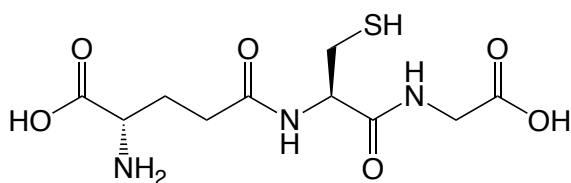


Figure 3.2. Structure of GSH, an endogenous intracellular antioxidant.

The free thiol group and comparatively larger cytosolic concentrations compared to other endogenous small chelators makes GSH an efficient chelator for free metal ions, including copper(I) ($K_D = 9 \times 10^{-12}$ M at pH 7.5 and 25°C)⁶ and zinc(II).⁷

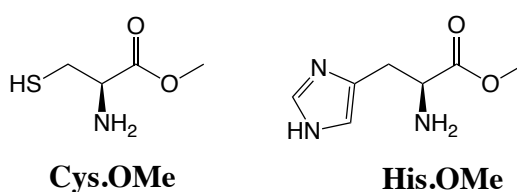


Figure 3.3. Structure of cysteine and histidine methyl esters.

Cys·OMe and His·OMe (Figure 3.3) were also selected to challenge the complex due to their prevalence in protein metal binding pockets.⁸

EDTA^{4-} (Figure 3.4) is a non-specific high affinity metal chelator, with an association constant, K_A , of $3.2 \times 10^{15} \text{ M}^{-1}$ at pH 7.0 for the formation of the copper(II) complex, $[\text{Cu}(\text{EDTA})]^{2-}$.⁹ Its indiscriminate binding to various metal ions and the highly stable chelate that forms makes it an excellent competitor for metal binding.

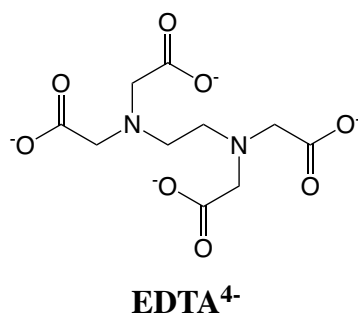


Figure 3.4. Structure of the tetraanionic chelator, EDTA^{4-} .

3. Assessing the Stability of CuL²

A preformed sample of CuL² in DMSO was treated with each competitor, with a final competitor concentration of 500 μ M. Spectroscopic changes are seen over the one hour analysis window (Figure 3.5a), constituting a slight variation in peak λ_{max} and intensity, tentatively attributed to weak axial interactions at the copper(II) centre. In all cases, after one hour of the challenge experiment, no significant change to the spectra is seen (Figure 3.5b). In addition, heating the EDTA challenge experiment to both 37°C and 80°C for 1 hour yielded no decomplexation (data not shown).

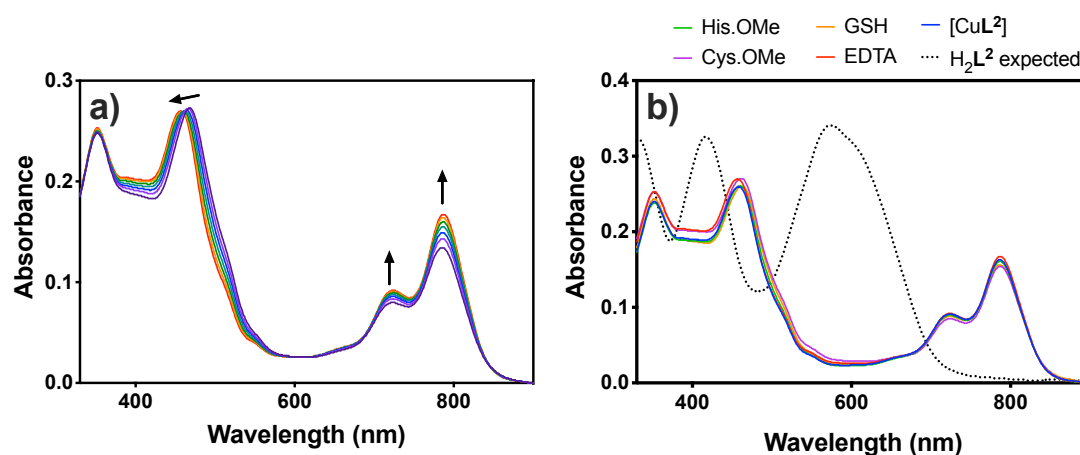


Figure 3.5. UV-Visible absorption spectra of CuL² (15 μ M in 10 mM HEPES buffer (pH 7.4) with 10% DMSO) in the presence of 500 μ M competitors. a) EDTA over 60 minutes, and b) His.OMe, Cys.OMe EDTA and GSH after incubating at room temperature for 60 minutes (expected spectrum for H₂L² given by dotted line).

A titration of Cu(II) into a solution of H₂L² with 20 μ M EDTA was then conducted to identify the kinetic product and determine the stability constant, K_D , for CuL².

3. Assessing the Stability of CuL^2

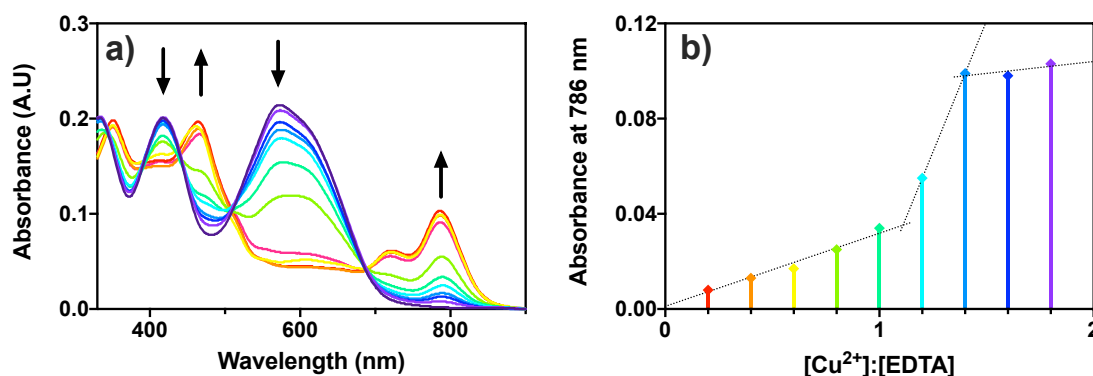


Figure 3.6. a) UV-Visible spectra for H_2L^2 ($8.21 \mu\text{M}$, 10% DMSO in 10 mM HEPES buffer, pH 7.4) with $20 \mu\text{M}$ EDTA following titration with Cu^{2+} (AAS Standard; HNO_3).
b) Change in absorbance with increasing $[\text{Cu}^{2+}]:[\text{EDTA}]$ ratio at 786 nm.

Using an $8.21 \mu\text{M}$ solution of H_2L^2 , two distinct linear increases in absorbance at 786 nm are shown. At ca. 1.15 equivalents ($23 \mu\text{M}$) saturation of EDTA with copper(II) is evident. The equilibrium concentrations of each species in the titration were then calculated for the values which satisfy the following conditions: $[\text{EDTA}]_{\text{tot}} > [\text{CuEDTA}]$, and $[\text{L}^2]_{\text{tot}} > [\text{CuL}^2]$ (Table 3.2).

Table 3.2. Equilibrium concentrations used in the estimation of $K_D(\text{CuL}^2)$ for H_2L^2 through EDTA challenge (10% DMSO in 10 mM HEPES buffer, pH 7.4).
 $[\text{EDTA}]_{\text{tot}} = 20 \mu\text{M}$; $[\text{L}^2]_{\text{tot}} = 8.21 \mu\text{M}$.

$[\text{Cu}^{2+}]$ (μM)	$[\text{CuL}]_{\text{eq}}$ (μM) ^a	$[\text{CuEDTA}]_{\text{eq}}$ (μM) ^b
0	0.00	0.00
4	0.62	3.38
8	0.93	7.07
12	1.16	10.84

^a Values measured using $A_{786 \text{ nm}}$ and $\epsilon = 1.27 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

^b Value calculated using mass balance ($[\text{CuEDTA}]_{\text{eq}} = [\text{Cu}^{2+}]_{\text{tot}} - [\text{CuL}^2]_{\text{eq}}$).

3. Assessing the Stability of CuL²

Using Equation 3.1,⁹ a plot of $([EDTA]_{tot}/[CuEDTA]) - 1$ versus $([L]_{tot}/[CuL]) - 1$ produces a linear line of gradient $K_D K'_A$, where K_D is the dissociation constant for CuEDTA, and K'_A is the experimentally determined association constant for CuL².

$$\text{Equation 3.1} \quad K_D K'_A = \frac{\left(\frac{[EDTA]_{tot}}{[CuEDTA]_{eq}}\right) - 1}{\left(\frac{[L]_{tot}}{[CuL]_{eq}}\right) - 1}$$

Table 3.3. Values of $([EDTA]_{tot}/[CuEDTA]) - 1$ and $([L]_{tot}/[CuL]) - 1$ used in the estimation of $K_D K'_A$.

[Cu ²⁺] (μM)	$([EDTA]_{tot}/[CuEDTA]) - 1$	$([L]_{tot}/[CuL]) - 1$
0	-	-
4	4.92	12.2
8	1.83	7.83
12	0.845	6.07
	$K_D K'_A$	1.5 ± 0.4

$$\text{Equation 3.2} \quad K'_D = \frac{1}{K'_A} = \frac{K_D}{K_D K'_A}$$

Taking the reciprocal value of $K'_A(\text{CuL}^2)$ produces the corresponding dissociation constant, K'_D , for CuL². Using the relationship given in Equation 3.2 and $K_D(\text{CuEDTA}) = 8.61 \times 10^{-15}$ M at pH 7.4,⁹ this was calculated to be $6 \pm 1 \times 10^{-15}$ M at pH 7.4.

Comparing this value to the corresponding K_D values for copper complexes with analogous ligands shows a decrease in stability for the 2,2'-bisdipyrrin. Similar ligands *N,N'*-bis(3-aminopropyl)-1,3-ethylenediamine (3,2,3-tet), *N,N'*-bis(3-aminopropyl)-1,3-propylenediamine (3,3,3-tet), and *meso*-tetra(*p*-sulfonatophenyl)porphyrin (TSPP) (Figure 3.7) have K_D values of 2×10^{-22} , 5×10^{-18} and 8×10^{-39} M, respectively (0.1 M ionic strength, 25°C).¹⁰⁻¹²

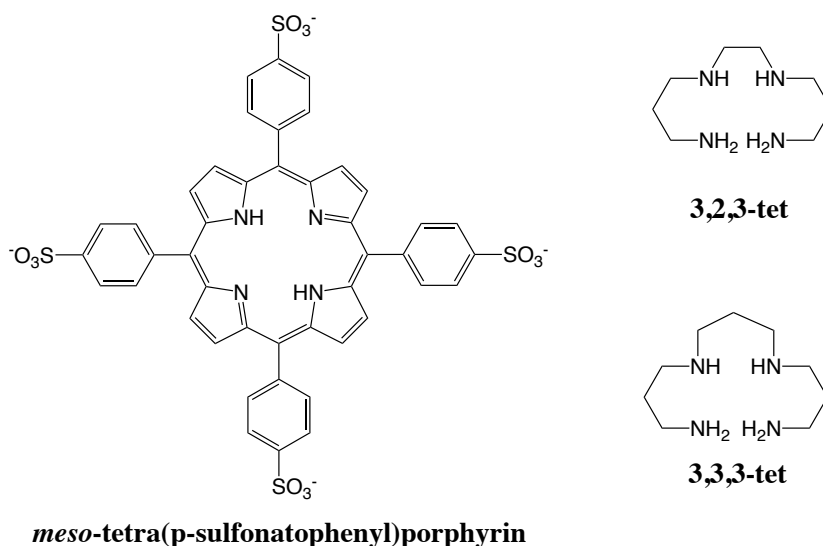


Figure 3.7. Structure of analogous ligands with known K_D values for the corresponding copper(II) complexes.

These values suggest that incorporation of a 5-membered chelate ring in 3,2,3-tet increases the stability compared to the three 6-membered chelate rings of 3,3,3-tet. The CuTSPP complex displays a stability approximately 24 orders of magnitude higher than the 2,2'-bisdipyrin copper(II) complex, a result of the macrocyclic structure. The latter is yet more unstable than 3,2,3-tet by 8 orders of magnitude, likely a result of the steric clash revealed by crystallographic experiments.

While there is a clear cost of thermodynamic stability on adoption of an acyclic structure, the favourable complexation kinetics compared to porphyrins and corroles may still outweigh this detriment. The inability of the above small molecule competitors to remove Cu(II) from the complex is therefore due to a high barrier to decomplexation and supports their use in biological systems. The presence of large proteins with copper(II) binding sites and the redox non-innocence of the ligands, however, may reduce their application potential. Accordingly, the stability of the complex in the presence of a serum albumin was next investigated.

3.3 INTERACTIONS OF H₂L² AND CuL² WITH BOVINE SERUM ALBUMIN

Serum albumins are large multi-domain plasma proteins present in the blood of animals. They are known for their ability to bind a range of endogenous and exogenous compounds, including fatty acids, hormones, drugs, metabolites and metal ions and complexes.¹³⁻¹⁵ Serum albumins are ~66 kDa proteins present in the blood serum at up to 40 mg/mL.¹⁶ Mature proteins contain approximately 580 amino acid residues, forming three homologous domains and containing 17 disulphide bridges.¹⁷ Their abundance in blood plasma is owed largely to the high proportions of ionisable amino acids in the protein chain, increasing solubility. Although structurally analogous, subtle differences in the protein sequence for each of the three domains leads to differences in binding affinities for endogenous and exogenous compounds.

Human serum albumin (HSA) and bovine serum albumin (BSA) are ~80% homologous in sequence.¹⁸ Characterisation of the two drug binding sites in HSA, site I and site II, has demonstrated that drugs show different binding affinities for each.¹⁹⁻²⁰ Other interactions distinct from these binding sites are also important for the transport of free fatty acids (FA). HSA:FA ratios have been observed as high as 1:5, although values in the range of 10:1 to 1:2 ratio is considered standard *in vivo*.¹³ The binding of FA to HSA and its analogues is suggested to alter binding affinities at the drug binding pockets to varying degrees, and restrict hydrophobic interactions with the protein surface.²¹

Comparing HSA and BSA, site I shows similar binding affinities for a pair of representative site I-specific drugs, warfarin and phenylbutazone, but show a decrease in the affinity for the site II-specific ibuprofen.¹⁸ This suggests a conservation of the general structure of the site I pocket, but some variation in site II. Nevertheless, BSA is commonly used as a model protein in place of HSA for drug binding studies and will be employed herein.

3. Assessing the Stability of CuL^2

Addition of BSA to aqueous mixtures extends the solubility of CuL^2 from 60 minutes to 3 to 4 hours before precipitation occurs. To further this, a sample comprising approximately $5\ \mu\text{M}$ of either H_2L^2 or CuL^2 with 0.01% BSA ($1.5\ \mu\text{M}$) and 10% DMSO, was analysed at various time points over two hours to examine the effects of BSA on the respective absorption spectra (Figure 3.8). Importantly, CuL^2 is stable in the presence of BSA based on the lack of change in the absorption spectra associated with decomplexation.

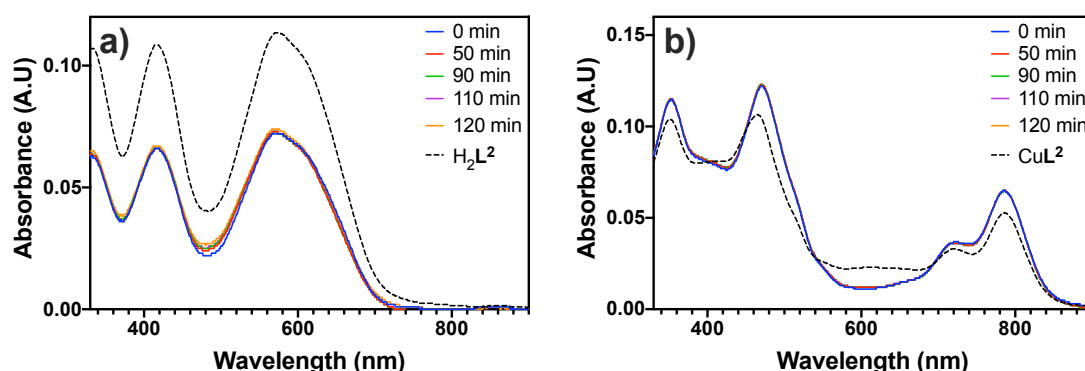


Figure 3.8. UV-Visible absorption spectra of a) H_2L^2 and b) CuL^2 in the presence of 0.01% BSA over 2 hours. Dotted line represents BSA-free sample in the same buffer at approximately $5\ \mu\text{M}$ concentrations for comparison.

Subsequent investigation was conducted to explore the nature of the interactions between BSA and H_2L^2 and CuL^2 through hydrophobic interactions. A fluorescent approach was adopted, with the intention to use the intrinsic fluorescence of BSA due to the presence of phenylalanine, tyrosine and tryptophan residues. For example, fluorescence of a tryptophan residue in one of the drug binding pockets (λ_{ex} 280 nm; λ_{em} 340 nm,) is commonly used to measure interactions between drugs and BSA.¹⁶

The absorbance of H_2L^2 and CuL^2 at 280 nm and 340 nm has limited the ability to use this fluorescence without inner filter effects (IFE) becoming considerable. Instead, the fluorescent properties of the ligand were explored in the presence of BSA to detect any binding interactions. Although this approach is not capable of discerning interactions of

H_2L^2 and CuL^2 with the drug binding sites, it was used to reveal the presence of *any* interactions that may alter their fluorescence in the presence of BSA.

As previously shown in section 2.7.2, H_2L^2 is fluorescent in ACN, with excitations at the Q-like peaks (550 and 580 nm) producing an emission at 638 nm. Excitation of the same ligand in the HEPES buffered solvent system at the Q-like bands, which have undergone a shift in relative intensity and an overall red-shift to 573 nm and 615 nm, displays no resulting emission.

Following addition of 0.01% BSA to the solution, recovery of fluorescence is observed. Excitation between 573 to 600 nm, however, produces no emission. Instead, fluorescence results from excitation at 503 nm, corresponding to neither a peak maximum in the ligand or BSA spectra, with an emission at 610 nm.

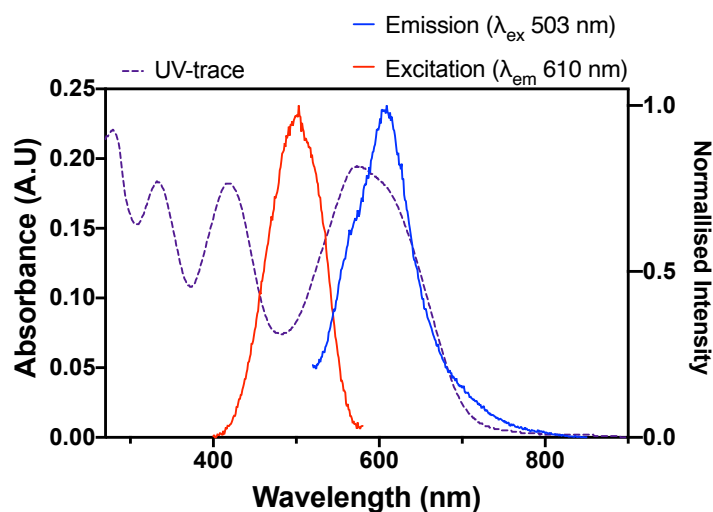


Figure 3.9. Normalised fluorescence and excitation spectra of H_2L^2 ($5\ \mu\text{M}$) bound to 0.01% BSA in 10 mM HEPES buffer (pH 7.4) with 10% DMSO. Dotted line indicates corresponding UV-visible spectrum of H_2L^2 ($10\ \mu\text{M}$) in the same buffer with BSA.

The fluorescence response in the presence of BSA is indicative of a change to the environment about the ligand through conformational restrictions or solvation changes. This supports the formation of a ligand-BSA conjugate.

CuL^2 lacks fluorescence in both the presence and absence of BSA across the entire spectrum, and sufficiently diminishes fluorescence of the tryptophan residue through IFE. While hydrophobic interactions of complex and BSA seem likely, it is unclear as to whether a similar interaction with the corresponding copper(II) complex is present.

3.4 INTERACTIONS OF CuL^2 WITH SODIUM DITHIONITE

Beyond investigating any interactions of CuL^2 with BSA, the redox non-innocence of both ligand and complex discussed in Section 2.8 suggests reduction-based decomplexation processes may occur. Given the high intracellular concentration of low molecular weight reductants like GSH, the ability of these complexes to resist reduction is fundamental to their role as stable copper(II) imaging tools.

Sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) was used to mimic the intracellular reducing environment in order to examine the implications of ligand-based reduction processes on complex stability. The dithionite anion in solution is detectible through absorption spectroscopy, with a strong absorbance below 400 nm, restricting analysis of the spectra to 600–900 nm.

Solutions containing 15 μM of CuL^2 were prepared in the presence and absence of 1 mM $\text{Na}_2\text{S}_2\text{O}_4$, both with and without BSA (Figure 3.10a). Post-analysis, each sample was purged with air to quench excess $\text{Na}_2\text{S}_2\text{O}_4$ in solution, enabling the analysis of the full spectral window (Figure 3.10b).

3. Assessing the Stability of CuL^2

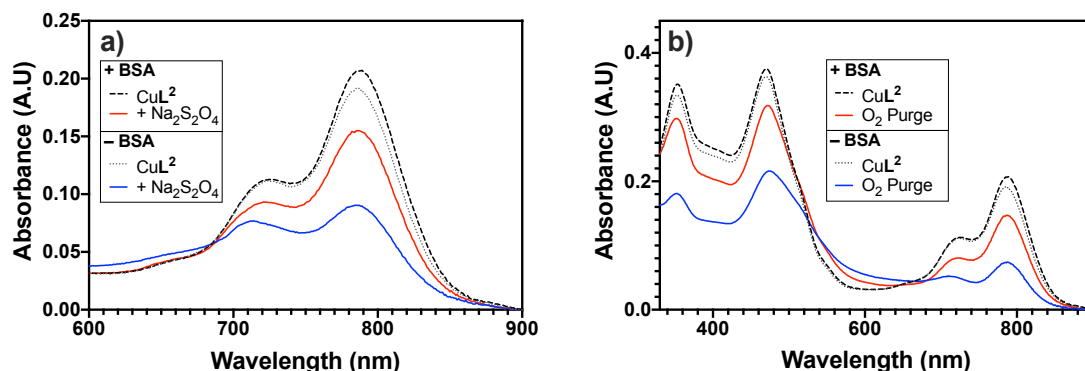


Figure 3.10. Absorbance spectrum of CuL^2 (15 μM) in HEPES buffer (10 mM, pH 7.4 with 10% DMSO) a) post-treatment with $\text{Na}_2\text{S}_2\text{O}_4$ (1 mM) and b) post purge of the samples with air, with and without BSA, as indicated.

In the absence of BSA, a 53% reduction in the absorbance at 786 nm is observed following the addition of dithionite, although no corresponding increase at 573 nm, corresponding to H_2L^2 , is evident. Sparging with air caused only a subtle increase in this value, but examination of the full spectra highlights changes centred around the Soret-like band at 464 nm. These results indicate ligand-based reduction, which is seemingly insufficient to effect decomplexation measurable through a spectroscopic approach.

$\text{Na}_2\text{S}_2\text{O}_4$ was only able to cause a 26% reduction in absorbance following addition of BSA to the media, indicating a decrease in the effects of dithionite on the complex. The large excess of dithionite compared to both the complex and BSA eliminates any likelihood that dithionite is consumed by preferential reduction of the protein. Instead, the CuL^2 is proposed to experience hydrophobic interactions with BSA, as evidenced by the improvement to solubility, that results in apparent protection against the effects of the reductant. This effect is limited by the 10-fold excess of complex in the sample relative to BSA, representing a saturation point of the BSA with complex, to which the unbound complex is susceptible to reduction, resulting in the observed decline in absorbance.

Under these conditions, the liberation of copper(I) following ligand reduction may be inhibited by the steric demands of the ligand being unable to stabilise the copper(I) species in solution. In the intracellular environment, such stabilisation is afforded by an abundance of copper(I) chelators in the intracellular environment, including metallothioneins and metallochaperone proteins. The presence of these proteins specific for copper(I) might therefore be expected to increase the occurrence of reductive decomplexation events. To model this herein, a small protein with high affinity for copper(I) ions in solution was employed as a copper(I) sink.

3.4.1.1 Dithionite and Atox1

Human antioxidant protein 1, Atox1, is a small (ca. 7.4 kDa) soluble cytosolic protein that shows high levels of ubiquity in nature. Its secondary structure consists of a ferredoxin fold, which is a common motif involving the alternation of α and β secondary structures in the sequence $\beta\alpha\beta\beta\alpha\beta$ (Figure 3.11).²² One of its primary roles in the organism is the trafficking of copper(I) from the copper transporter, CTR1, which resides in the plasma membrane, to one of two copper transporting ATPases, ATP7A or ATP7B.²³⁻²⁴ The transportation of copper from CTR1 to ATP7A/B, and indeed the other intracellular proteins in the cell that bind to copper for enzyme incorporation or secretion, is dependent on an affinity cascade from low- to high-affinity proteins.⁶ ATP-hydrolysis provides the ATP7A/B with the required energy to transition into a high energy, low copper-affinity state to allow subsequent release of copper to further downstream proteins or secretion pathways.²⁵

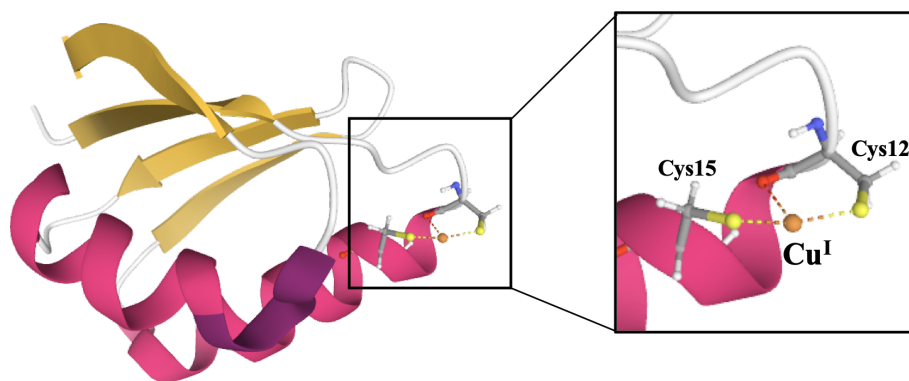


Figure 3.11. Ribbon structure of the ferredoxin fold ($\beta\alpha\beta\beta\alpha\beta$) present in copper(I)-bound Atox1 (Protein data bank code 1TL4). Pink represents the α -helices and yellow represents β -sheets in the secondary structure. Cu(I) ion indicated in orange, with coordinating cysteine residues of the CXXC motif given as ball/stick structures.

Its role as an antioxidant is proposed to result from the highly stable Cu(I)-Atox1 complex (binding affinity, $K_D = 5.6 \times 10^{17} \text{ M}^{-1}$ at pH 7.0)²⁶ through a CXXC binding motif in the protein sequence incorporating Cys12 and Cys15. Along with the millimolar concentrations of GSH present in the cytosol, Atox1 cooperatively binds any free copper ions, preventing their catalytic production of ROS. Atox1, the yeast orthologue of Atox1, was also shown to inhibit ROS activity in strains where normal antioxidant machinery, including the superoxide dismutase 1, SOD1, were diminished or absent, suggesting these small proteins confer their own antioxidant activity.²⁷ As such, Atox1 can be considered crucial to both regulation of intracellular copper levels and ROS, as well as correct processing and synthesis of cuproenzymes.

Atox1 is a copper(I)-specific metalloprotein, and so direct transfer of copper from CuL^2 to the CXXC binding site is unlikely due to the oxidation state of copper. Dithionite was again used to attempt reduction of the complex to measure any transfer of the ion and liberation of the free ligand.

Subtle variations associated with the introduction of a protein can again be seen from the resulting spectra in both buffers. 50 μM of Atox1 was used in this study, which is

3. Assessing the Stability of CuL^2

retrospectively likely to be capable of binding a larger proportion of complex, associated with an additional increase in absorbance of the sample (Figure 3.12a).

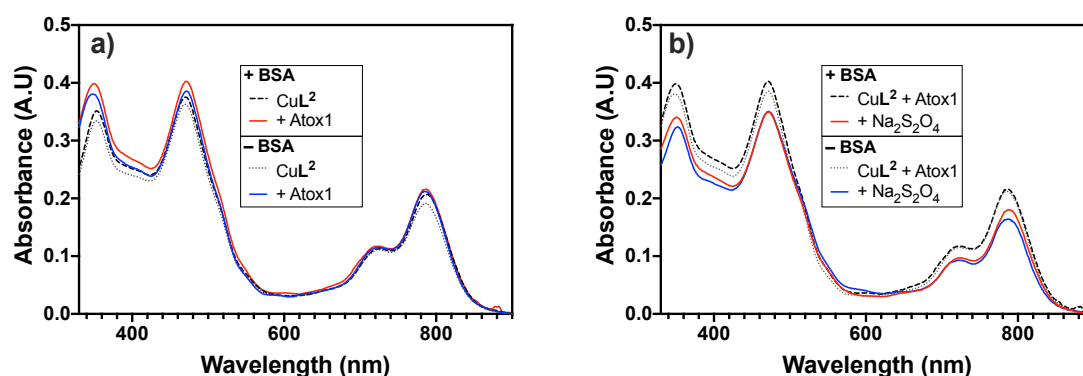


Figure 3.12. Absorbance spectrum of CuL^2 (15 μM) in HEPES buffer (10 mM, pH 7.4 with 10% DMSO) a) pre- and post-treatment with Atox1 (50 μM) and b) Atox1 samples pre- and post-treatment with $\text{Na}_2\text{S}_2\text{O}_4$ (1 mM) after purging the samples, with and without BSA (0.01%), as indicated.

Addition of dithionite to the sample and purging the samples after analysis produced near-identical spectra (Figure 3.12b), with only minor deviations in absorbance. CuL^2 in the presence and absence of BSA with Atox1 following treatment of dithionite yielded a reduction in absorbance of 11% and 21%, respectively. Again, a lack of corresponding H_2L^2 signals suggests no decomplexation, and the further inhibition of the dithionite's activity relative to the Atox1-free samples is suggestive of additional protein-complex interactions. The smaller size of Atox1 (~7.5 kDa) compared to BSA (~66 kDa) suggests reduced capacity for binding interactions, as does the absence of drug binding sites like those seen on BSA.

These results are in agreement with the dithionite control samples. That is, protein-complex interactions are inhibitory of any reduction events, and the addition of a protein-based copper(I) high affinity binding site makes no appreciable difference to the spectra.

The presence of protein in solution efficiently inhibits the action of the reductant and may limit a decomplexation response. The use of protein-free media with a small-molecule high-affinity copper(I) sink was therefore investigated, with the added ability to probe the formation of a new copper(I) complex rather than only measuring the decrease in CuL^2 absorbance.

3.4.1.2 Dithionite and Bicinchoninic Acid

Bicinchoninic acid (Bca), or 2,2'-biquinoline-4,4'-dicarboxylic acid (Figure 3.13), is a ligand used to quantify protein in solution. Specifically, the Bca assay uses the formation of a 1:2 complex of Cu(I) with Bca, $[\text{Cu}(\text{Bca})_2]^{3-}$, which is strongly absorbing in solution at 562 nm to monitor the production of copper(I) in solution hitherto unseen.²⁸

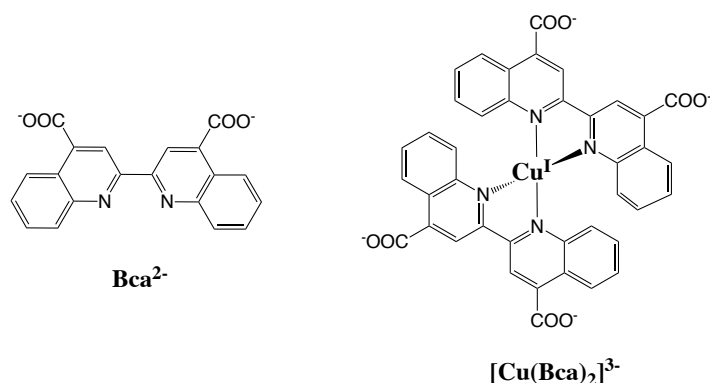


Figure 3.13. Structures of Bca²⁻ anion and the $[\text{Cu}(\text{Bca})_2]^{3-}$ complex.

Once again, controls in the absence of dithionite indicate no significant shift in the CuL^2 spectra above 600 nm, although Bca²⁻ exhibits significant absorbance below 400 nm at the concentrations used (1 mM). Dramatic changes to the spectrum, on top of a marked reduction in the absorbance at 786 nm, suggests the overlap of the complex's spectrum with the resulting $[\text{Cu}(\text{Bca})_2]^{3-}$ spectrum (Figure 3.14).

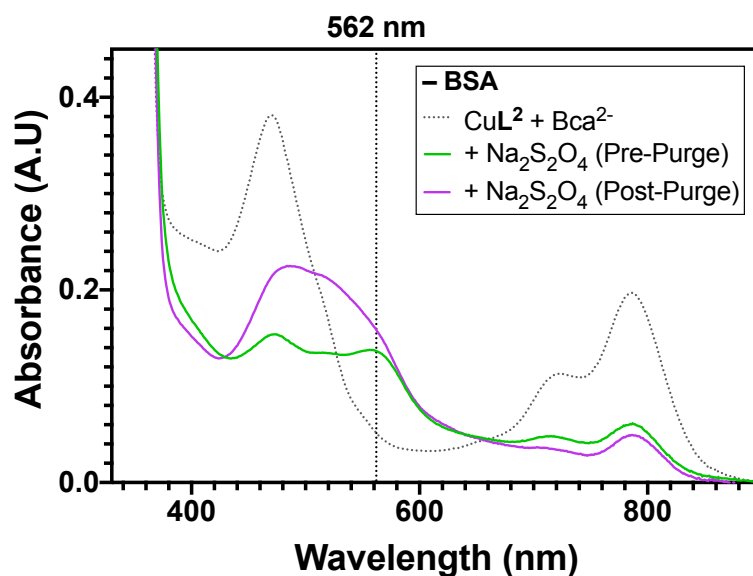


Figure 3.14. Absorbance spectra of CuL^2 ($15\ \mu\text{M}$) in HEPES buffer ($10\ \text{mM}$, pH 7.4 with 10% DMSO) without BSA, in the presence of Bca^{2-} ($1\ \text{mM}$) and $\text{Na}_2\text{S}_2\text{O}_4$ ($1\ \text{mM}$), before and after purging the samples with air.

There is a 68 and 74% reduction in the absorbance relative to the controls for the pre-purge and post-purge samples, respectively. This, matched with a clear increase absorbance at 562 nm, a feature not observed in any other sample analysed, suggests the formation of the $[\text{Cu}(\text{Bca})_2]^{3-}$ complex, proving reduction-mediated decomplexation of CuL^2 in the absence of BSA. No obvious increase in the wavelengths corresponding to the ligand's absorbance spectrum, however, suggests decomplexation is not independent of ligand reduction, which would be coupled with spectroscopic changes thus far uncharacterised.

3.5 SUMMARY

It has already been well-established that CuL^2 is sensitive to proton-mediated decomplexation. Aqueous solutions of at least pH 5 were shown to sufficiently minimise protonation and decomplexation, with DMSO required as an additive to promote solubility.

Complexation of copper(II) by H_2L^2 is rapid in aqueous solutions supplemented with DMSO for solubility at pH 7.4. In the presence of small molecule chelators, EDTA, GSH and select amino acids, the complex is able to resist kinetic challenge, with a corresponding K_D of $6 \pm 1 \times 10^{-15}$ M at pH 7.4. Solubility was improved with the addition of BSA, which changes the fluorescent properties of H_2L^2 in solution.

As well as enhancing solubility, BSA inhibits the reduction of CuL^2 by $\text{Na}_2\text{S}_2\text{O}_4$ by more than 50% (from 53% to 26% decrease in absorbance at 786 nm). This is observed to a larger extent following the addition of Atox1, a small copper(I) metallochaperone, due to a large excess of protein facilitating enhanced hydrophobic interactions. Decomplexation by reduction with $\text{Na}_2\text{S}_2\text{O}_4$ was only definitively observed by the formation of a spectroscopically detectable copper(I) complex, $[\text{Cu}(\text{Bca})_2]^{3-}$, in the absence of protein. This suggests that, in the absence of hydrophobic interactions with proteins, CuL^2 is susceptible to decomplexation following ligand reduction.

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4 CELL UPTAKE OF BISDIPYRRINATO COPPER(II) COMPLEXES

4.1 BACKGROUND

Mammalian cell membranes are dynamic lipid bilayers forming a physical barrier between the intra- and extracellular environments, housing the required cell-surface proteins for normal biological function.¹ Passage of metal complexes across cell membranes is influenced by ionic charge, lipophilicity of the complexes as well as membrane structure (fluidity, composition, membrane potential/charge). Typically, the net negative charge of the intra- and extracellular membrane surfaces facilitates better interaction with positively charged hydrophobic complexes, enhancing their diffusion across the membrane.²

4.1.1 Chapter Aims

The new (2,2'-bisdipyrinato) copper(II) complexes presented in this thesis exhibit planar charge-neutral structures. Despite previous investigation of synthesis and electrochemistry (section 2.8), there have been no reports describing the biological behaviour of such complexes *in vitro* or *in vivo*. The work presented in this chapter will address the ability of our new (2,2'-bisdipyrinato) copper(II) complexes to cross cell membranes using four different cell lines, and examine substituent effects on any observed membrane permeability. This work was conducted in a variety of cell lines in collaboration with other researchers. The preliminary nature of this work required varied selection of compounds for analysis, and each cell line was selected opportunistically.

4.2 UPTAKE OF CuL^2 IN HEK293 CELLS

Cellular experiments presented in this section were conducted by Dr. Karla Acevedo and Assoc. Prof. Tony White using compounds synthesised by the candidate.

The uptake of CuL^2 was initially measured using the primary human embryonic kidney cells transformed with sheared human adenovirus type 5 DNA (HEK293). Cells were treated with CuL^2 (5 μM) for 0.5 hours or 4 hours, and intracellular copper(II) levels measured by ICP-MS following HNO_3 digestion. Copper concentrations ($\mu\text{mol/L}$) were standardised to protein content in the cell pellet (μg) (Figure 4.1).

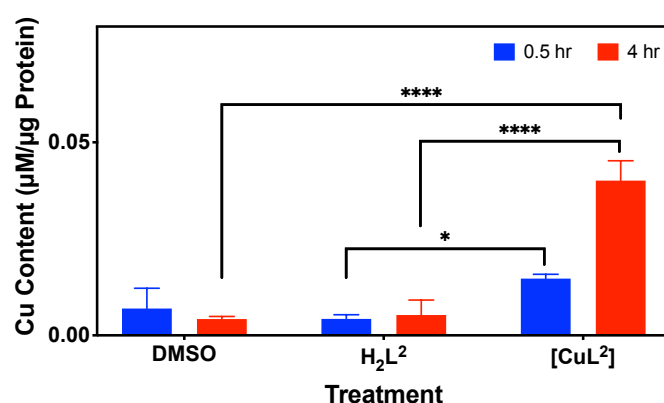


Figure 4.1. HEK293 intracellular copper levels ($\pm$ SD) following 30 minute and 4 hour treatments with 5 μM H_2L^2 and CuL^2 . (* $p < 0.05$; **** $p < 0.0001$).

A significant increase in the measured copper content is observed following 4 hours incubation for CuL^2 compared to both the vehicle control and treatment with H_2L^2 . A significant difference is also observed after treatment for 0.5 hours relative to the free ligand control. Additionally, a three-fold increase of copper concentration from 0.014 ± 0.001 to 0.040 ± 0.005 $\mu\text{M}/\mu\text{g}$ protein is evident between the experimental groups (0.5 vs. 4 hours), indicating a time-dependant uptake response in these cells, and a modest degree of cell uptake for CuL^2 .

4.3 UPTAKE OF CuL^2 AND CuL^3 IN SKOV-3 CELLS

Cell experiments presented in this section were conducted by Dr. Karla Acevedo and Assoc. Prof. Tony White using compounds synthesised by the candidate.

Differences in solubility were first observed following ester hydrolysis of the ligand H_2L^2 to produce H_2L^3 . The lower logD value of the complex CuL^3 (1.51 ± 0.15) compared to CuL^2 (1.64 ± 0.65), as well as the expectant dianionic form of CuL^3 following deprotonation, suggests that these complexes may exhibit different membrane permeabilities (Figure 4.2).

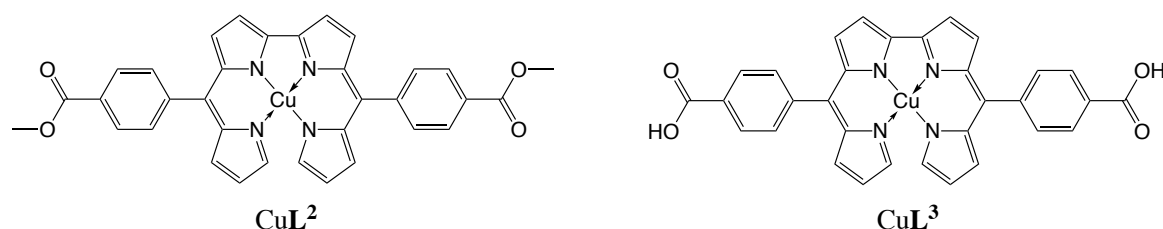


Figure 4.2. Structures of CuL^2 and CuL^3 .

To investigate these differences, analysis was carried out using SKOV-3 (human ovarian cancer) cells. Cells were treated with $1\ \mu\text{M}$ of either CuL^2 or CuL^3 for 1 hour or 2 hours before analysis by ICP-MS.

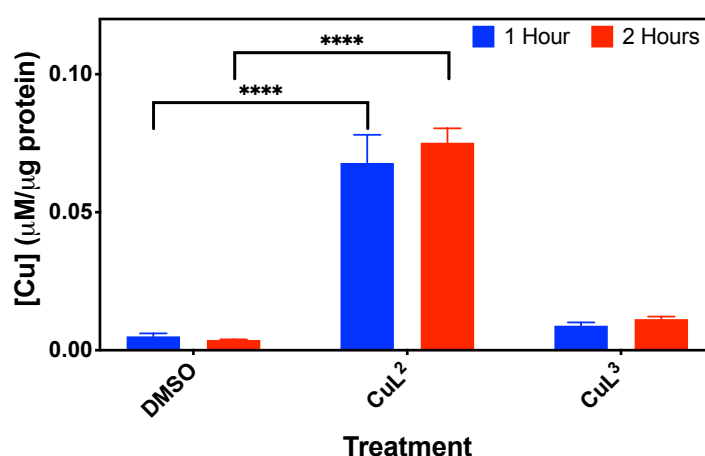


Figure 4.3. SKOV-3 intracellular copper levels ($\pm$ SD) following 1- and 2-hour treatments with $1\ \mu\text{M}$ CuL^2 and CuL^3 . (**** $p < 0.0001$).

Compared to the previous study, SKOV-3 cells show only minor differences in uptake of CuL^2 between the two time points (0.068 ± 0.01 and 0.075 ± 0.01 μM per μg protein for 1 hour and 2 hours, respectively), with significant increases compared to the vehicle control. The uptake following treatment with CuL^3 for both 1 hour and 2 hours was not statistically significant relative to the DMSO vehicle control ($p > 0.05$), due to either poor solubility in the cell media or poor membrane permeability.

Poor membrane permeability is ascribed to result from the loss of neutrality. The pK_a of the carboxylic acid groups in CuL^3 is estimated to be less than 5 based on the pK_a of benzoic acid (4.2).³ Accordingly, CuL^3 would exist primarily as an anionic species in the cell media of pH 7.4, and interactions with the negatively charged membrane surface would therefore be electrostatically unfavourable.

4.4 *ATP7A LOCALISATION STUDIES WITH CuL^2 IN SKOV-3 CELLS*

Cell treatment and imaging presented in this section was conducted by Dr. Karla Acevedo and Assoc. Prof. Tony White using compounds synthesised by the candidate.

Following uptake, the fate of the complexes was uncertain. Using electrochemistry as a guide, it was unlikely that these complexes would undergo reduction in normoxic cells to promote release of copper(I). However, in the complex intracellular environment, with an assortment of reductants and high-affinity copper chaperone proteins, it remained possible that the complexes were donating the copper for incorporation into copper-based enzymes or efflux from the cell.

In order to appreciate how the redox non-innocence of the complex CuL^2 translates into the intracellular environment, the use of an indirect copper(I) probe was employed. This involved monitoring changes in the distribution of a copper(I) metallochaperone, ATP7A, within the cell using confocal laser scanning microscopy. ATP7A is responsible for

incorporation of copper into metalloproteins and efflux of excess copper(I) from cells.⁴ Following an increase to 'free' copper within cells, ATP7A migrates from the Golgi to the cell membrane to affect copper(I) efflux. As such, changes in ATP7A localisation has been exploited as an indirect probe to monitor intracellular 'free' copper levels.⁵ This approach has been used herein to investigate the stability of CuL² following uptake into SKOV-3 cells.

In DMSO control cells with normal copper levels, the ATP7A mostly resides within the Golgi apparatus (Figure 4.3, Panel A: Golgin-97 stain [green]). Following an increase in bioavailable copper levels, ATP7A migrates to the cell membrane (Figure 4.3, Panel B: F-actin indirect stain [white]). Changes in the ATP7A localisation in these cells (Figure 4.3, Panel C: ATP7A immunofluorescent probe [red]) following treatment with CuL² and appropriate controls were used to qualitatively assess if the complexes were liberating free copper into the cytoplasm. Glyoxalbis(*N*(4)-methylthiosemicarbazonato) copper(II) (CuGTSM) was used as a potent ATP7A activator, given its marked ability to cross cell membranes and release copper intracellularly.⁶

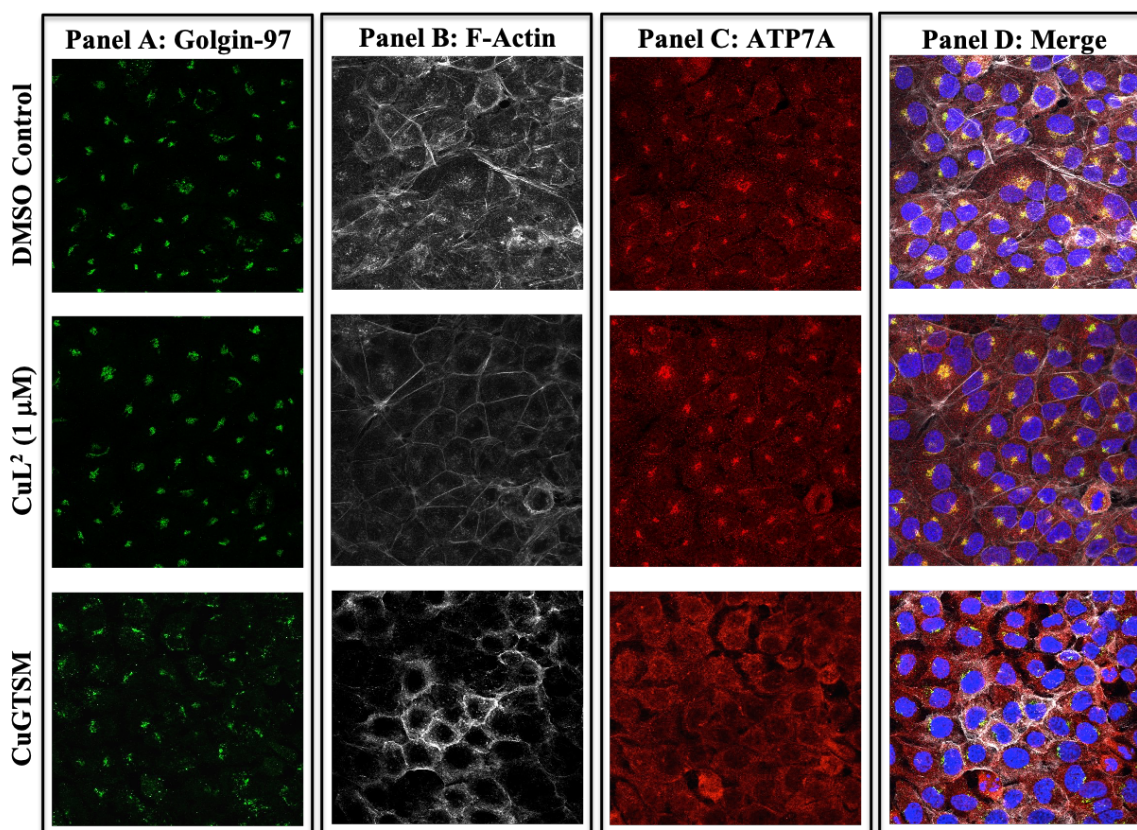


Figure 4.4. Confocal laser scanning microscopy images of fixed SKOV-3 cells. Cells were treated with either DMSO (top), CuL² (middle; 1 μ M for 2 hours) and CuGTSM (bottom; positive control) before staining with Golgin-97 (panel A), F-Actin (panel B) and an immunofluorescent probe for ATP7A (panel C). Images overlaid with the nuclear Hoechst stain (blue, panel D).

Treatment with CuGTSM (positive control) is characterised by translocation of ATP7A from the Golgi to the cell membrane, associated with the release of copper intracellularly and consequential loss of Golgi/ATP7A co-localisation. Co-localisation of ATP7A and the Golgi apparatus, however, is evident for both the DMSO control and CuL² by the yellow pixels closely associated with the nuclear stain (blue) (see Figure 4.3, Panel D). Accordingly, it is concluded that CuL² is stable following uptake into these cells, such that free copper is not released into the intracellular environment. These results are in accordance with the electrochemistry of CuL², which highlights ligand-based one-electron transfer processes that do not necessarily result in decomplexation.

4.5 UPTAKE OF CuL^2 AND CELL VIABILITY IN ReN NEURAL STEM CELLS

Cell experiments presented in this section were conducted by Dr. Lotta Oikari and Assoc. Prof. Tony White using compounds synthesised by the candidate.

Dose-dependent cell toxicity and metal uptake was also assessed for CuL^2 . Human neural progenitor cells (ReN), which differentiate into glial cells and neurons within the nervous system, were incubated for 72 hours with CuL^2 , and viability in the presence of increasing concentration in solution was determined (Figure 4.5). Cell viability was measured using propidium iodide (PI) and fluorescein diacetate (FDA) to image dead and live cells respectively using fluorescence microscopy.⁷ The viability was then determined *via* mean fluorescence intensity for both fluorophores using ImageJ software⁸ and calculating the proportion of live cells in the sample.

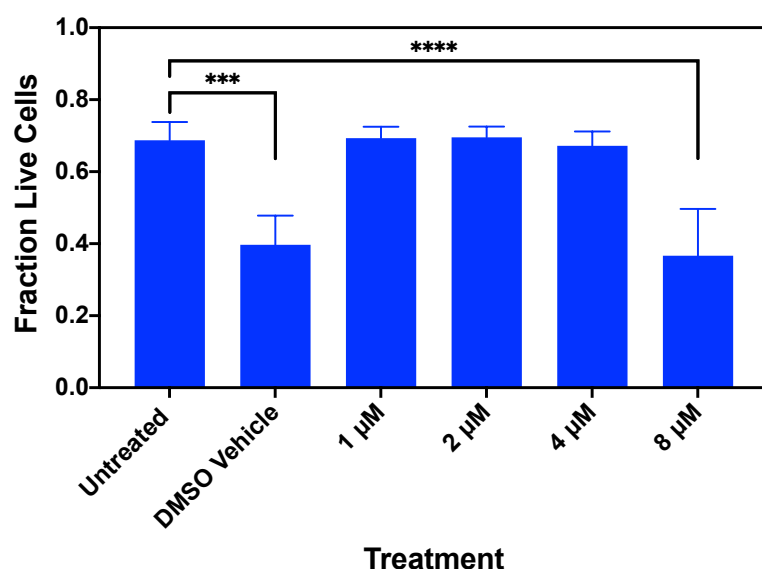


Figure 4.5. ReN cell viability ($\pm$ SD) following incubation for 72 hours with CuL^2 (0 – 8 μM). Cell viability measured using the PI/FDA assay. DMSO vehicle control applied at the same concentration as 8 μM treatment (*** $p < 0.001$; **** $p < 0.0001$).

A decrease in viability is observed at 8 μM treatment with CuL^2 , although it is noted that the same level of decrease is observed for the DMSO vehicle control relative to the untreated control. A single stock solution was used to deliver the CuL^2 into cell media for each treatment concentration (*for these studies only*), and as such the DMSO used increases with increasing $[\text{CuL}^2]$. Since the DMSO vehicle control was applied at the same concentration used in the 8 μM sample, the decreased cell viability is attributed to DMSO toxicity rather than toxicity of CuL^2 .

Treatments using 1 μM and 2 μM CuL^2 were then investigated for copper(II) uptake, given no effect on cell viability was observed. In both cases, a significant increase in the intracellular copper(II) levels is noted compared to each of the untreated control and the vehicle control (Figure 4.6). The observed difference in uptake for both concentrations, however, is not significant after 72 hours.

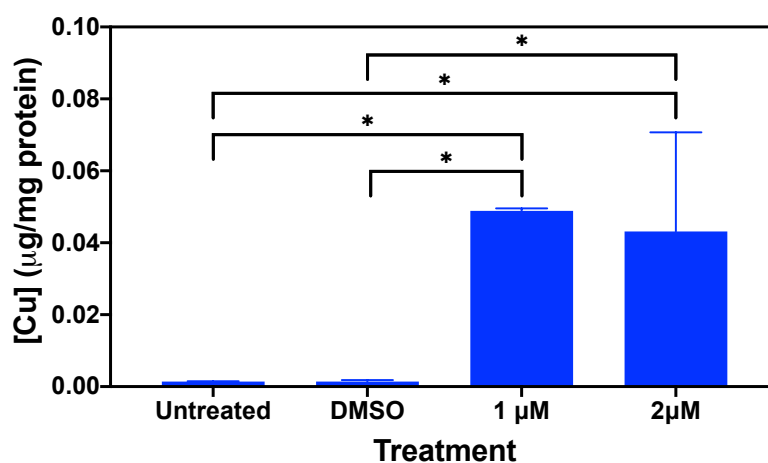


Figure 4.6. Copper cellular uptake in ReN cells ($\pm$ SD) following treatment with CuL^2 for 72 hours. Analysis by ICP-MS. Values standardised to μg metal per mg protein, determined using the Bca assay (* $p < 0.05$).

4.6 *REN CELL VIABILITY AND METAL UPTAKE: COMPLEXES OF L² AND L¹²*

Cell experiments presented in this section were conducted by Dr. Lotta Oikari and Assoc. Prof. Tony White using compounds synthesised by the candidate.

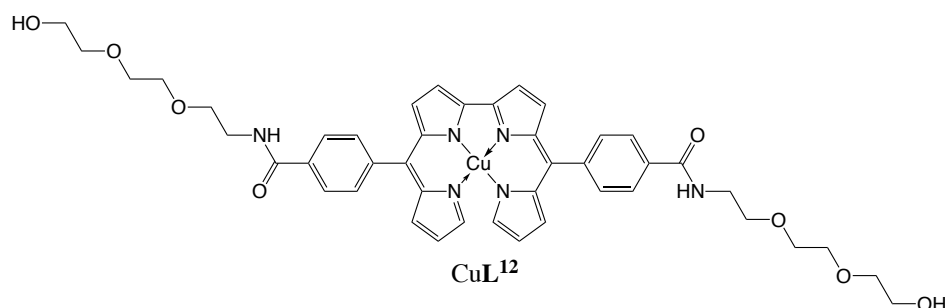


Figure 4.7. Structure of CuL¹².

The compounds CuL², NiL² and H₂L², as well as CuL¹² and its free ligand (Figure 4.7) were also analysed for toxicity in ReN cells (Figure 4.8). Free ligands H₂L² and H₂L¹² reduced cell viability by ~22% following treatment with 1 μ M for 72 hours, while the same concentration of metal complexes shows no loss of viability compared to the untreated control. This is likely a chelation response, whereby nutrient metals in the media or intracellular environment are made unavailable for normal metabolic processing, leading to cell death, although no further investigation has been made.

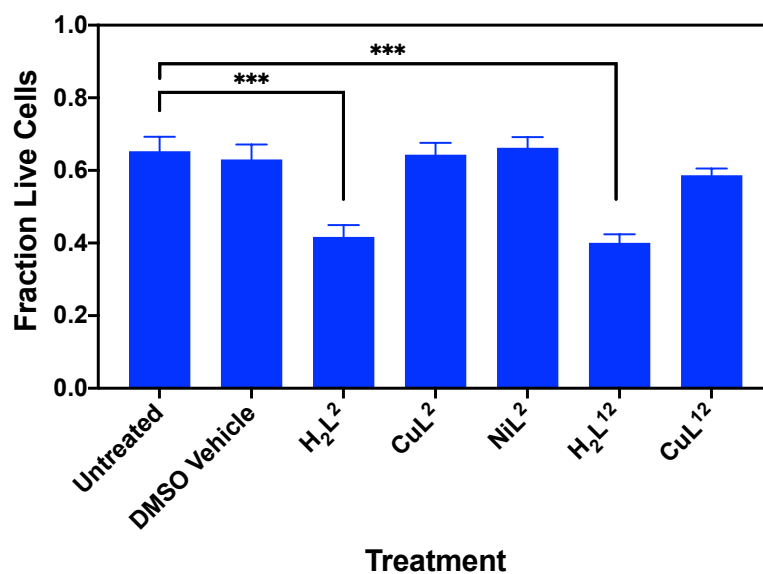


*Figure 4.8. Fractional ReN cell viability ($\pm$ SD) following 72-hour incubation with 1 μ M test compounds. DMSO applied at 0.003% (***) $p < 0.001$.*

In addition to not reducing cell viability in ReN cells, complexes CuL^2 , CuL^{12} and NiL^2 increase the level of intracellular metal (either copper or nickel) following treatment (Figure 4.9).

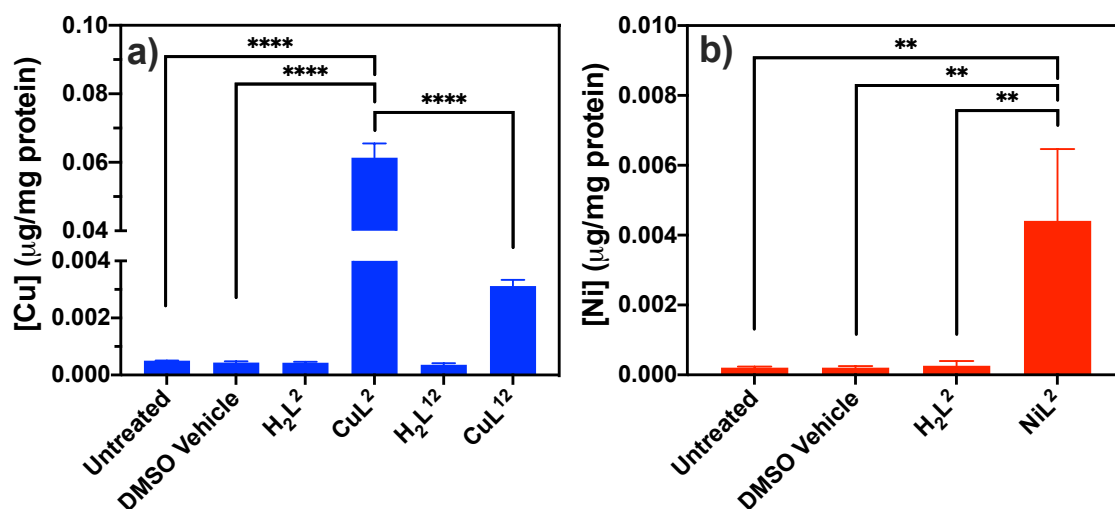


Figure 4.9. a) Copper and b) nickel cellular uptake in ReN cells ($\pm$ SD) following $1 \mu\text{M}$ treatment for 2 hours. Analysis by ICP-MS. Values standardised to μg metal per mg protein, determined using the Bca assay (** $p < 0.01$; **** $p < 0.0001$).

For CuL^2 , the resulting increase in copper levels to $0.061 \pm 0.004 \mu\text{g/mg protein}$ was 20-fold larger than that of CuL^{12} ($0.0031 \pm 0.0002 \mu\text{g/mg protein}$). The poor membrane permeability of CuL^{12} is attributed to a high logD and the presence of two oligo(ethylene glycol) groups, which may inhibit membrane interactions or increase solvation in the treatment media.

The corresponding nickel uptake experiment also shows a statistically significant increase in intracellular nickel levels following treatment compared to the untreated and vehicle controls. Compared to the untreated control, CuL^2 represents a 122-fold increase in copper, while the analogous NiL^2 produces only a 21-fold increase compared to nickel levels in the untreated cells.

This extended time point introduces the complexity of cellular efflux through either active or passive mechanisms, and as such it is unclear as to whether the observed results are a result of this. Future studies were therefore adapted to examining the 1-hour uptake profiles with this in mind.

4.7 LNCaP CELL UPTAKE DOSE-RESPONSE USING CuL^2 , CuL^{13} AND CuL^{14}

Cell experiments presented in this section were conducted by the candidate. Cells were cultured to confluency by Dr. Stacey Rudd.

Based on the previous results, which suggest that oligo(ethylene glycol) substituents impair membrane permeability, a final analysis of copper uptake was investigated using complexes CuL^2 , CuL^{13} and CuL^{14} (Figure 4.10) in LNCaP cells derived from the metastases of human prostate adenocarcinoma. Complexes CuL^{13} and CuL^{14} were selected as analogues to CuL^2 , with enhanced solubility compared to CuL^{10-12} .

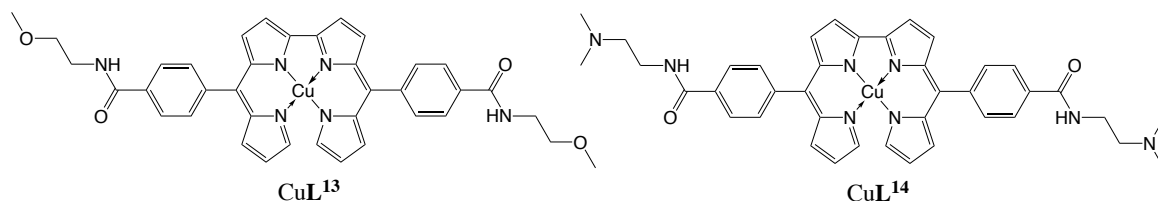


Figure 4.10. Structures of CuL^{13} and CuL^{14} .

The cells were treated with 0 (vehicle control), 5, 10, 25 or 50 μM concentrations of complex, maintaining a final DMSO concentration of 1% in PBS supplemented with 1% foetal bovine serum for incubation at 37°C for 1 hour (Figure 4.11).

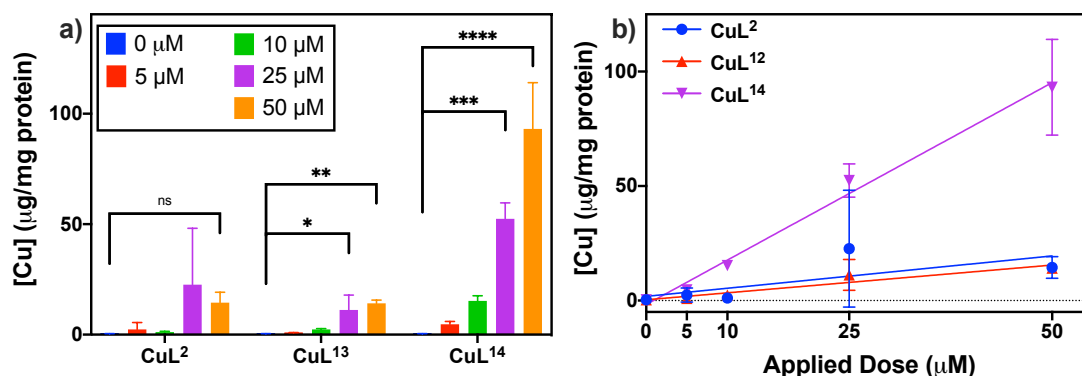


Figure 4.11. Copper cellular uptake in LNCaP cells ($\pm$ SD) following treatment for 1 hour with 0 to 50 μ M concentrations of CuL², CuL¹³ and CuL¹⁴. Analysis by ICP-MS and resulting values standardised to μ g copper per mg protein, determined using the Bca assay (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ **** $p < 0.0001$).

Treatment with 50 μ M concentrations of each complex gave intracellular copper concentrations of 14.5 ± 4.71 , 14.1 ± 1.52 and 93.1 ± 20.9 μ g copper per mg protein for CuL², CuL¹³ and CuL¹⁴ respectively. These correspond to approximately a 40-fold increase in intracellular copper levels for CuL² and CuL¹³, and a 270-fold increase for CuL¹⁴ compared to the vehicle (0 μ M) control.

In all cases, increasing the dosage concentration produces a linear increase in intracellular copper levels, with no saturation of uptake observed up to 50 μ M, suggesting passive diffusion across the membrane is the dominating uptake mechanism for each of the complexes.

Differences in uptake due to peripheral substitution is considered a result of the changing pK_b of the terminal groups (-OH and -NMe₂). Based on the pK_b values of diethyl ether (17.5)⁹ and *N,N*-dimethyl ethylamine (3.84)¹⁰, which have been selected as structural analogues to the discrete functionality of the side chains, the tertiary amine in CuL¹⁴ is expected to exist primarily in a protonated form at physiological pH (7.4) (Figure 4.12), whereas CuL¹³ and CuL² are not.

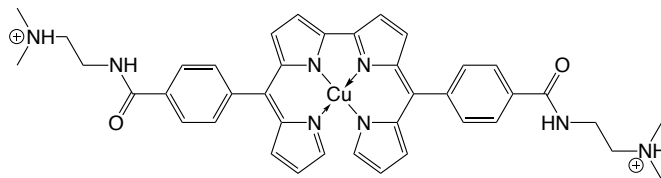


Figure 4.12. Proposed protonation event in CuL¹⁴ at physiological pH.

The resulting electrostatic attraction between the protonated CuL¹⁴ with the cell membranes is currently the best explanation for the increased cellular uptake given the similarity of CuL¹³ and CuL¹⁴.

4.8 SUMMARY

As the first studies investigating membrane permeability and cell toxicity for the (2,2'-bisdipyrrinato) complexes, it is clear that, unsurprisingly, uptake is largely governed by the peripheral substitution of the ligands. The simplest of these square-planar and charge-neutral complexes, CuL² causes a significant increase in intracellular copper levels in the four cell lines after various incubation periods (0 – 72 hours). Additionally, it is, at least up to concentrations of 4 μ M, non-cytotoxic in ReN neural cells, although evidence of toxicity at 8 μ M is attributed to higher DMSO content of the samples. In fact, all complexes analysed are non-cytotoxic in ReN cells at 1 μ M after 72 hours. Ligands H₂L² and H₂L¹², however, reduce cell viability by up to 22% compared to the untreated control.

In SKOV-3 cells, trafficking studies of ATP7A highlight possible intracellular stability following cell uptake, which coincides with the electrochemistry of CuL². Applications involving a radioisotope of copper could therefore be reasonably assumed to be similarly stable in the intracellular environment after 2 hours.

Generally speaking, complexes CuL³ and CuL¹² represent the extremes of peripheral substitution, and both exhibit poor cell membrane permeability. Poor solubility and the negative charge of CuL³ at pH 7.4 is the likely cause of its poor cell uptake, while the

lipophilicity and incorporation of two oligo(ethylene glycol) substituents may inhibit passage across the membrane of SKOV-3 cells for CuL¹².

Rather, the best membrane permeability in LNCaP cells was achieved using CuL¹⁴, the 2-(dimethylamino)ethyl amide ligand, producing intracellular copper concentrations over 6-fold higher than CuL² following 50 μ M treatment. This is attributed to the either better solubility or the low pK_b for the tertiary amines in this complex, facilitating electrostatic interactions with the cell membrane and enhancing uptake.

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5 RADIOLABELLING OF 2,2'-BISDIPYRRINS

5.1 BACKGROUND

5.1.1 PET Imaging

Positron Emission Tomography (PET) imaging is a technique employed clinically in the diagnosis and progression monitoring of various diseases. Its diagnostic potential is attributed to the tailored design of molecular tracers capable of selectively imaging changes in metabolism, cell surface receptor expression, or blood perfusion, for example.¹ Combining PET imaging with structural imaging modalities such as magnetic resonance imaging (MRI) or computed tomography (CT) allows for the localisation of radionuclide to be correlated to structural features, giving anatomical information required for medical interventions.²⁻³

PET is a non-invasive imaging technique that requires pre-administration of a molecular tracer that has been radiolabelled with a positron emitting radionuclide into the patient. Positrons are the antiparticle of electrons, exhibiting the same mass but opposite charge, and result from the decay of a neutron-deficient radioisotope.⁴ A proton in the nucleus of the radionuclide is converted to a neutron, which is coupled to the release of an electron neutrino and a positron. The emitted positron travels a short distance before it collides with an electron in the surrounding environment, causing annihilation of both (Figure 5.1).

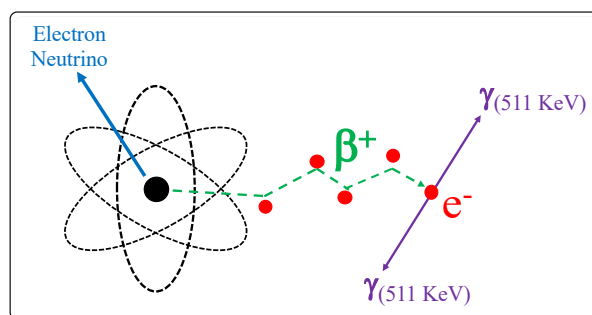


Figure 5.1. Schematic for positron emission from decay of a neutron-deficient radioisotope.

Positron-electron annihilation releases two 511 KeV γ -photons in opposite directions, which are measurable by scintillation detectors (Figure 5.2). In PET scanners, these are arranged in a ring such that each pair of colinear γ -photons can be detected.⁵ The resulting data provides information regarding the localisation of radionuclide in the detection field and concentration of radionuclide at each point.

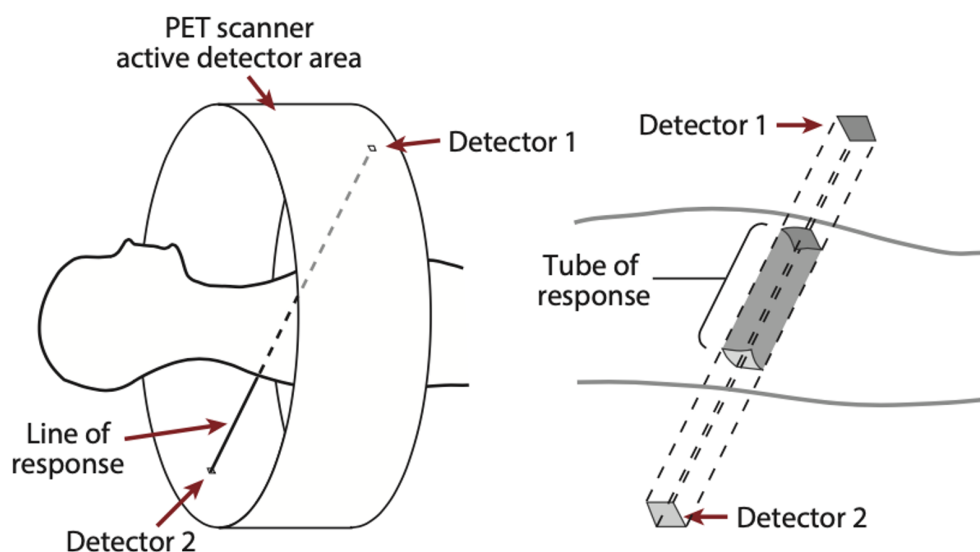


Figure 5.2. Schematic of a typical PET scanner detection ring (Reproduced with permission from Vaquero, J. J.; Kinahan, P., Annu. Rev. Biomed. Eng. 2015, 17 (1), 385-414).²

5.1.2 Copper(II) Radionuclides

Among the 32 isotopes of copper, five have been identified as useful in nuclear imaging and therapies. These isotopes range in half-lives from 9 minutes to 3 days and exhibit various modes of decay (Table 5.1), making them applicable for use in both diagnostics and therapeutics. In particular, the copper-64 radioisotope decays through β^+ emission, β^- emission and electron capture, and is of increasing interest in the development of PET imaging agents.

Table 5.1. Nuclear decay properties of various copper isotopes.⁶

Isotope	Half-Life	Mode of Decay (%) [#]	Major Radiation [KeV]
⁶⁰ Cu	23.7 min	β^+ (93)	1981
		EC (7)	1333
⁶¹ Cu	3.33 h	β^+ (62)	1215
		EC (38)	283, 656
⁶² Cu	9.67 min	β^+ (98)	2926
		EC (2)	
⁶⁴ Cu	12.7 h	β^+ (19)	653
		EC (41)	1346
		β^- (40)	579
⁶⁷ Cu	62.0 h	β^- (100)	395

[#] EC: Electron capture; β^+ decay: positron and electron neutrino emission; β^- decay: electron and electron antineutrino emission.

Design of suitable radiotracers through incorporation of copper-64 requires careful ligand design. A review into the favourable complexation of radiometals has described four key requirements for effective radiotracers incorporating transition metal radionuclides. These are:⁷

- Rapid complexation kinetics
- Mild complexation conditions
- High *in vivo* stability
- Ability for conjugation to targeting groups

A fine balance between kinetic inertness and rapid complexation must be achieved to ensure the labelling is rapid and efficient, while ensuring that the complex doesn't degrade following administration into the patient. Moreover, the stability and reactivity of the ligand must be considered to ensure that a large presence of excess chelator has no adverse side effects.

Labelling with copper-64 is typically conducted below pH 7 due to the precipitation of $\text{Cu}(\text{OH})_2$ limiting the radiolabelling.⁸⁻⁹ The addition of phosphate buffers also promotes precipitation of the radionuclide, and so many examples use buffers incorporating the acetate anion are often used. In the pH range of 3.8 to 5.8, the copper(II) remains soluble as either the aquo or acetate complex.¹⁰

5.1.3 Chapter Aims

2,2'-Bisdipyrins represent a new class of tetrapyrrole which, although structurally similar to the macrocyclic porphyrins, display a much lower kinetic barrier to complexation. We have shown these ligands exhibit rapid complexation kinetics, under mild conditions. Moreover, the activated ester present in $\text{H}_2\text{L}^{3-\text{OSu}}$ has already been shown to facilitate the ability to conjugate targeting groups, and the previously discussed cell work has supported our claims for sufficient stability *in vitro*.

There are, however, currently no examples of these ligands being radiolabelled, including with copper-64. The work presented in this chapter will explore the ability for the 2,2'-bisdipyrins synthesised herein to efficiently and rapidly chelate copper-64. Additional stability of the radiolabelled $[\text{}^{64}\text{Cu}]\text{CuL}^2$ will be investigated, and preliminary biodistribution of this complex will be assessed, to determine whether this family of complexes may indeed be suitable for *in vivo* imaging.

A pH of 5.5 will be used herein to favour complexation with respect to the needs of the ligand, as the complex is unstable below pH 5, while ensuring the pH is sufficiently low to maintain solubility of the copper-64.

5.2 RADIOLABELLING OF H_2L^2 WITH COPPER-64

H_2L^2 was radiolabelled with $[^{64}\text{Cu}]\text{CuCl}_2$ buffered in NH_4OAc (100 mM, pH 5.5), using 5 nmol of ligand in DMSO. A final concentration of 50% DMSO in the labelling mixture was used. The reaction mixture was analysed by TLC (SiO_2) using ethanol as the mobile phase. The activity of the plate was then measured as counts/min over 2 minutes, and then normalised to maximum activity. Under these conditions, unlabelled $[^{64}\text{Cu}]\text{Cu}^{2+}$ remains at the baseline ($R_f = 0$), and the lipophilic ligand and complex moves with the solvent front ($R_f = 1$). After 30 minutes of labelling at room temperature using 5 MBq of activity, a ~93% radiochemical yield was obtained (Figure 5.3) for $[^{64}\text{Cu}]\text{Cu}L^2$ ($R_f = 0.95$).

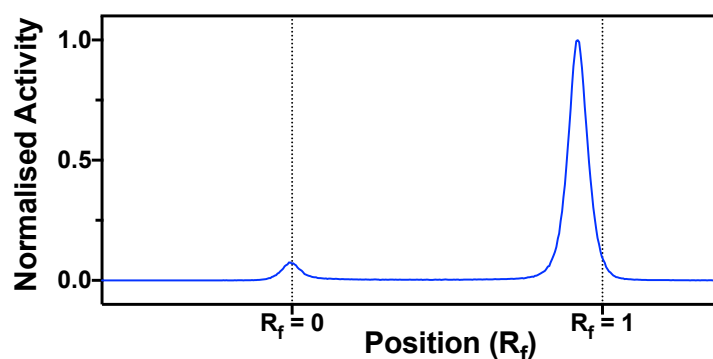


Figure 5.3. Radio TLC chromatogram of $[^{64}\text{Cu}]\text{Cu}L^2$ after 30 minutes incubation at room temperature (SiO_2 , ethanol).

This rapid complexation under mild conditions (room temperature, pH 5.5) is a distinct advantage compared to porphyrins investigated previously for radiochemical applications.¹¹⁻¹² The measured radiochemical yield also supports the hypothesis that these ligands may prove more versatile and useful in diagnostic imaging. A high radiochemical yield and purity is essential for translation into *in vivo* studies. This is to ensure that the localisation of radionuclide *in vivo* is due to the effect of the carrier ligand rather than contaminants or unlabelled copper-64.

5.2.1 Stability of $[^{64}\text{Cu}]\text{CuL}^2$

The stability of the radiolabelled complex $[^{64}\text{Cu}]\text{CuL}^2$ in solution was investigated in the presence of competing ligands. A sample of $[^{64}\text{Cu}]\text{CuL}^2$ (~ 1.3 nmol and ~ 1.3 MBq) was introduced into three separate solutions containing 25 nmol of either $\text{Na}_2\text{H}_2\text{EDTA}$, glutathione (GSH) or Cys.OMe, and incubated for a further 1 hour at room temperature before analysis by TLC (Figure 5.4).

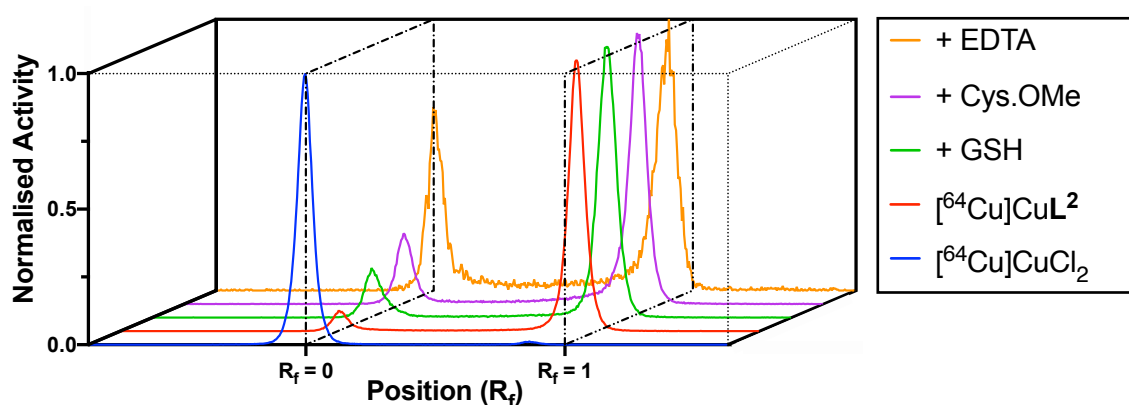


Figure 5.4. Radio TLC chromatograms (SiO_2 , ethanol) of $[^{64}\text{Cu}]\text{CuCl}_2$, $[^{64}\text{Cu}]\text{CuL}^2$, and $[^{64}\text{Cu}]\text{CuL}^2$ after 60 minutes incubation at room temperature with either GSH, $\text{Na}_2\text{H}_2\text{EDTA}$ or Cys.OMe.

Radiolabelled competitor controls were prepared under the same conditions as the labelling of H_2L^2 . Again, labelled $[^{64}\text{Cu}]\text{CuL}^2$ corresponds to the signal at R_f 0.95, while all other active species (unlabelled copper-64, competitor-bound copper-64) correspond to an R_f of 0. The increase in integration at $R_f = 0$ following administration of each competitor compared to the positive control (Figure 5.4, red line) is indicative of an increase in the free or competitor-bound copper-64 following incubation. The percent integration of each sample is given in Figure 5.5.

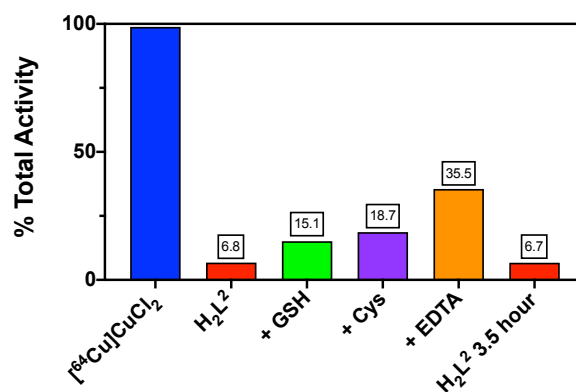


Figure 5.5. Percent total activity for unlabelled or competitor-bound copper-64 ($R_f = 0$) in each run as % total activity. TLC: SiO_2 , Ethanol.

The change in baseline integration ($\Delta\%$) compared to the competitor-free control is 8.3, 11.9 and 28.7% for GSH, Cys.OMe and EDTA, respectively. In the absence of competitors, $[^{64}\text{Cu}]\text{CuL}^2$ was stable under these conditions for 3.5 hours.

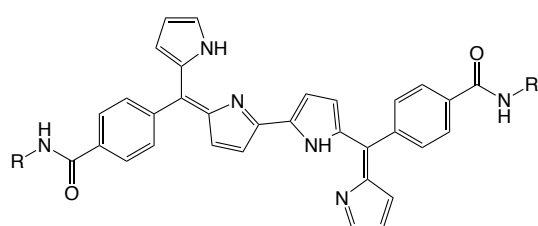
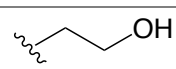
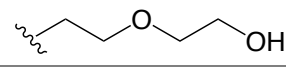
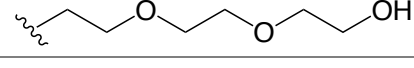
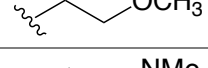
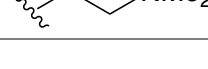
These results contradict the stability measurements discussed in Section 3.2.2, in which the selected challengers were unable to extract Cu(II) from CuL^2 when applied at ~ 33 -fold excess compared to both Cu^{2+} and $(\text{L}^2)^{2-}$. In this case, a lower excess of competitor (~ 20 -fold) compared to L^2 was not expected to cause decomplexation. The observed decomplexation under these conditions is therefore attributed to the lower working pH used during competitions. This would promote ligand protonation and assist in competitor-mediated decomplexation.

While this provides insight into the pH dependence of complex stability in the (2,2'-bisdipyrrinato) copper(II) complexes, it does not suggest these complexes would be less suitable for biological applications. Under normal physiological conditions, blood necessarily maintains a pH of between 7.35 and 7.45. It is therefore reasonable to claim, at physiological pH following intravenous administration, that CuL^2 would not release the radionuclide into general circulation and is therefore capable of carrying out a diagnostic imaging role.

5.3 RADIOLABELLING OF H_2L^{10-14} WITH COPPER-64

The precedent set by the labelling of H_2L^2 is indicative of the ability of 2,2'-bisdipyrins to coordinate the diminishingly small quantities of radionuclide in solution. Ligands H_2L^{10-14} incorporate additional ligand donor atoms in their peripheral substitution (Table 5.2). These ligands were radiolabelled under the same conditions as H_2L^2 (5 nmol ligand, 50% DMSO in NH_4OAc [100 mM, pH 5.5]) using $[^{64}Cu]CuCl_2$. For each ligand, the radiochemical purity was determined using reverse-phase HPLC (unchelated copper-64 is expected to elute with the solvent front at 2 – 3 minutes).

Table 5.2. Benzamidyl-ligands H_2L^{10-14} and corresponding R-groups used in radiolabelling experiments herein.

 H_2L^{10-14}	Ligand	R-group
	H_2L^{10}	
	H_2L^{11}	
	H_2L^{12}	
	H_2L^{13}	
	H_2L^{14}	

Labelling of H_2L^{10} showed incomplete labelling after 30 minutes with a peak at 5.95 minutes, tentatively attributed to the radiolabelled $[^{64}Cu]CuL^{10}$. Reanalysis after 140 minutes yields no peak at 5.95 minutes, instead producing a chromatogram representative of free copper-64 (Figure 5.6a).

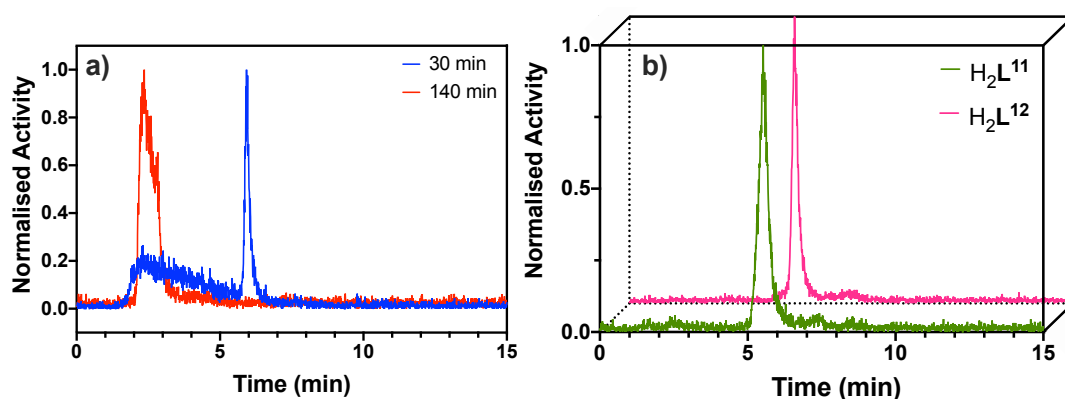


Figure 5.6. a) HPLC scintillation chromatograms of H_2L^{10} labelled with $[^{64}Cu]Cu^{2+}$ after 30 minutes (blue) and 140 minutes (red) of labelling. b) HPLC scintillation chromatograms of H_2L^{11-12} labelled with $[^{64}Cu]Cu^{2+}$ after 30 minutes (50–100% ACN in H_2O over 10 minutes; 100% ACN for additional 5 minutes).

The same labelling conditions for H_2L^{11} and H_2L^{12} showed negligible free $[^{64}Cu]Cu^{2+}$ after 30 minutes of labelling, eluting at 5.56 and 5.58 minutes, respectively (Figure 5.6b). In both cases, however, these peaks do not correlate to the cold standards of CuL^{11} or CuL^{12} analysed under the same conditions, suggesting a different species is being formed in the labelling experiment. Labelling of H_2L^{13} , on the other hand, produces a scintillation peak at 12.05 minutes with a radiochemical purity of more than 93% after 30 minutes of labelling, coinciding with the cold trace of CuL^{13} represented in the UV-chromatogram at 11.65 minutes (Figure 5.7).

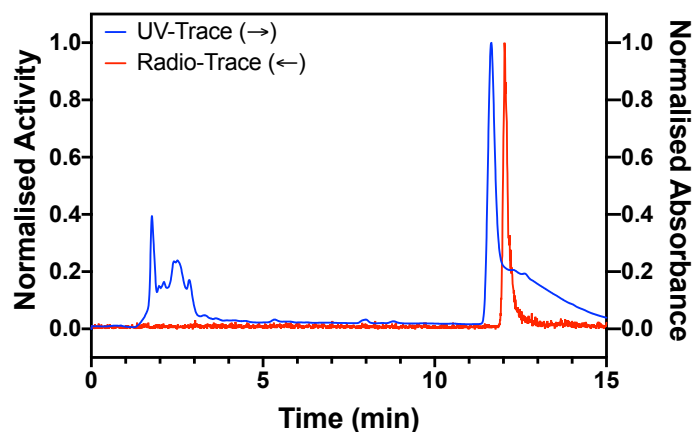


Figure 5.7. HPLC chromatograms of $[^{64}\text{Cu}]\text{CuL}^{13}$ (scintillation trace: red) and CuL^{13} (UV: blue) (50–100% ACN in H_2O over 10 minutes; 100% ACN for additional 5 minutes).

Differences in these peak retention times is a result of the sequential analysis within the instrumentation, and a difference of 0.4 minutes is typical for this set-up. From these results, methylation of the terminal hydroxyl groups in H_2L^{13} has enabled efficient labelling compared to $\text{H}_2\text{L}^{10-12}$.

To date, attempts at labelling H_2L^{14} have yet to produce definitive results. The radio TLC chromatogram (SiO_2 , ethanol) reveals that all activity corresponds to an R_f of 0, which is indistinguishable from unlabelled $[^{64}\text{Cu}]\text{Cu}^{2+}$ under these conditions (Figure 5.8).

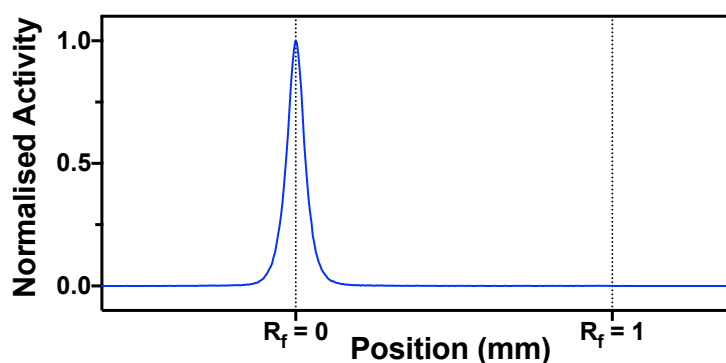


Figure 5.8. Radio TLC chromatogram of $[^{64}\text{Cu}]\text{CuL}^{14}$ after 30 minutes incubation at room temperature (SiO_2 , ethanol).

Further labelling investigations are required for H_2L^{14} , utilising different TLC conditions or reverse phase HPLC to differentiate $[^{64}\text{Cu}]\text{CuL}^{14}$ from free copper-64.

5.4 BIODISTRIBUTION OF CuL^2

Work conducted in this section was conducted Ms Jessica Van Zuylekon, Dr. Carlene Cullinane and Dr. Lachlan McInnes using ligands synthesised by the candidate.

The data presented herein for CuL^2 suggests the formation of a complex able to permeate biological membranes and resist decomplexation at physiological pH. As the first example of a (2,2'-bisdipyrinato) copper(II) complex labelled with copper-64, there have accordingly been no reports for the biodistribution *in vivo*.

Six female C57BL/6 mice were injected with $[\text{}^{64}\text{Cu}]\text{CuL}^2$ (2 – 3 MBq) in PBS (100 mM, pH 7.4) with 8% ethanol and 10% DMSO. Three mice were culled and dissected at 2 minutes post-injection (mpi) for organ radioactivity counting. The remaining three mice were imaged at 5 mpi and then culled for organ activity counting at 60 mpi. Results are presented as maximum standard uptake values (SUV_{max}) or percent injected dose per gram of tissue (%ID/g), as indicated. MicroPET images of the three mice 5 mpi are given below (Figure 5.9).

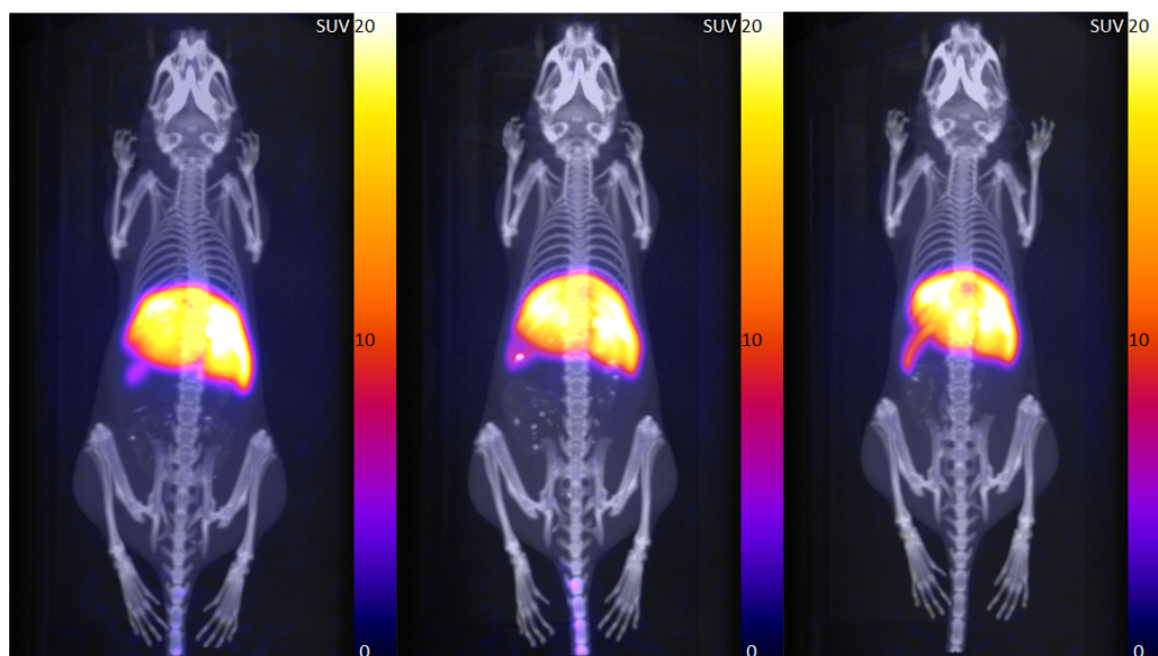


Figure 5.9. MicroPET images at 5 mpi of $[\text{}^{64}\text{Cu}]\text{CuL}^2$ of three female C57BL/6 mice.

The SUV_{max} is indicative of low accumulation in the target tissue through normalisation of activity concentration with respect to administered dose and subject body weight.¹³ An SUV_{max} of 1 would represent uniform distribution of activity across the entire body, while values of 2 or more indicates high specific uptake in the target organ or tissue.

Following administration of $[^{64}Cu]CuL^2$, residual radioactivity observed in the tail is a result of tail-vein injection for administration of the radiolabelled species. Low brain uptake is indicated by an SUV_{max} of 0.39 ± 0.07 , which suggests poor blood-brain barrier (BBB) permeability.

Inspection of the PET images reveals a significant accumulation the radionuclide in the liver. These results were compared directly to the biodistribution of $[^{64}Cu]Cu(OAc)_2$, which was administered under the same conditions, and analysed at both 2 and 60 mpi (Figure 5.10 and Table 5.3). The difference in brain uptake between $[^{64}Cu]CuL^2$ and $[^{64}Cu]CuCl_2$ is insignificant at both timepoints, further indicating poor BBB permeability.

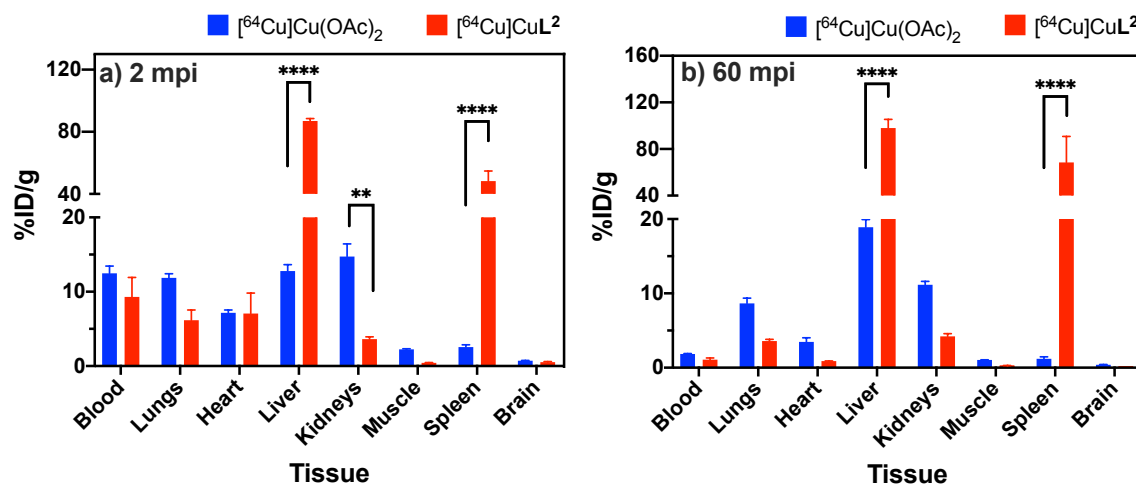


Figure 5.10. Biodistribution of $[^{64}Cu](OAc)_2$ and CuL^2 biodistribution (%ID/g $\pm$ SD) at a) 2 mpi and b) 60 mpi (** $p < 0.01$; **** $p < 0.0001$; $N = 3$).

Table 5.3. $[^{64}\text{Cu}](\text{OAc})_2$ and $[^{64}\text{Cu}]\text{CuL}^2$ biodistribution (%ID/g $\pm$ SD) at 2 mpi and 60 mpi.

	$[^{64}\text{Cu}]\text{Cu}(\text{OAc})_2$ (%ID/g)		$[^{64}\text{Cu}]\text{CuL}^2$ (%ID/g)	
	2 min	60 min	2 min	60 min
Blood	12.47 $\pm$ 1.69	1.84 $\pm$ 0.10	9.31 $\pm$ 4.54	1.08 $\pm$ 0.40
Lungs	11.86 $\pm$ 0.96	8.65 $\pm$ 1.24	6.16 $\pm$ 2.38	3.59 $\pm$ 0.40
Heart	7.17 $\pm$ 0.66	3.46 $\pm$ 0.95	7.07 $\pm$ 4.76	0.88 $\pm$ 0.03
Liver	12.78 $\pm$ 1.49	18.91 $\pm$ 1.77	86.99 $\pm$ 2.65	98.01 $\pm$ 12.84
Kidneys	14.73 $\pm$ 2.96	11.15 $\pm$ 0.81	3.62 $\pm$ 0.55	4.21 $\pm$ 0.64
Muscle	2.25 $\pm$ 0.10	1.05 $\pm$ 0.04	0.43 $\pm$ 0.07	0.29 $\pm$ 0.01
Spleen	2.55 $\pm$ 0.54	1.19 $\pm$ 0.47	48.32 $\pm$ 11.22	68.42 $\pm$ 38.66
Brain	0.72 $\pm$ 0.07	0.39 $\pm$ 0.06	0.52 $\pm$ 0.16	0.15 $\pm$ 0.01

Liver and spleen uptake of $[^{64}\text{Cu}]\text{CuL}^2$ is significantly ($p < 0.0001$) higher compared to $[^{64}\text{Cu}]\text{Cu}(\text{OAc})_2$ at both 2 and 60 mpi. At 2 mpi, liver and spleen uptake is $\sim$ 7-fold and $\sim$ 19-fold higher for $[^{64}\text{Cu}]\text{CuL}^2$, respectively. At 60 mpi, these values become $\sim$ 5-fold and $\sim$ 57-fold larger for $[^{64}\text{Cu}]\text{CuL}^2$, indicating superior hepatobiliary accumulation and metabolism for the complex relative to $[^{64}\text{Cu}]\text{Cu}(\text{OAc})_2$. This is seen in addition to a lower renal uptake, reflected by approximately 4- and 2-fold lower %ID/g at 2 and 60 mpi, respectively.

Hepatobiliary accumulation and excretion is characteristic of complexes with high lipophilicity or a reasonable affinity for blood-proteins,¹⁴⁻¹⁵ while accumulation in the renal system is often observed for ionic and hydrophilic species for excretion. The marked difference in renal and hepatobiliary uptake between the $[^{64}\text{Cu}]\text{CuL}^2$ and $[^{64}\text{Cu}]\text{Cu}(\text{OAc})_2$ suggests stability of the former *in vivo* and indicates the retention of the charge-neutral and lipophilic complex following administration.

5.5 SUMMARY

H_2L^2 was radiolabelled with copper-64 after 30 minutes in NH_4OAc (100 mM, pH 5.5) at room temperature, using 5 nmol of ligand in DMSO, with ~93% radiochemical purity. The resulting $[^{64}Cu]CuL^2$ is stable in solution for 3.5 hours, although the addition of competing ligands was able to increase free or competitor-bound copper-64 by up to 29%, attributed to the lower working pH of the sample.

Radiolabelling attempts using ligands H_2L^{10-12} produced results inconsistent with cold HPLC traces for the premade complexes. $[^{64}Cu]CuL^{10}$ was additionally shown to decompose through an unknown mechanism after 2 hours of labelling, indicating their reduced capacity for radiolabelling under these conditions.

Ligand H_2L^{13} was radiolabelled with relative ease under the same conditions used for H_2L^2 to produce $[^{64}Cu]CuL^{13}$ with ~93% radiochemical purity. The terminal hydroxyl groups in ligands H_2L^{10-12} may impair their labelling compared to H_2L^2 and H_2L^{13} . Unfortunately, H_2L^{14} has yet to be categorically labelled, potentially due to changes in ligand and complex solubility.

Nevertheless, the rapid complexation of H_2L^2 under mild conditions is distinguished from previous reports of porphyrin labelling, in which harsh or complex labelling conditions were required. Despite the loss of stability associated with the macrocyclic nature of porphyrin ligands, $[^{64}Cu]CuL^2$ was expected to retain its structure under physiological conditions. Significant differences in kidney and liver uptake at 2 and 60 minutes post-injection when compared with $[^{64}Cu]Cu(OAc)_2$ further support this. These results suggest either interactions with blood proteins or a lipophilicity-driven accumulation in the hepatobiliary system for metabolism and excretion.

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6 CONCLUDING REMARKS

Macrocyclic tetrapyrroles are characterised by their unique spectroscopic properties and their ability to form highly stable metal complexes, essential for carrying out substrate binding and catalysis *in vivo*. Their prevalence in biology suggested they may be useful in the development of highly stable radiotracers for imaging disease. Unfortunately, the difficulty with which metalloporphyrins are synthesised has limited this somewhat. Radiolabelling porphyrins has, thus far, typically required conditions which are considered incompatible for biological targeting groups.

The 2,2-bisdipyrins have been explored herein as an alternative to their macrocyclic counterparts. Synthetically, (2,2'-bisdipyrinato) metal(II) complexes were obtainable from room temperature complexation reactions. These complexes were found to show a high degree of planarity, indicated by a refined τ_4 parameter in the range of 0.05 to 0.13, representing a square-planar coordination geometry about the metal ion. The planarity deviates from formal square-planar geometry due to distortions imposed by metal ionic radius and steric clashing of two terminal hydrogen atoms, as indicated by crystallography.

CuL^2 forms a 1:1 complex, even when synthesised in aqueous solutions, with a K_D of $6 \pm 1 \times 10^{-15}$ M at pH 7.4 and ambient temperature. Cysteine, histidine, glutathione and EDTA were all unable to cause decomplexation under these conditions, suggesting a high barrier to decomplexation for these complexes.

Electrochemistry reveals conservation of a number of ligand-based processes for all *meso*-phenyl substituted (2,2'-bisdipyrinato) copper(II) complexes at ca. -1.1 V and -1.7 V vs. Fc^+/Fc . These processes were expected to confer some stability to reduction intracellularly, and indeed ATP7A localisation imaging experiments in SKOV-3 suggest this to be true.

Decomplexation by reduction has only been demonstrated once herein. In this case, the use of $\text{Na}_2\text{S}_2\text{O}_4$ and Bca to effect decomplexation was only observable to a small extent. The use of spectroscopy in this instance is somewhat limited by the assumption that a reduced form of the complex would be considerably distinct spectroscopically from its native form. It is clear however, that the presence of proteins in solution offers some protection against the $\text{Na}_2\text{S}_2\text{O}_4$ -mediated reduction in absorbance at 786 nm. Hydrophobic interactions between BSA and H_2L^2 have been evidenced by fluorescence spectroscopy, and the effect on solubility for both H_2L^2 and CuL^2 is further indicative of such interactions.

Based on these findings, we hoped to explore the ability for this new family of copper(II) complexes to cross cell membranes. Unsurprisingly, modifications to peripheral substituents resulted in dramatic alterations to membrane permeability. For CuL^3 , the presence of ionisable carboxylic acid substituents would result in a dianionic complex at physiological pH which is incapable of crossing the cell membrane of SKOV-3 cells. Incorporation of the longest oligo(ethylene glycol) side group in CuL^{12} also resulting in lower membrane permeability, for reasons that are largely unclear. Complexes CuL^{10-12} incorporating these oligo(ethylene glycol) side groups were also unable to be radiolabelled under the conditions explored, making them poor candidates for future study as biological imaging agents.

On the other hand, complexes CuL^2 , CuL^{13} and CuL^{14} all showed membrane permeability in LNCaP cells. CuL^2 and CuL^{13} show similar degrees of uptake at all concentrations, representing a 40-fold increase in intracellular copper levels following treatment with 50 μM of complex for 1 hour compared to the vehicle control. CuL^{14} produced a response 270-fold higher than the control at the same treatment concentration, which is ascribed a result of the enhanced solubility or the low pK_b of the tertiary amines facilitating electrostatic interactions with the cell membrane prior to uptake.

The rapid complexation kinetics of H_2L^2 under mild conditions favour incorporation of the copper-64 radionuclide that would preclude the need for extensive radiolabelling purification. Assuming that the various copper(II) complexes would exhibit similar stability to CuL^2 , modulation of substitution would be sufficient in some cases to alter biodistribution *in vivo*. The activated ester derivative, $\text{H}_2\text{L}^{3\text{-OSu}}$ would also facilitate conjugation of this tetrapyrrole to biological targeting groups equipped with primary amines, which would expectantly confer selectivity for a specific target tissue in disease. Indeed, work has already begun to show that small peptides bearing a non-essential primary amine can be linked to this chelator with dramatic effects on solubility.

The *in vivo* stability of $[\text{}^{64}\text{Cu}]\text{CuL}^2$ is an excellent indicator of the potential efficacy of the bisdipyrins as molecular tracers. Biodistribution experiments using $[\text{}^{64}\text{Cu}]\text{CuL}^2$ revealed marked change in distribution compared to a $[\text{}^{64}\text{Cu}]\text{Cu}(\text{OAc})_2$ control. This is characterised by enhanced hepatic uptake and reduced kidney uptake, attributed to retention copper-64 chelation following administration. Poor brain uptake was consistent for both studies, although this may simply be a result of the high liver accumulation.

Future research into the 2,2'-bisdipyrins requires further investigations of H_2L^{13} and H_2L^{14} , the two less-lipophilic analogues of H_2L^2 with similar and superior cell uptake, respectively. An enhanced understanding of the intracellular redox processing and metabolism of these complexes is also of interest, given reports of copper(I) release following administration of $\text{Na}_2\text{S}_2\text{O}_4$ and subsequent ligand-based reduction.

7 EXPERIMENTAL

7.1 GENERAL METHODS

All reagents and solvents were obtained from standard commercial chemical suppliers, and unless indicated otherwise, were used as received. DMSO and ACN used in spectroscopy experiments were of spectroscopic grade. An atomic absorption spectroscopy (AAS) standard of Cu^{2+} in 0.1% HNO_3 (Fluka Analytical) was used in the copper(II) titration experiments.

ESI-HRMS spectra were collected using a Thermo Scientific Q ExactiveTM Plus Hybrid Quadrupole-OrbitrapTM Mass Spectrometer (Thermo Scientific, Australia) equipped with a Dionex UltiMate 3000 UHPLC pump and autosampler. Analysis was performed using Qual Browser, Thermo Xcalibur 2.2 SP1.48 (Thermo Scientific, 2011), and calculated exact masses obtained using the isotope simulation function.

^1H and ^{13}C $\{^1\text{H}\}$ NMR spectra were recorded with either an Agilent FT-NMR 400 MHz spectrometer with autosampler, an Agilent FT-NMR 500 MHz spectrometer (Agilent, CA, USA), a Varian INOVA FT-NMR 600 MHz spectrometer (Varian, CA, USA) or a Bruker Avance III 600 MHz spectrometer with a TCI cryoprobe and BACS-60 autosampler (Bruker, MA, USA). ^1H NMR were acquired at 400, 500 or 600 MHz, and ^{13}C NMR were acquired at 101, 126 or 151 MHz, as indicated. All NMR spectra were acquired at ambient temperature and referenced to the residual solvent shifts, relative to TMS. Solvent impurities were assigned according to reported signals in deuterated solvents.¹

Elemental analysis was conducted on samples dried *in vacuo* at 45°C overnight. Samples were analysed by the Campbell Microanalytical Laboratory, Chemistry Department, University of Otago, Dunedin, New Zealand.

Cyclic voltammetry was conducted using an AUTOLAB PGSTAT100 alongside GPES V4.9 software. Measurements were carried out at approximately 1.0 mM concentrations in dry, deoxygenated DMF with $[\text{Bu}_4\text{N}]\text{PF}_6$ electrolyte (100 mM), as indicated. A glassy carbon working electrode, a platinum wire counter electrode and a leakless Ag/Ag^+ reference electrode were used. Ferrocene was used as an internal reference ($E_{\text{m}}(\text{Fc}^+/\text{Fc})_{\text{DMF}} = 0.00 \text{ V}$).

UV-Visible absorption spectra were acquired with a Shimadzu UV-1650PC UV-Visible Spectrophotometer equipped with a Shimadzu TCC-240A Temperature controlled cell holder (Shimadzu, Kyoto, Japan). Glove box assays were analysed using a Varian Cary50 Bio UV-Visible spectrophotometer (Varian, CA, USA). Unless otherwise stated, all spectra were acquired at ambient temperature.

Fluorescence spectra were acquired using a Varian CARY50 Eclipse Fluorescence spectrophotometer at ambient temperature. All spectra were recorded at ambient temperature, and absorbance of the peak used for excitation was maintained below an absorbance of 0.1 to minimise inner-filter effects. Slit widths of 5 and 10 nm were selected for the excitation and emission filters, respectively, using a medium photomultiplier tube voltage and slow scan speed.

ICP-MS analysis was conducted on an Agilent 8900 Triple Quadrupole ICP-mass spectrometer. Samples were introduced using an Agilent Integrated Autosampler and Micromist nebuliser (Agilent, CA, USA) using a helium gas flow rate of 5 mL/min. Concentrations were determined using an Agilent Multi Element Calibration Standard (2A, PN 8500-6940, Lot # 13-05YPY2). Matrix interference was monitored by recovery of an internal standard (Agilent Yttrium Standard; 1000 $\mu\text{g/mL}$ Y in 5% HNO_3 , PN 5190-8555, Lot # 0016818672) which was diluted to a concentration of 100 ppb using

1% HNO₃. Data was processed using Agilent MassHunter 4.5 Workstation software (vC.01.05, build 588.3) (Agilent, CA, USA).

Single crystal X-ray diffraction data for all samples were collected as follows: a typical crystal was mounted on a MiTeGen Micromount using high viscosity oil and cooled rapidly to the indicated temperature in a stream of nitrogen gas using an Oxford Cryostream cooling device. Diffraction data were collected (ω -scans) on a Rigaku XtaLAB Synergy diffractometer equipped with a HyPix detector using Cu-K α radiation ($\lambda = 1.54184 \text{ \AA}$). Raw frame data were reduced using CrysAlisPro. The structures were solved using SHELXT,² and the refinement was carried out with SHELXL (version 2018/3)³ employing full-matrix least-squares on F² using the OLEX2 software package.⁴ All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were placed in geometrically calculated positions and refined using a riding model.

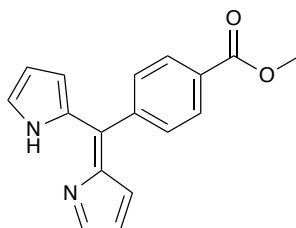
All spectra and graphs of data presented herein were prepared using GraphPad Prism version 8.4.3 for Macintosh, (GraphPad Software, San Diego, California USA).

7.2 SYNTHESIS

7.2.1 Previous Work

The work contained in Section 7.2.1 was conducted by the candidate during an honours project.

5-(4-methoxycarbonylphenyl)-4,6-dipyrin, HL¹:



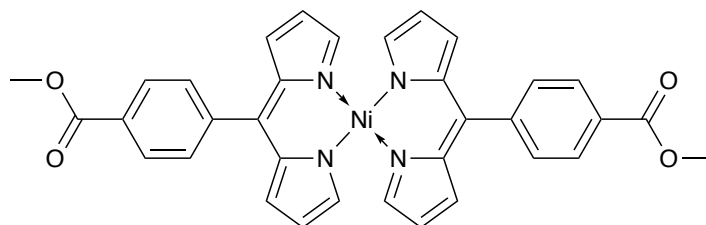
The precursor dipyrin was synthesised per a general procedure adapted from literature methods as follows.⁵⁻⁶

A solution of 0.18 M HCl (100 mL) containing pyrrole (0.70 g, 10 mmol, 3 eq.) was treated over 15 minutes with methyl-4-formyl benzoate (0.57 g, 3.5 mmol, 1 eq.) in methanol (25 mL). The mixture was stirred at room temperature until the aldehyde was consumed, as indicated by TLC (SiO₂, CH₂Cl₂). The lavender-grey 5-(4-methoxycarbonylphenyl)-4,6-dipyrromethane was collected by filtration, washing with H₂O (2 x 250 mL) and petroleum spirits (1 x 250 mL). This was carried through to the next step without further purification.

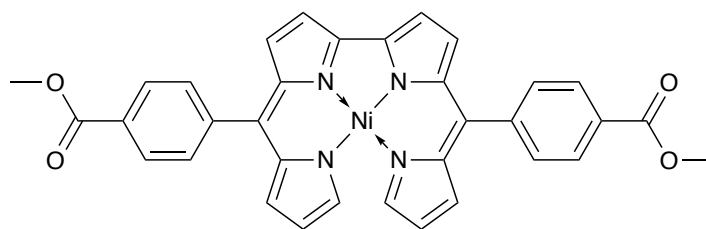
The dipyrromethane was dissolved in a 2:1 CHCl₃/acetone mixture (60 mL) and cooled on ice. To this, 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (1.45 g, 6.4 mmol, 1.1 eq. to crude) in acetone (20 mL) was added and the reaction was stirred on ice for 1 hour. The solvent was removed under reduced pressure, and the resulting solids triturated with a cold CHCl₃/CH₂Cl₂ mixture (4:1) before collecting by filtration. The crude was washed with cold CHCl₃ and dried, after which it was adsorbed onto alumina using a 5% NEt₃ solution in CH₂Cl₂ (10 g of Al₂O₃ per 1 g of crude material). Purification by column chromatography

(Al₂O₃ [Basic, Brockmann Activity V], CH₂Cl₂) gave analytically pure dipyrin as a dark yellow solid (0.502 g, 52% yield over two steps). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 8.15 – 8.06 (m, 2H), 7.65 (t, J = 1.2 Hz, 2H), 7.61 – 7.53 (m, 2H), 6.52 (dd, J = 4.3, 1.1 Hz, 2H), 6.40 (dd, J = 4.2, 1.4 Hz, 2H), 3.97 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ (ppm) 166.9, 144.3, 142.1, 140.8, 140.6, 130.9, 130.7, 129.1, 128.7, 118.2, 52.5. ESI-HRMS (*m/z*) [M+H]⁺: 279.1125, calculated for (C₁₇H₁₅N₂O₂)⁺: 279.1128.

bis(5-(4-methoxycarbonylphenyl)-4,6-dipyrinato) nickel(II), [Ni(L¹)₂]:

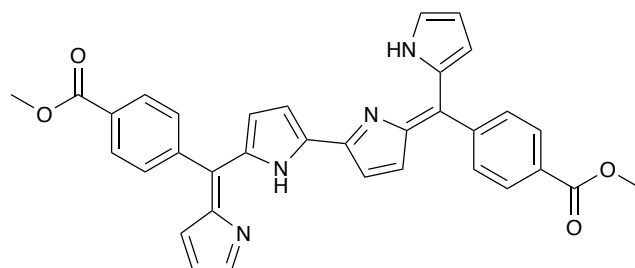


The 5-(4-methoxycarbonylphenyl)-4,6-dipyrin (0.300 g, 1.1 mmol) was dissolved in min. methanol and stirred at room temperature. To this, Ni(OAc)₂·4H₂O (0.134 g, 0.54 mmol, 0.5 eq.) in min. methanol was added slowly, and the reaction stirred at room temperature for 4 hours. The reaction was concentrated under reduced pressure to half the original volume and cooled on ice. The resulting solids were collected by filtration, washed twice with water, followed by a small portion of cold methanol, and dried to give [Ni(L¹)₂] as a dark red solid (0.201 g, 61%). ¹H NMR (400 MHz, CDCl₃) δ (ppm) 10.45 (s, 4H), 8.10 – 8.01 (m, 8H), 7.45 – 7.43 (m, 4H), 6.68 (s, 4H), 3.96 (s, 6H). ¹³C NMR (126 MHz, CDCl₃) δ (ppm) 178.43, 166.81, 151.55, 142.01, 141.26, 140.12, 138.98, 130.83, 130.66, 128.78, 52.46.

2,2'-bis(6-(4-methoxycarbonylphenyl)-5,7-dipyrrinato) nickel(II), NiL².

The nickel(II) bisdipyrrin complex was synthesised according to a literature procedure.⁷ Identity was confirmed by comparison to reported analytical data.

Dark brown solid; 55% yield. ¹H NMR (500 MHz, CDCl₃) δ (ppm) 8.18 – 8.12 (m, 4H), 7.67 – 7.61 (m, 4H), 6.74 (dd, J = 4.4, 1.2 Hz, 2H), 6.68 (d, J = 4.4 Hz, 2H), 6.64 (d, J = 4.4 Hz, 2H), 6.45 (dd, J = 4.4, 1.6 Hz, 2H), 5.96 (t, J = 1.5 Hz, 2H), 3.99 (s, 6H). ¹³C NMR (126 MHz, CDCl₃) δ (ppm) 166.84, 161.65, 154.02, 142.25, 141.41, 138.75, 135.65, 134.75, 131.04, 130.85, 130.07, 129.05, 117.58, 115.80, 52.51. ESI-HRMS (*m/z*) [M+H]⁺: 611.1220, calculated for (C₃₄H₂₅N₄NiO₄)⁺: 611.1224.

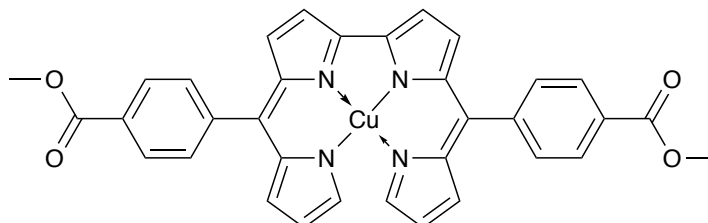
2,2'-bis(6-(4-methoxycarbonylphenyl)-5,7-dipyrrin), H₂L².

Ligand H₂L² was synthesised following the reported protocol.⁷ Identity was confirmed by comparison to reported analytical data.

Dark blue solid; 92% yield. ¹H NMR (500 MHz, CDCl₃) δ (ppm) 8.18 – 8.11 (m, 4H), 7.63 – 7.60 (m, 6H), 7.00 (d, J = 4.3 Hz, 2H), 6.70 (d, J = 4.3 Hz, 2H), 6.52 (dd, J = 4.1, 1.2 Hz, 2H), 6.43 – 6.41 (m, 2H), 3.98 (s, 6H). ¹³C NMR (126 MHz, CDCl₃) δ (ppm) 166.69, 152.84, 145.12, 141.82, 139.27, 139.11, 138.99,

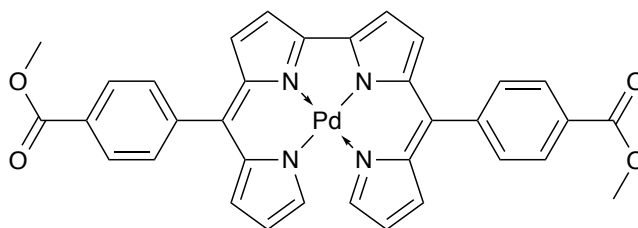
131.55, 130.87, 130.58, 128.97, 126.42, 120.41, 116.36, 52.33. ESI-HRMS (m/z) $[M+H]^+$: 555.2029, calculated for $(C_{34}H_{27}N_4O_4)^+$: 555.2027.

2,2'-bis(6-(4-methoxycarbonylphenyl)-5,7-dipyrinato) copper(II), CuL².



To a 25 mL solution of H_2L^2 (200 mg, 0.36 mmol) in CH_3OH , solid $Cu(OAc)_2 \cdot H_2O$ (80 mg, 0.40 mmol) was added. The reaction was stirred at ambient temperature overnight, after which the solvent was removed under reduced pressure. The resulting solids were triturated with a methanol/water mixture (approx. 1:20), before filtering. The solids were washed thoroughly with petroleum spirits and dried *in vacuo* to give CuL^2 as a dark brown solid (150 mg, 68%). ESI-HRMS (m/z) $[M+H]^+$: 616.1175, calculated for $(C_{34}H_{25}CuN_4O_4)^+$: 616.1166. Elemental Analysis: C, 65.98; H, 4.09; N, 8.99. Calc. for $C_{34}H_{24}N_4O_4Cu$: C, 66.28; H, 3.93; N, 9.09. Crystals of suitable quality for x-ray crystallography were grown by slow evaporation of a toluene solution of CuL^2 .

7.2.2 Current Work

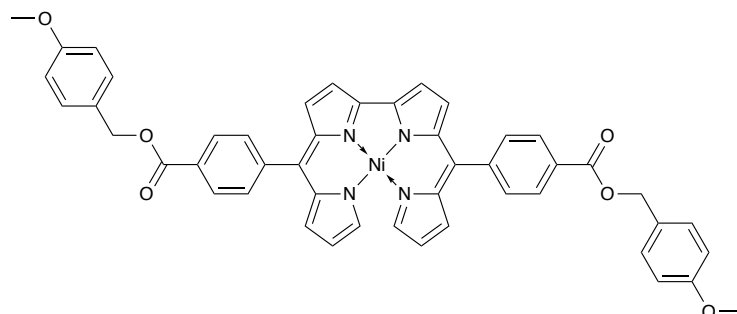
2,2'-bis(6-(4-methoxycarbonylphenyl)-5,7-dipyrrinato) palladium(II), PdL².

A solution of H₂L² (50 mg, 0.090 mmol) in CHCl₃ (5 mL) was added to a solution of Pd(OAc)₂ (30 mg, 0.13 mmol, 1.5 eq.) in methanol (5 mL), and the reaction left to stir at room temperature for 2 hours. The solvent was removed under reduced pressure, and the resultant solids triturated with cold methanol (2 mL), before filtering, washing with cold methanol (4 x 2 mL) and drying to give PdL² as a dark brown solid (48 mg, 81%). ¹H NMR (500 MHz, CDCl₃) δ (ppm) 8.12 (d, *J* = 7.9 Hz, 4H), 7.57 (d, *J* = 7.9 Hz, 4H), 7.17 (s, 2H), 6.52 (d, *J* = 4.4 Hz, 2H), 6.47 (d, *J* = 4.4 Hz, 2H), 6.36 (d, *J* = 4.4 Hz, 2H), 6.31 (d, *J* = 4.4 Hz, 2H), 3.98 (s, 6H). ¹³C NMR (126 MHz, CDCl₃) δ (ppm) 166.80, 157.17, 153.63, 145.11, 141.80, 138.28, 135.06, 134.97, 130.81, 130.68, 128.97, 128.48, 117.57, 115.88, 52.50. ESI-HRMS (*m/z*) [M+H]⁺: 659.0924, calculated for (C₃₄H₂₅N₄O₄Pd)⁺: 659.0905.

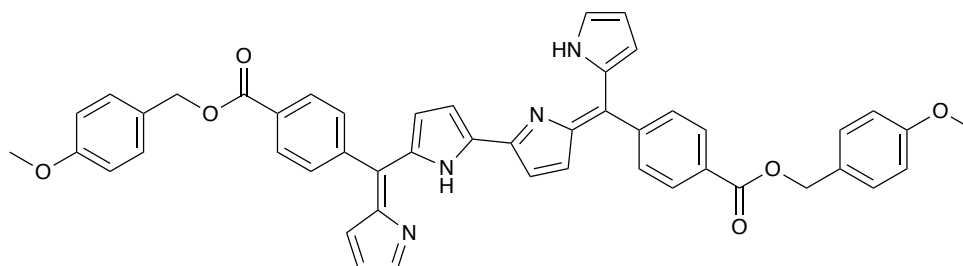
Towards H_2L^3 .

2,2'-bis(6-(4-(*p*-methoxybenzyloxy)carbonylphenyl)-5,7-dipyrrinato) nickel(II),

NiL^{2-PMB} .

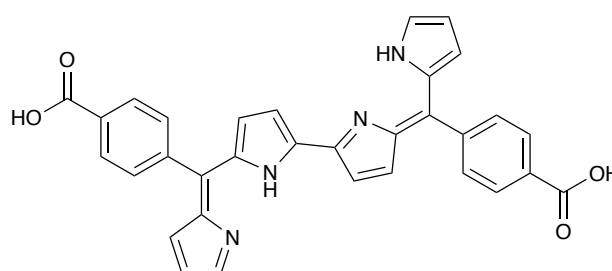


A solution of NiL^2 (0.503 g, 0.867 mmol) in toluene (50 mL) was combined with a separate toluene solution (10 mL) containing Otera's catalyst (0.224 g, 0.173 mmol).⁸ To this, *p*-methoxybenzyl alcohol (2.63 g, 19.0 mmol) in toluene (50 mL) was added, and the reaction stirred at reflux for 48 hours. The crude reaction mixture was taken to dryness, and purified by flash chromatography (SiO_2 , CH_2Cl_2), to give the *p*-methoxybenzylester derivative, NiL^{2-PMB} , as a dark brown solid (0.512 g, 76%). Crystals were grown by slow evaporation of a $CHCl_3$ solution of NiL^{2-PMB} . ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.16 (d, $J = 7.9$ Hz, 4H), 7.62 (d, $J = 8.0$ Hz, 4H), 7.43 (d, $J = 8.2$ Hz, 4H), 6.94 (d, $J = 8.4$ Hz, 4H), 6.72 (d, $J = 4.3$ Hz, 2H), 6.67 – 6.61 (m, 4H), 6.47 – 6.40 (m, 2H), 5.97 – 5.91 (m, 2H), 5.36 (s, 4H), 3.83 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm) 166.05, 161.44, 159.73, 153.82, 142.06, 141.26, 138.55, 135.46, 134.55, 130.83, 130.78, 130.16, 129.86, 128.95, 127.98, 117.38, 115.60, 114.01, 66.81, 55.29. ESI-HRMS (m/z) $[M]^+$: 822.1980, calculated for $(C_{48}H_{36}N_4O_6Ni)^+$: 822.1981.

2,2'-bis(6-(4-(*p*-methoxybenzyloxy)carbonylphenyl)-5,7-dipyrrin), H_2L^{2-PMB} .

Ligand H_2L^{2-PMB} was synthesised according to reported procedures for the methoxycarbonylphenyl bisdipyrrin, H_2L^2 (quant.).⁷

^1H NMR (500 MHz, $CDCl_3$) δ (ppm) 8.18 – 8.13 (m, 4H), 7.64 – 7.57 (m, 6H), 7.46 – 7.40 (m, 4H), 6.99 (d, $J = 4.4$ Hz, 2H), 6.98 – 6.91 (m, 4H), 6.68 (d, $J = 4.4$ Hz, 2H), 6.50 (dd, $J = 4.2, 1.2$ Hz, 2H), 6.41 (dd, $J = 4.1, 1.8$ Hz, 2H), 5.36 (s, 4H), 3.83 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm) 166.08, 159.73, 152.83, 145.10, 141.84, 139.23, 139.11, 138.96, 131.53, 130.83, 130.69, 130.17, 129.05, 128.01, 126.39, 120.39, 116.33, 114.02, 66.81, 55.30. ESI-HRMS (m/z) $[M+H]^+$: 767.2853, calculated for $(C_{48}H_{39}N_4O_6)^+$: 767.2863.

2,2'-bis(6-(4-carboxylphenyl)-5,7-dipyrrin), H_2L^3 .Method A:

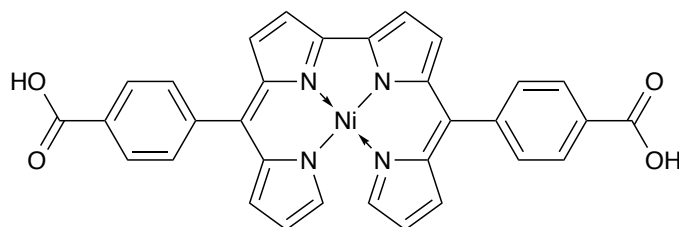
H_2L^{2-PMB} (0.219 g, 0.285 mmol) was dissolved in a 10% TFA in CH_2Cl_2 solution (50 mL), and the reaction was stirred at room temperature for 4 hours. The reaction mixture was diluted with Et_2O (150 mL), and the product extracted into 1 M aqueous NaOH, washing twice with Et_2O (2 x 50 mL). The coloured product was then extracted into EtOAc

following acidification using 1 M HCl. The organics were washed twice with water (50 mL) and once with brine (50 mL) before drying over MgSO₄. The solvent was removed under reduced pressure to give H₂L³ as a dark green solid (0.149 g, 99%).

Method B:

To 70 mL of CH₂Cl₂ containing H₂L² (774 mg, 1.4 mmol), 7.5 mL of NaOH in CH₃OH (3 N, 16 eq.) was added and the reaction was stirred at room temperature for 48 hours. The solvent was removed under reduced pressure, and the resulting solids dissolved in 20 mL H₂O, before washing with Et₂O (2 x 20 mL). The aqueous layer was acidified using 1 M HCl until the intensely coloured product extracted into EtOAc. The aqueous layer was washed with EtOAc (2 x 10 mL), before washing the organics with brine (1 x 25 mL) and drying over MgSO₄. The solvent was removed under reduced pressure to give H₂L³ as a dark green/blue solid (0.693 g, 94%). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm) 8.09 (d, *J* = 7.9 Hz, 4H), 7.70 (s, 2H), 7.65 (d, *J* = 7.8 Hz, 4H), 7.53 (d, *J* = 4.5 Hz, 2H), 6.76 (d, *J* = 4.5 Hz, 2H), 6.40 (s, 4H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm) 166.99, 147.32, 141.12, 139.45, 133.26, 131.31, 130.82, 130.74, 128.88, 128.78, 128.65, 124.59, 123.46, 114.08. ESI-HRMS (*m/z*) [M+H]⁺: 527.1721, calculated for (C₃₂H₂₃N₄O₄)⁺: 527.1714.

2,2'-bis(6-(4-carboxyphenyl)-5,7-dipyrrinato) nickel(II), NiL³.



A solution of NiL² (322 mg, 0.526 mmol) in 27 mL of CH₂Cl₂ was treated with 3 mL of a 3 N solution of NaOH in CH₃OH. The reaction was stirred at room temperature

overnight, at which point TLC indicated consumption of starting material (SiO_2 , CH_2Cl_2 ; $R_f[\text{NiL}^2] = 0.8$, $R_f[\text{NiL}^3] = 0$). The reaction mixture was diluted with 70 mL of CH_2Cl_2 , and the product was extracted into H_2O (150 mL). After further extraction with H_2O (2 x 50 mL) the combined aqueous layer was neutralised using 9 mL of 1 M HCl. The solution was allowed to stand at room temperature for 30 minutes, and the resultant precipitate was filtered, washed with small portions of H_2O and dried to give NiL^3 as a dark brown solid (286 mg, 93%). Crystals were obtained by passing DMF through the sintered-glass frit used in the filtration and allowing the solution to stand open to air overnight. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ (ppm) 8.09 (d, $J = 7.8$ Hz, 4H), 7.68 (d, $J = 7.8$ Hz, 4H), 6.81 (d, $J = 4.3$ Hz, 2H), 6.73 (d, $J = 4.4$ Hz, 2H), 6.69 (d, $J = 4.3$ Hz, 2H), 6.55 (d, $J = 4.4$ Hz, 2H), 6.00 (s, 2H). ESI-HRMS (m/z) $[\text{M}+\text{H}]^+$: 583.0908, calculated for $(\text{C}_{32}\text{H}_{21}\text{N}_4\text{NiO}_4)^+$: 583.0911.

General Synthesis of Dipyrins, HL^{4-6} :

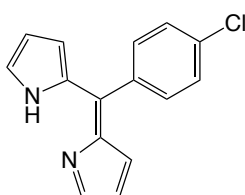
Dipyrins were synthesised per a general procedure adapted from literature procedures as follows.⁵⁻⁶

A solution of 0.18 M HCl (50 mL) containing pyrrole (1.05 g, 21 mmol, 3 eq.) was treated with the selected aldehyde (7 mmol, 1 eq.) in methanol (25 mL). The mixture was stirred vigorously at room temperature until the aldehyde was consumed, as indicated by TLC (SiO_2 , CH_2Cl_2). The solid dipyrromethane was collected via vacuum filtration, washed twice with water, followed by petroleum spirits and dried. This was carried through to the next step without further purification.

The dipyrromethane was dissolved in a 2:1 CHCl_3 /acetone mixture (60 mL) and cooled on ice. To this, DDQ (1.75 g, 7.7 mmol, 1.1 eq) in acetone (20 mL) was added and the reaction was stirred on ice for 1 hour. The solvent was removed under reduced pressure,

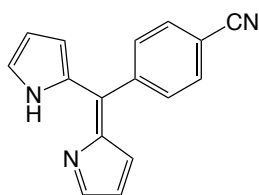
and the resulting solids triturated with a cold $\text{CHCl}_3/\text{CH}_2\text{Cl}_2$ mixture (4:1) before filtering. The crude was washed with cold CHCl_3 , dried and then adsorbed onto alumina using a 5% NEt_3 solution in CH_2Cl_2 . Purification by column chromatography (Al_2O_3 [Basic, Brockmann Activity V], CH_2Cl_2) gave analytically pure dipyrin as a dark yellow solid or oil, as indicated

5-(4-chlorophenyl)-4,6-dipyrin, HL^4 .

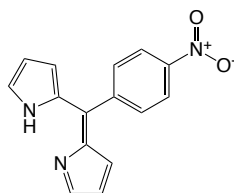


Dark yellow oil which crystallised on standing; 24% yield. ^1H NMR (600 MHz, CDCl_3) δ (ppm) 7.65 (t, $J = 1.3$ Hz, 2H), 7.43 (s, 4H), 6.57 (dd, $J = 4.1, 1.1$ Hz, 2H), 6.41 (dd, $J = 4.2, 1.5$ Hz, 2H). ^{13}C NMR (151 MHz, CDCl_3) δ (ppm) 144.05, 140.89, 140.48, 135.84, 135.21, 132.15, 128.63, 128.07, 117.99. ESI-HRMS (m/z) $[\text{M}+\text{H}]^+$: 255.0685, calculated for $(\text{C}_{15}\text{H}_{12}\text{N}_2\text{Cl})^+$: 255.0684.

5-(4-cyanophenyl)-4,6-dipyrin, HL^5 .



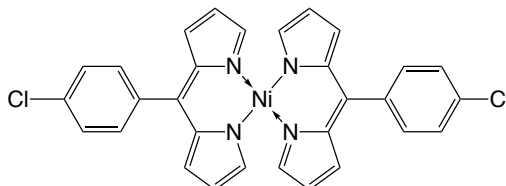
Dark yellow solid; 12% yield. ^1H NMR (400 MHz, CDCl_3) δ (ppm) 7.77 – 7.74 (m, 2H), 7.67 (d, $J = 1.3$ Hz, 2H), 7.63 – 7.59 (m, 2H), 6.47 (dd, $J = 4.2, 1.1$ Hz, 2H), 6.41 (dd, $J = 4.2, 1.4$ Hz, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ (ppm) 144.47, 141.96, 140.35, 138.99, 131.47, 131.27, 128.19, 118.42, 118.31, 112.76. ESI-HRMS (m/z) $[\text{M}+\text{H}]^+$: 246.1026, calculated for $(\text{C}_{16}\text{H}_{12}\text{N}_3)^+$: 246.1026

5-(4-nitrophenyl)-4,6-dipyrrin, HL^6 .

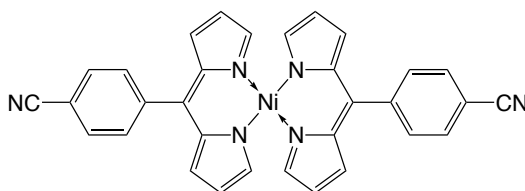
Dark yellow solid; 42% yield. ^1H NMR (500 MHz, CDCl_3) δ (ppm) 8.36 – 8.30 (m, 2H), 7.75 – 7.65 (m, 4H), 6.48 (dd, $J = 4.2, 1.1$ Hz, 2H), 6.43 (dd, $J = 4.2, 1.5$ Hz, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ (ppm) 148.03, 144.48, 143.73, 140.21, 138.46, 131.41, 128.06, 122.80, 118.31. ESI-HRMS (m/z) $[\text{M}+\text{H}]^+$: 266.0924, calculated for $(\text{C}_{15}\text{H}_{12}\text{N}_3\text{O}_2)^+$: 266.0924.

General Synthesis of NiL_2 Dipyrrin Complexes, $[\text{Ni}(\text{L}^{4-6})_2]$.

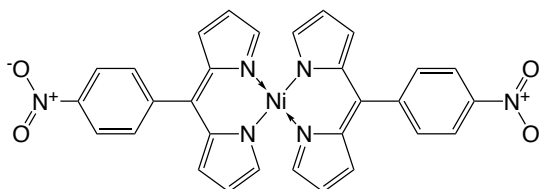
The dipyrrins were dissolved in min. methanol and stirred at room temperature. To this, 0.5 eq. of $\text{Ni}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}$ in min. methanol was added slowly, and the reaction stirred at room temperature for 4 hours. The reaction was concentrated under reduced pressure to half the original volume and cooled on ice. The resulting solids were filtered, washed twice with water, followed by a small portion of cold methanol, and dried to give $[\text{Ni}(\text{L})_2]$ as a dark red solid.

bis(5-(4-chlorophenyl)-4,6-dipyrrinato) nickel(II), $[\text{Ni}(\text{L}^4)_2]$.

Dark red powder; 75% yield. ^1H NMR (400 MHz, CDCl_3) δ (ppm) 9.81 (s, 4H), 7.71 (s, 4H), 7.42 – 7.29 (m, 8H), 6.71 (s, 4H). ^{13}C NMR (101 MHz, CDCl_3) δ (ppm) 171.45, 146.03, 142.18, 138.91, 135.36, 133.53, 133.45, 131.94, 127.77.

bis(5-(4-cyanophenyl)-4,6-dipyrinato) nickel(II), [Ni(L⁵)₂].

Dark red solid; 62% yield. ¹H NMR (400 MHz, CDCl₃) δ (ppm) 10.84 (s, 4H), 8.26 (s, 4H), 7.69 (d, *J* = 7.6 Hz, 4H), 7.45 (d, *J* = 7.9 Hz, 4H), 6.64 (s, 4H). ¹³C NMR (151 MHz, CDCl₃) δ (ppm) 181.88, 153.98, 141.82, 141.18, 140.56, 140.47, 131.41, 131.10, 118.67, 113.15.

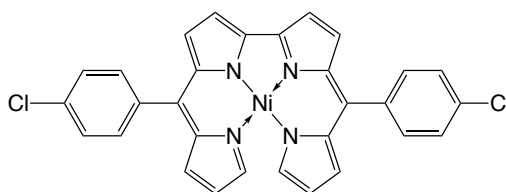
bis(5-(4-nitrophenyl)-4,6-dipyrinato) nickel(II), [Ni(L⁶)₂].

Dark red solid; 79% yield. ¹H NMR (500 MHz, CDCl₃) δ (ppm) 10.93 (s, 4H), 8.33 – 8.24 (m, 8H), 7.55 – 7.50 (m, 4H), 6.67 – 6.63 (m, 4H). ¹³C NMR (126 MHz, CDCl₃) δ (ppm) 182.67, 154.72, 154.55, 148.24, 142.82, 140.42, 139.88, 131.17, 122.69.

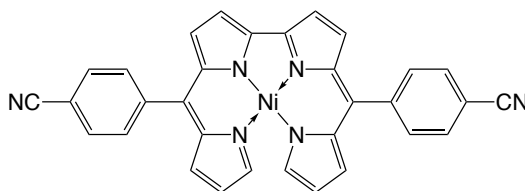
General synthesis of (2,2'-bisdipyrinato) nickel(II) complexes

The (2,2'-bisdipyrinato) nickel(II) complexes were synthesised according to a literature procedure for the synthesis of the methoxycarbonylphenyl bisdipyrin NiL^2 as follows.⁷

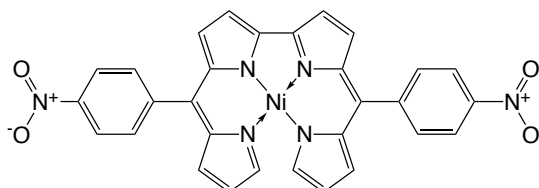
To a toluene solution containing the nickel(II) dipyrin complex (1 eq.) was added a toluene solution of DDQ (1.1 eq.), and the reaction stirred at reflux for 48 hours. After removing the solvent under reduced pressure, the residue was purified by column chromatography (SiO_2 , CHCl_3) to afford the (2,2'-bisdipyrinato) nickel(II) complexes as dark brown solids.

2,2'-bis(6-(4-chlorophenyl)-5,7-dipyrinato) nickel(II), NiL^7 .

Dark brown solid; 51% yield. ^1H NMR (400 MHz, CDCl_3) δ (ppm) 7.50 (d, $J = 8.3$ Hz, 4H), 7.46 (d, $J = 8.3$ Hz, 4H), 6.76 (dd, $J = 4.4, 1.3$ Hz, 2H), 6.71 (d, $J = 4.4$ Hz, 2H), 6.63 (d, $J = 4.3$ Hz, 2H), 6.45 (dd, $J = 4.4, 1.5$ Hz, 2H), 5.96 (t, $J = 1.4$ Hz, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ (ppm) 161.13, 153.76, 142.06, 138.67, 135.60, 135.33, 135.14, 134.53, 132.09, 129.81, 127.98, 117.26, 115.47. ESI-HRMS (m/z) $[\text{M}]^+$: 562.0264, calculated for $(\text{C}_{30}\text{H}_{18}\text{N}_4\text{Cl}_2\text{Ni})^+$: 562.0257.

2,2'-bis(6-(4-cyanophenyl)-5,7-dipyrrinato) nickel(II), NiL⁸.

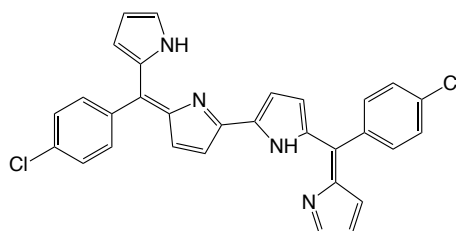
Dark brown solid; 42% yield. ¹H NMR (600 MHz, CDCl₃) δ (ppm) 7.81 – 7.76 (m, 4H), 7.69 – 7.67 (m, 4H), 6.69 (dd, *J* = 4.4, 1.2 Hz, 2H), 6.67 – 6.61 (m, 4H), 6.46 (dd, *J* = 4.4, 1.5 Hz, 2H), 5.93 (d, *J* = 1.5 Hz, 2H). ¹³C NMR (151 MHz, CDCl₃) δ (ppm) 162.25, 154.19, 141.45, 140.90, 138.57, 135.44, 134.63, 131.68, 131.56, 129.92, 118.53, 117.99, 116.21, 113.16. ESI-HRMS (*m/z*) [*M*]⁺: 544.0948, calculated for (C₃₂H₁₈N₆Ni)⁺: 544.0941.

2,2'-bis(6-(4-nitrophenyl)-5,7-dipyrrinato) nickel(II), NiL⁹.

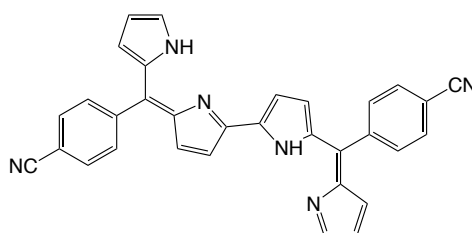
Dark brown solid; 57% yield. ¹H NMR (400 MHz, CDCl₃) δ (ppm) 8.40 – 8.33 (m, 4H), 7.78 – 7.71 (m, 4H), 6.71 (dd, *J* = 4.4, 1.2 Hz, 2H), 6.68 (d, *J* = 4.4 Hz, 2H), 6.65 (d, *J* = 4.4 Hz, 2H), 6.48 (dd, *J* = 4.4, 1.5 Hz, 2H), 5.95 (t, *J* = 1.4 Hz, 2H). ¹³C NMR (101 MHz, CDCl₃) δ (ppm) 162.42, 154.22, 148.40, 143.27, 140.40, 138.53, 135.40, 134.60, 131.77, 129.90, 123.09, 118.08, 116.32. ESI-HRMS (*m/z*) [*M*]⁺: 584.0745, calculated for (C₃₀H₁₈N₆O₄Ni)⁺: 584.0738.

General Synthesis of Ligands H_2L^{8-11} .

Ligands H_2L were synthesised according to reported procedures for the methoxycarbonylphenyl bisdipyrin, H_2L^2 (quant.).⁷

2,2'-bis(6-(4-chlorophenyl)-5,7-dipyrin), H_2L^7 .

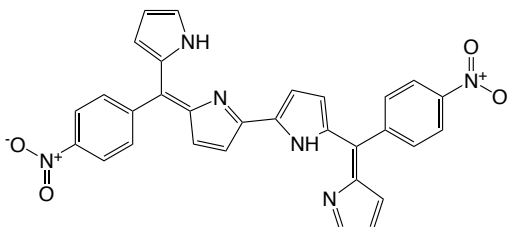
Dark blue solid; 60% yield. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.62 (t, $J = 1.4$ Hz, 2H), 7.50 – 7.44 (m, 8H), 7.00 (d, $J = 4.4$ Hz, 2H), 6.74 (d, $J = 4.4$ Hz, 2H), 6.55 (dd, $J = 4.1, 1.1$ Hz, 2H), 6.42 (dd, $J = 4.1, 1.7$ Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm) 152.98, 145.40, 139.25, 139.21, 135.85, 135.33, 132.31, 131.77, 128.23, 126.56, 120.43, 116.37. ESI-HRMS (m/z) $[M+H]^+$: 507.1144, calculated for $(C_{30}H_{21}N_4Cl_2)^+$: 507.1138.

2,2'-bis(6-(4-cyanophenyl)-5,7-dipyrin), H_2L^8 .

Dark blue solid; 100% yield. ^1H NMR (600 MHz, $CDCl_3$) δ (ppm) 7.81 – 7.76 (m, 4H), 7.68 – 7.63 (m, 6H), 7.01 (d, $J = 4.4$ Hz, 2H), 6.65 (d, $J = 4.4$ Hz, 2H), 6.47 (dd, $J = 4.1, 1.1$ Hz, 2H), 6.44 (dd, $J = 4.1, 1.7$ Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm) 153.46, 145.10, 142.06, 139.96, 138.93, 138.04, 131.78, 131.60, 131.46, 126.49,

120.96, 118.57, 116.92, 113.04. ESI-HRMS (m/z) $[M+H]^+$: 489.1824, calculated for $(C_{32}H_{21}N_6)^+$: 489.1822.

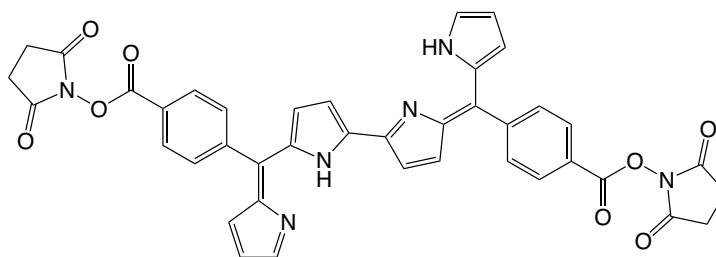
2,2'-bis(6-(4-nitrophenyl)-5,7-dipyrrin), H_2L^9 .



Dark blue solid; 79% yield. ^1H NMR (500 MHz, $CDCl_3$) δ (ppm) δ 8.40 – 8.34 (m, 4H), 7.77 – 7.71 (m, 4H), 7.67 (dd, J = 1.7, 1.1 Hz, 2H), 7.04 (d, J = 4.4 Hz, 2H), 6.67 (d, J = 4.4 Hz, 2H), 6.50 (dd, J = 4.2, 1.2 Hz, 2H), 6.46 (dd, J = 4.2, 1.8 Hz, 2H). ESI-HRMS (m/z) $[M+H]^+$: 529.1627, calculated for $(C_{30}H_{21}N_6O_4)^+$: 529.1619.

Synthesis of H_2L Amides

2,2'-bis(6-(4-succinimidylphenyl)-5,7-dipyrrin), H_2L^{3-OSu} .

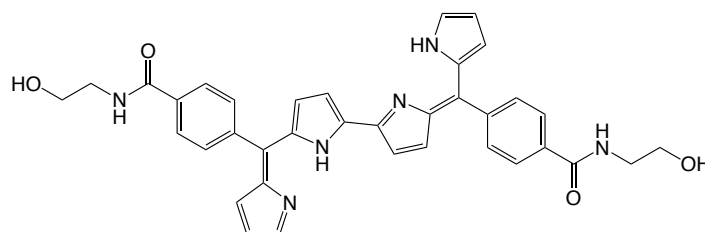


EDC·HCl (530 mg, 2.8 mmol, 2.1 eq.) and N-hydroxysuccinamide (325 mg, 2.8 mmol, 2.1 eq.) in DMF (20 mL) were added to a solution of H_2L^3 (693 mg, 1.3 mmol) in DMF (30 mL) and the reaction stirred at room temperature for 16 hours. The reaction mixture was diluted with ethyl acetate (500 mL) and the organics washed with a 9:1 mixture of H_2O and saturated Na_2CO_3 (5 x 250 mL). The combined aqueous layers were back extracted with ethyl acetate (2 x 250 mL). The organic layers were combined, washed with a 9:1

7. Experimental

H₂O/brine mixture (2 x 250 mL), brine (2 x 250 mL) and dried over MgSO₄. The solvent was removed under reduced pressure to give H₂L^{3-OSu} as a dark blue solid (0.904 g, 95%). ¹H NMR (600 MHz, CDCl₃) δ (ppm) 8.28 – 8.23 (m, 4H), 7.72 – 7.67 (m, 4H), 7.64 (s, 2H), 7.03 (d, *J* = 4.4 Hz, 2H), 6.67 (d, *J* = 4.4 Hz, 2H), 6.49 (dd, *J* = 4.1, 1.1 Hz, 2H), 6.44 (dd, *J* = 4.2, 1.7 Hz, 2H), 2.95 (s). ¹³C NMR (151 MHz, CDCl₃) δ (ppm) 169.53, 161.88, 153.44, 145.34, 144.33, 139.88, 139.19, 138.63, 131.81, 131.61, 130.40, 126.68, 125.83, 121.17, 116.98, 26.08. ESI-HRMS (*m/z*) [M+H]⁺: 721.2037, calculated for (C₄₀H₂₉N₆O₈)⁺: 721.2040.

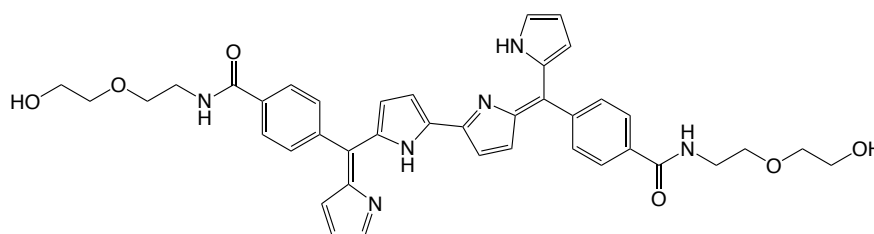
2,2'-bis(6-(4-(N-[2-hydroxyethyl]benzamidyl))-5,7-dipyrin), H₂L¹⁰.



H₂L^{3-OSu} (106 mg, 0.147 mmol) was added to a solution of CHCl₃ (10 mL) containing K₂CO₃ (193 mg, 1.40 mmol, 10 eq.), to which was added ethanolamine (142 μL, 2.36 mmol, 16 eq.) and the solution was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the resulting solids dissolved in 50 mL 1 M HCl, which was washed with EtOAc (1 x 100 mL). The organics were back extracted into H₂O (2 x 100 mL) and the aqueous layers (H₂O and HCl) were combined. EtOAc (125 mL) was added to the aqueous layer, followed by 100 mL of a saturated NaHCO₃ solution, and the purple product was extracted into the organics. The aqueous layer was further extracted with 125 mL EtOAc, before combining the organics and washing with H₂O (1 x 100 mL) and brine (2 x 100 mL). The organics were dried over MgSO₄ before the solvent was removed, yielding H₂L¹⁰ as a dark blue solid (57 mg, 63%). ¹H NMR

(600 MHz, Acetone- d_6) δ (ppm) 8.10 – 8.06 (m, 4H), 7.97 (s, 2H), 7.75 – 7.72 (m, 2H), 7.67 – 7.62 (m, 4H), 7.30 (d, J = 4.4 Hz, 2H), 6.78 (d, J = 4.4 Hz, 2H), 6.49 (dd, J = 4.1, 1.2 Hz, 2H), 6.41 (dd, J = 4.0, 1.9 Hz, 2H), 3.75 (t, J = 5.7 Hz, 4H), 3.58 (q, J = 5.7 Hz, 4H). ^{13}C NMR (51 MHz, Acetone- d_6) δ (ppm) 167.29, 147.70, 141.24, 140.53, 137.87, 137.75, 136.24, 133.43, 131.57, 127.64, 126.04, 122.69, 115.69, 75.83, 61.91, 43.66. ESI-HRMS (m/z) $[\text{M}+\text{H}]^+$: 613.2547, calculated for $(\text{C}_{36}\text{H}_{33}\text{N}_6\text{O}_4)^+$: 613.2558.

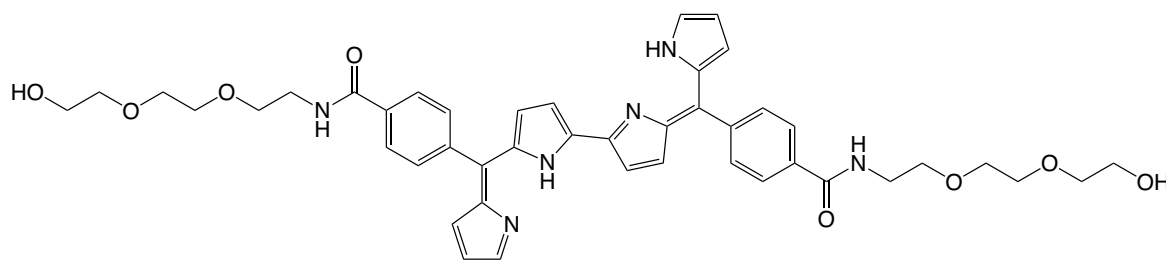
2,2'-bis(6-(4-(N-[2-(2-hydroxyethoxy)ethyl]benzamidyl))-5,7-dipyrrin), H_2L^{11} .



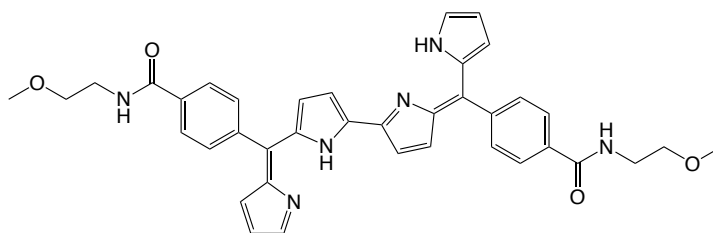
$\text{H}_2\text{L}^{3\text{-OSu}}$ (52 mg, 0.072 mmol) was dissolved in CHCl_3 (10 mL) and treated with K_2CO_3 (103 mg, 0.75 mmol, 10 eq.). To this, 2-(2-aminoethoxy)ethanol (230 μL , 2.30 mmol, 32 eq.) was added, and the solution was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the resulting solids dissolved in 50 mL 1 M HCl, which was washed with EtOAc (1 x 100 mL). The organics were back extracted into H_2O (2 x 100 mL) and the aqueous layers (H_2O and HCl) were combined. EtOAc (125 mL) was added to the aqueous layer, followed by 100 mL of a saturated NaHCO_3 solution, and the purple product was extracted into the organics. The aqueous layer was further extracted with 125 mL EtOAc, before combining the organics and washing with H_2O (1 x 100 mL) and brine (2 x 100 mL). The organics were dried over MgSO_4 before the solvent was removed, yielding H_2L^{11} as a dark blue solid (34 mg, 72%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) 7.90 (d, J = 7.9 Hz, 4H), 7.67 – 7.55 (m, 6H), 7.00 (d, J = 4.4 Hz, 2H), 6.71 (d, J = 4.4 Hz, 4H), 6.53 (d, J = 4.1 Hz, 2H),

6.42 (dd, $J = 4.2, 1.7$ Hz, 2H), 3.84 – 3.78 (m, 4H), 3.75 (d, $J = 2.6$ Hz, 8H), 3.67 (t, $J = 4.5$ Hz, 4H). ^{13}C NMR (151 MHz, CDCl_3) δ (ppm) 167.54, 153.21, 145.55, 140.84, 139.59, 139.54, 139.42, 135.26, 131.96, 131.45, 126.83, 120.75, 116.69, 72.61, 70.27, 62.22, 40.29. ESI-HRMS (m/z) $[\text{M}+\text{H}]^+$: 701.3088, calculated for $(\text{C}_{40}\text{H}_{41}\text{N}_6\text{O}_6)^+$: 701.3079.

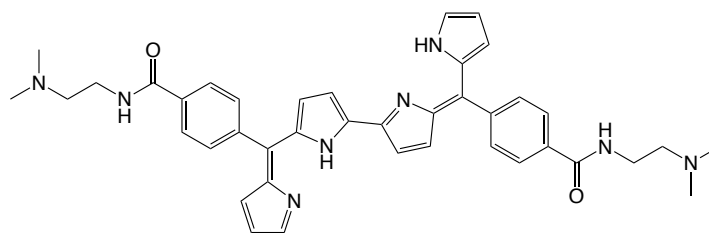
2,2'-bis(6-(4-(N-(2-[2-[2-hydroxyethoxy]ethoxy]ethyl)benzamidyl))-5,7-dipyrrin), H_2L^{12} .



$\text{H}_2\text{L}^{3\text{-OSu}}$ (290 mg, 0.402 mmol) was dissolved in CHCl_3 (30 mL) and treated with K_2CO_3 (205 mg, 1.48 mmol, 4 eq.). To this, 2-(2-[2-aminoethoxy]ethoxy)ethanol (123 μL , 0.885 mmol, 2.2 eq.) was added, and the reaction stirred at room temperature for 16 hours. The reaction mixture was diluted with 170 mL CHCl_3 and washed with a 3:1 H_2O /brine mixture (2 x 200 mL). The aqueous layers were combined and extracted with CHCl_3 (1 x 50 mL each). All the organics were combined before washing with brine (2 x 250 mL) and drying over MgSO_4 . The solvent was removed under reduced pressure to give H_2L^{12} as a dark blue solid (215 mg, 68%). ^1H NMR (500 MHz, CDCl_3) δ (ppm) 7.95 – 7.91 (m, 4H), 7.63 – 7.59 (m, 6H), 6.99 (dd, $J = 7.7, 3.5$ Hz, 4H), 6.71 (d, $J = 4.4$ Hz, 2H), 6.53 (dd, $J = 4.1, 1.2$ Hz, 2H), 6.42 (dd, $J = 4.1, 1.8$ Hz, 2H), 3.76 – 3.69 (m, 22H), 3.66 – 3.63 (m, 4H). ^{13}C NMR (126 MHz, CDCl_3) δ (ppm) 167.22, 153.02, 145.38, 140.56, 139.42, 139.31, 139.20, 135.07, 131.79, 131.20, 126.67, 126.59, 120.53, 116.44, 72.70, 70.58, 70.55, 70.18, 61.96, 40.02. ESI-HRMS (m/z) $[\text{M}+\text{H}]^+$: 789.3602, calculated for $(\text{C}_{44}\text{H}_{49}\text{N}_6\text{O}_8)^+$: 789.3603.

2,2'-bis(6-(4-(N-[2-methoxyethyl]benzamidyl))-5,7-dipyrrin), H_2L^{13} .

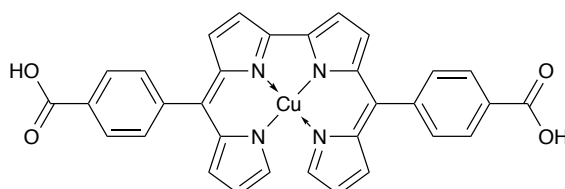
H_2L^{3-OSu} (103 mg, 0.143 mmol) was added to a solution of $CHCl_3$ (10 mL) containing K_2CO_3 (196 mg, 1.42 mmol, 10 eq.), to which was added 2-methoxyethylamine (199 μ L, 2.29 mmol, 16 eq.) and the solution was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the resulting solids dissolved in 50 mL 1 M HCl, which was washed with EtOAc (1 x 100 mL). The organics were back extracted into H_2O (2 x 100 mL) and the aqueous layers (H_2O and HCl) were combined. EtOAc (125 mL) was added to the aqueous layer, followed by 100 mL of a saturated $NaHCO_3$ solution, and the purple product was extracted into the organics. The aqueous layer was further extracted with 125 mL EtOAc, before combining the organics and washing with H_2O (1 x 100 mL) and brine (2 x 100 mL). The organics were dried over $MgSO_4$ before the solvent was removed, yielding H_2L^{13} as a dark blue solid (60 mg, 66%). ^1H NMR (600 MHz, $CDCl_3$) δ (ppm) 7.91 – 7.87 (m, 4H), 7.64 – 7.60 (m, 6H), 7.01 (d, J = 4.3 Hz, 2H), 6.71 (d, J = 4.3 Hz, 2H), 6.61 (t, J = 5.6 Hz, 2H), 6.53 (dd, J = 4.1, 1.2 Hz, 2H), 6.42 (dd, J = 4.1, 1.7 Hz, 2H), 3.72 (q, J = 5.3 Hz, 4H), 3.62 (dd, J = 5.6, 4.5 Hz, 4H), 3.43 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm) 167.17, 152.94, 145.38, 140.58, 139.36, 139.33, 139.23, 135.16, 131.76, 131.23, 126.58, 120.54, 116.46, 71.33, 59.06, 39.98. ESI-HRMS (m/z) $[M+H]^+$: 641.2869, calculated for $(C_{38}H_{37}N_6O_4)^+$: 641.2871.

2,2'-bis(6-(4-(*N*-(2-[*N,N*-dimethylamino]ethyl)benzamidyl))-5,7-dipyrrin), *H₂L*¹⁴.

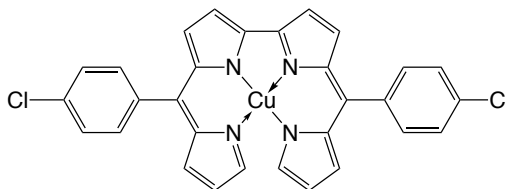
H₂L^{3-Osu} (104 mg, 0.144 mmol) was added to a solution of CHCl_3 (10 mL) containing K_2CO_3 (193 mg, 1.40 mmol, 10 eq.), to which was added *N,N*-dimethylethylenediamine (252 μL , 2.31 mmol, 16 eq.) and the solution was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the resulting solids dissolved in 100 mL 1 M HCl, which was washed with EtOAc (1 x 200 mL). The organics were back extracted into H_2O (2 x 200 mL) and the aqueous layers (H_2O and HCl) were combined. EtOAc (200 mL) was added to the aqueous layer, followed by 100 mL of a saturated NaHCO_3 solution, and the purple product was extracted into the organics. The aqueous layer was further extracted with 200 mL EtOAc, before combining the organics and washing with 10% sat. $\text{NaHCO}_3/\text{H}_2\text{O}$ (1 x 200 mL) and 10% sat. $\text{NaHCO}_3/\text{brine}$ (2 x 200 mL). The organics were dried over MgSO_4 before the solvent was removed, yielding *H₂L*¹⁴ as a dark blue solid (59 mg, 61%). ¹H NMR (600 MHz, CDCl_3) δ (ppm) 7.92 – 7.88 (m, 4H), 7.64 – 7.57 (m, 6H), 7.00 (d, $J = 4.3$ Hz, 2H), 6.97 (t, $J = 4.8$ Hz, 2H), 6.71 (d, $J = 4.3$ Hz, 2H), 6.53 (dd, $J = 4.1, 1.1$ Hz, 2H), 6.42 (dd, $J = 4.1, 1.8$ Hz, 2H), 3.61 – 3.56 (m, 4H), 2.57 (t, $J = 5.9$ Hz, 4H), 2.31 (s, 12H). ¹³C NMR (151 MHz, CDCl_3) δ (ppm) 167.15, 153.02, 145.38, 140.41, 139.46, 139.27, 139.21, 135.27, 131.79, 131.17, 126.61, 120.49, 116.42, 57.87, 45.34, 37.40. ESI-HRMS (m/z) [$\text{M}+\text{H}$]⁺: 667.3501, calculated for $(\text{C}_{40}\text{H}_{43}\text{N}_8\text{O}_2)^+$: 667.3503.

General synthesis of copper(II) complexes, CuL.

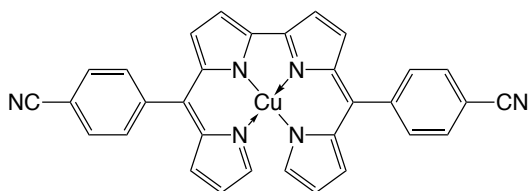
A solution of ligand H₂L in CHCl₃ was treated with Cu(OAc)₂·H₂O (1.1 eq.) in an equal volume of methanol. The mixture was stirred for 4 hours at room temperature before removing the solvent under reduced pressure. The resultant solids were triturated with small portions of cold methanol (**L**³, **L**⁷⁻⁹) or cold acetone (**L**¹⁰⁻¹⁴) and collected by filtration, affording the copper(II) complexes as dark brown solids.

2,2'-bis(6-(4-carboxyphenyl)-5,7-dipyrrinato) copper(II), CuL³.

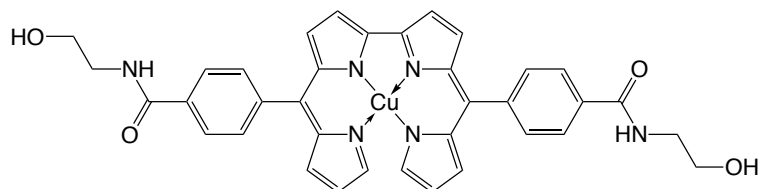
Dark brown microcrystalline solid; 76%. ESI-HRMS (*m/z*) [M+H]⁺: 588.0854, calculated for (C₃₂H₂₁CuN₄O₄)⁺: 588.0852. Elemental Analysis: Found C, 57.88; H, 3.46; N, 7.88. Calculated for C₃₂H₂₀N₄CuO₄·Cu·CH₃OH: C, 57.98; H, 3.54; N, 8.20. Crystals of suitable quality for x-ray diffraction were grown by slow evaporation of a DMF solution of CuL³.

2,2'-bis(6-(4-chlorophenyl)-5,7-dipyrrinato) copper(II), CuL⁷.

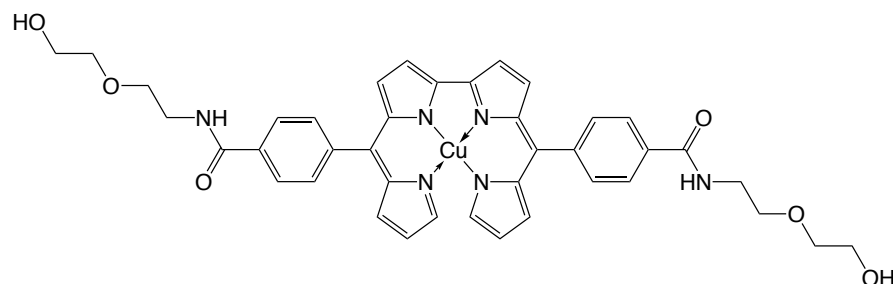
Dark brown solid; 63% yield. ESI-HRMS (*m/z*) [M+H]⁺: 568.0278, calculated for (C₃₀H₁₉N₄Cl₂Cu)⁺: 568.0277. Elemental Analysis: Found C, 60.69; H, 3.49; N, 8.48. Calculated for C₃₀H₁₈N₄Cl₂Cu·2CH₃OH: C, 60.72; H, 4.14; N, 8.85.

2,2'-bis(6-(4-cyanophenyl)-5,7-dipyrrinato) copper(II), CuL⁸.

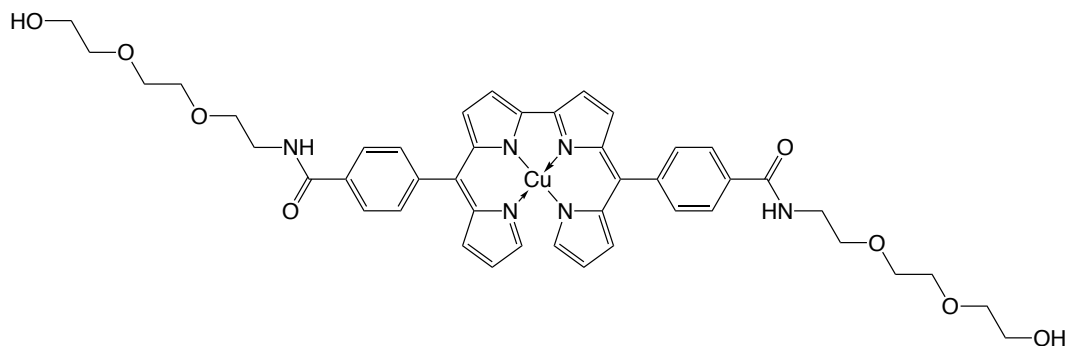
Dark brown solid; 43% yield. ESI-HRMS (m/z) $[M+H]^+$: 550.0941, calculated for $C_{32}H_{18}N_6Cu$: 550.0962. Elemental Analysis: Found C, 67.71; H, 3.42; N, 15.08. Calculated for $C_{32}H_{18}N_6Cu \cdot H_2O$: C, 67.66; H, 3.55; N, 14.79.

2,2'-bis(6-(4-(N-[2-hydroxyethyl]benzamidyl))-5,7-dipyrrinato) copper(II), CuL¹⁰.

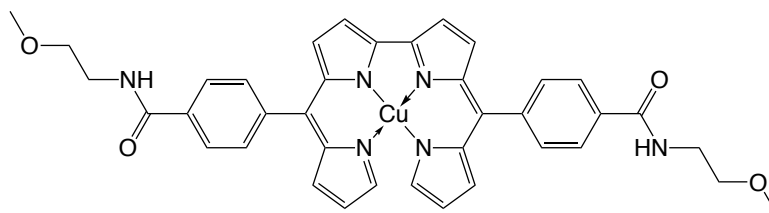
Dark brown solid; 71% yield. ESI-HRMS (m/z) $[M+H]^+$: 674.1696, calculated for $(C_{36}H_{31}CuN_6O_4)^+$: 674.1697. Elemental Analysis: Found C, 57.39; H, 4.38; N, 10.77. Calculated for $C_{36}H_{30}N_6CuO_4 \cdot Cu \cdot CH_3OH$: C, 57.73; H, 4.45; N, 10.93.

2,2'-bis(6-(4-(N-[2-[2-hydroxyethoxy]ethyl]benzamidyl))-5,7-dipyrrinato) copper(II),***CuL¹¹*.**

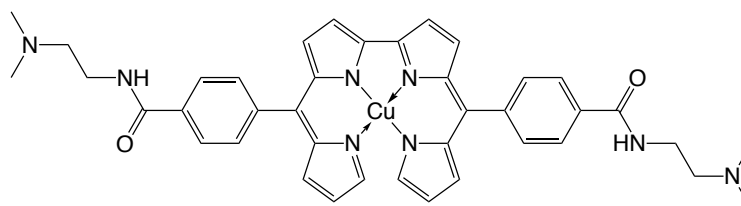
Dark brown solid; 59% yield. ESI-HRMS (m/z) $[M+H]^+$: 762.2212, calculated for $(C_{40}H_{39}CuN_6O_6)^+$: 762.2222. Elemental Analysis: Found C, 59.81; H, 5.08; N, 10.29. Calculated for $C_{40}H_{38}N_6CuO_6 \cdot 2H_2O$: C, 60.18; H, 5.30; N, 10.53.

2,2'-bis(6-(4-(N-(2-[2-[2-hydroxyethoxy]ethoxy]ethyl)benzamidyl))-5,7-dipyrrinato)***copper(II), CuL¹²*.**

Dark brown solid; 71% yield. ESI-HRMS (m/z) $[M+H]^+$: 850.2734, calculated for $(C_{44}H_{47}CuN_6O_8)^+$: 850.2746. Elemental Analysis: Found C, 59.29; H, 5.31; N, 9.47. Calculated for $C_{44}H_{46}N_6CuO_8 \cdot 2H_2O$: C, 59.62; H, 5.69; N, 9.48.

2,2'-bis(6-(4-(N-[2-methoxyethyl]benzamidyl))-5,7-dipyrrinato) copper(II), CuL¹³.

Dark brown solid; 26% yield. ESI-HRMS (m/z) $[M+H]^+$: 702.1946, calculated for $(C_{38}H_{35}CuN_6O_4)^+$: 702.2010. Elemental Analysis: Found C, 60.33; H, 4.92; N, 10.60. Calculated for $C_{38}H_{34}N_6CuO_4 \cdot 3H_2O$: C, 60.35; H, 5.33; N, 11.11.

2,2'-bis(6-(4-(N-(2-[N,N-dimethylamino]ethyl)benzamidyl))-5,7-dipyrrinato) copper(II), CuL¹⁴.

Dark brown solid; 56% yield. ESI-HRMS (m/z) $[M+H]^+$: 728.2580, calculated for $(C_{40}H_{41}CuN_8O_2)^+$: 728.2643. Elemental Analysis: Found C, 58.25; H, 5.24; N, 13.06. Calculated for $C_{40}H_{40}N_8CuO_2 \cdot CHCl_3$: C, 58.09; H, 4.87; N, 13.22.

7.3 *PROTEIN ASSAYS, CELL STUDIES, AND RADIOLABELLING*

7.3.1 *LogD Sample Preparation*

1-Octanol and PBS (1x, 0.01 M, pH 7.4) were pre-saturated with each other (all PBS and 1-octanol hereafter refers to the pre-saturated samples). An unknown mass of each compound was suspended in 2 mL of the 1-octanol and sonicated for 1 hour. The suspension was filtered through a 0.45 μm Durapore membrane and centrifuged (14,000 RCM, 5 minutes). A 1 mL aliquot of the supernatant was added to 1 mL of PBS. The samples were mixed for 5 minutes before standing for 30 minutes to complete partitioning, after which the compounds were mostly present within the octanol phase.

The following samples were prepared in triplicate from the stock solution of compound in 1-octanol. Aliquots of the 1-octanol (100 μL) were diluted to 500 μL in 1-octanol. PBS (500 μL) was added, and the samples mixed at 600 rpm on a BioSan Thermoshake TS-100 for 30 minutes. The samples were allowed to separate for 30 minutes before digestion.

Aliquots of the 1-octanol layers (100 μL) were added to 1 mL 35% HNO_3 . Aliquots of the PBS layers (100 μL) were added to 900 μL of 35% HNO_3 . Samples were left uncovered and heated to 95°C, shaking at 400 RPM for 60 minutes. 200 μL of aqueous layer in each digest were diluted to 2 mL with milliQ and analysed by ICP-MS.

Average values of the copper content corrected by subtraction of the average copper content in the blank sample. The ratio $[\text{Cu}]_{\text{org}}/[\text{Cu}]_{\text{aq}}$ was calculated, and the base-10 logarithm taken.

7.3.2 *Cu Titration, ϵ Determination and Kinetic Challenges*

An approximately 100 μM stock solution of H_2L^2 was prepared in DMSO. 100 μL of this was added to a solution containing 100 μL HEPES buffer (100 mM, pH 7.4) and 800 μL milliQ, and the absorbance spectrum acquired in the range 900–330 nm (Control). A Cu^{2+} standard solution (100 μM) was prepared using a Cu^{2+} AAS standard (1000 ± 4 mg/L; Fluka Analytical) in 0.1% HNO_3 using milliQ. This was added to 12 eppendorfs in 10 μL increments (up to 120 μL , 12 eq. Cu^{2+}) containing milliQ such that a total volume of 800 μL was achieved. HEPES buffer (100 μL , 100 mM, pH 7.4) was added, followed by 100 μL of the H_2L^2 stock in DMSO. Samples were mixed briefly before acquiring the spectra. Absorbance values at 786 nm were plotted against $[\text{Cu}^{2+}]$ (μM) in solution, and the gradients of the two lines needed to fit the data were used to determine the precise concentration of H_2L^2 and CuL^2 in solution.

Competitions studies were conducted using a stock of CuL^2 in DMSO (approx. 10 μM). Stock solutions of the competitors ($\text{Na}_2\text{H}_2\text{EDTA}$, cysteine methyl ester, glutathione and histidine methyl ester) were prepared in milliQ to a concentration of 500 μM . Final samples for analysis were comprised of 100 μL HEPES buffer (100 mM, pH 7.4), 700 μL milliQ, 100 μL competitor and 100 μL CuL^2 stock solution. Samples were analysed at 10-minute intervals for 1 hour. Data shown represents 60 minutes post addition of complex.

7.3.3 BSA Binding

BSA used herein was heat shock fractionated and fatty acid free and protease free. A stock solution of 0.1% w/v was formulated in milliQ immediately before using. A 100 μ L aliquot of this was diluted with 700 μ L MilliQ and 100 μ L HEPES buffer (100 mM, pH 7.4), before the addition of 100 μ L of DMSO containing either H_2L^2 or CuL^2 (50 and 56 μ M, respectively). This gave a final composition of 10% DMSO and 10 mM HEPES buffer (pH 7.4) containing 0.01% BSA w/v and a 1/10 dilution of analyte. Samples were mixed by pipette aspiration and analysed by UV-Visible spectrophotometry at 0-, 50-, 90-, 110- and 120-minutes post-addition.

Fluorescence samples were prepared in the same manner, using concentrations of H_2L^2 and CuL^2 , in the presence and absence of BSA, such that absorbance at the excitation wavelength was maintained at or below 0.1 absorbance units.

7.3.4 Dithionite, Atox1 and Bicinchoninic Acid Assays

The stock solutions of HEPES buffer (100 mM, pH 7.4), sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$; 10 mM), Bca (10 mM) and BSA (0.1% m/v) stocks were prepared under anoxic conditions in a glove box using degassed milliQ. Atox1 was obtained from colleagues in MOPS buffer (pH 7.4) and diluted to 500 μ M using milliQ. The DMSO was degassed before use to maintain low O_2 before being used in the preparation of a CuL^2 stock (150 μ M).

Samples were prepared in the glove box, as follows, before sealing under an inert atmosphere in the cuvette for analysis. BSA-free samples were prepared as described below. Samples containing 0.01% BSA in the final solution were prepared by replacing 100 μ L of milliQ with BSA stock.

The CuL² control was prepared by the addition of 100 µL of HEPES buffer (100 mM, pH 7.4) to 800 µL milliQ, followed by the addition of 100 µL of CuL² in DMSO (150 µM). The samples were mixed by pipette aspiration prior to analysis.

Negative controls (containing no sodium dithionite) were prepared by the addition of 100 µL of HEPES buffer (100 mM, pH 7.4) to 700 µL milliQ containing 100 µL of either Atox1 (500 µM) or Bca (10 mM). To this, 100 µL of CuL² in DMSO (150 µM) was added, and the samples mixed by pipette aspiration.

Sample runs (containing sodium dithionite) were prepared by the addition of 100 µL of HEPES buffer (100 mM, pH 7.4) to 600 µL milliQ followed by 100 µL of either Atox1 (500 µM) or Bca (10 mM). To this, 100 µL of CuL² in DMSO (150 µM) was added, followed by 100 µL of Na₂S₂O₄ (10 mM) and the samples mixed by pipette aspiration. Samples were sealed under an inert atmosphere and analysed within 15 minutes of preparation.

7.3.5 Cell Culture and Treatment

Cultures of LNCaP prostate cancer cells were grown at 37°C with 5% CO₂ using RPMI 1640 cell culture media (ThermoFisher) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were passaged using a 0.25% trypsin-EDTA solution (ThermoFisher Scientific) at 80-90% confluency no more than 10 times before analysis.

Cells were serum starved in RPMI 1640 media with 1% FBS for 1 hour at 37°C with 5% CO₂, before treating the cells with trypsin/EDTA to detach the cells from the culture flask. Cells were counted using a hemocytometer with trypan blue stain. Viability of cells was maintained above 85% for all analyses.

The bulk cell solution was washed with 5 mL of Na₂H₂EDTA (5 mM) in PBS, and the cells pelleted (300 RCF, 5 minutes). Dulbecco's PBS (~22 mL) was added to give

approximately 7×10^5 cells per mL of solution. Approximately 4×10^5 cells were transferred to eppendorfs (500 μ L). The cells were pelleted (300 RCF, 5 minutes), and the supernatant removed. To the resulting pellet was added 500 μ L of the pre-prepared solutions for analysis (1% DMSO and 1% FBS in PBS; 0 – 50 μ M copper complex; incubated at 37°C for 10 minutes prior to addition), and the samples incubated at 37°C with 5% CO₂ for 1 hour. The cells were then cooled on ice for 5 minutes, before pelleting the cells (300 RCF, 5 minutes).

The cells were washed with Na₂H₂EDTA (5 mM in PBS; 2 x 500 μ L) and PBS (2 x 500 μ L). The supernatant was removed, and the pellet was suspended in 200 μ L ICP-MS grade milliQ water. A 100 μ L aliquot of this was taken for protein determination by Bca assay, and the remaining 100 μ L aliquots were treated with 100 μ L 70% HNO₃. These samples were heated to 95°C for 1 hour, and then cooled to room temperature. ICP-MS grade milliQ water was added to a total volume of 1 mL, and the samples centrifuged at 14000 RCF for 5 minutes. The supernatant was added to 1 mL of ICP-MS grade milliQ water (total volume 2 mL) for ICP-MS analysis of copper levels.

7.3.6 Protein Quantification using Bca Assay

The pre-prepared standards of BSA (0 – 2000 μ g/mL) were diluted with NaOH to constitute the same conditions as the cell lysate, as follows. A 25 μ L aliquot of each BSA standard was added to 25 μ L of 2 M aqueous NaOH, giving a range of standard concentrations of 0 – 1000 μ g/mL. These samples were used directly using the Bca assay kit according to the assay protocol (Sigma Aldrich). Standard samples were analysed in duplicate, and the average value taken.

Cells in the 100 μ L aliquots were lysed using 100 μ L 2 M aqueous NaOH for 30 minutes at room temperature before analysis. Cell lysates were analysed using the Pierce™

Bca Protein Assay Kit (ThermoFisher Scientific) without further dilution. Lysates were analysed in duplicate, and the average protein concentration used in normalisation. The high pH of this assay required 24-hour incubation at room temperature before analysis. The standard curve data was fitted with a linear fit, and concentrations of protein in each cell lysate were calculated accordingly.

Cell protein content was calculated using the concentration in $\mu\text{g/mL}$ for the 100 μL sample of cells. ICP-MS analysis gave copper concentration as ppb. This was converted to $\mu\text{g/mL}$, and the total $m(\text{Cu})$ in the 2 mL sample, corresponding to the cell pellet, was calculated. The concentration of Cu was divided by the average protein mass, to give normalised values. One-way ANOVA followed by Tukey's multiple comparisons tests were performed to determine the statistical significance of the results between different concentrations. This was performed using GraphPad Prism version 8.4.3 for Macintosh, (GraphPad Software, San Diego, California USA). All statistical analyses were conducted under the assumptions of sphericity for unmatched/unpaired data and equal standard deviations.

7.3.7 Radiolabelling Optimisation

^{64}Cu samples were obtained as no carrier added $[^{64}\text{Cu}]\text{CuCl}_2$ in 0.2 M HCl by Rapid Laboratories, Medical Technology and Physics, Sir Charles Gardner Hospital, Western Australia, or as no carrier added $[^{64}\text{Cu}]\text{CuCl}_2$ in 0.1 M HCl by Austin Health Molecular Imaging and Therapy, Victoria, Australia. The radionuclide solution was neutralised using either NH_4OAc (0.1 M, pH 5.5) or 3 M aqueous NaOH followed by NH_4OAc (0.1 M, pH 5.5) to the desired pH (5.5 – 7).

HPLC analysis

HPLC analysis of radioactive samples was performed on a Shimadzu SCL-10A VP/LC-10AT VP HPLC system with a Shimadzu SPD-10A VP UV detector followed by a radiation detector (Ortec model 276 photomultiplier base with preamplifier, Ortec 925-SCINT ACE mate preamplifier, BIAS supply and SCA, Bicron 1M 11/2 photomultiplier tube) using a Phenomenex Luna 5 μm C18(2) 100 Å 150 x 4.6 mm column. A H₂O/ACN buffer free solvent system was used (50-100% ACN over 10 minutes) for analysis.

A typical labelling experiment utilised ~10 MBq of [⁶⁴Cu]CuCl₂ per ligand for repeat analysis as necessary. Each ligand for labelling (5 μL , 1 mM in DMSO) was added to the required volume of buffered [⁶⁴Cu]CuCl₂. Neat DMSO was added such that the final DMSO concentration was maintained at 25%. Labelling was conducted at room temperature for 30 minutes before a volume of sample corresponding to ~ 4 MBq of activity was diluted to 60 μL with ethanol for injection.

Radio TLC analysis

Radio-TLC analysis was performed using a Raytest Rita-Star TLC scanner and silica TLC plates. Ethanol was used as the eluent; $R_f(\text{free } [^{64}\text{Cu}]\text{CuCl}_2) = 0$.

A typical labelling experiment utilised ~5 MBq of $[^{64}\text{Cu}]\text{CuCl}_2$ per ligand for repeat analysis as necessary. H_2L^2 for labelling (5 μL , 1 mM in DMSO) was added to the required volume of buffered $[^{64}\text{Cu}]\text{CuCl}_2$. Neat DMSO was added such that the final DMSO concentration was maintained at 50%. Labelling was conducted at room temperature for 30 minutes before 10 μL was diluted with 90 μL with ethanol. These final samples were then analysed by radio TLC (SiO_2 , ethanol).

Competitors GSH, EDTA and cysteine methyl ester were prepared in milliQ as 1 mM stock solutions for analysis. Each was labelled in the absence of ligand with ~5 MBq of $[^{64}\text{Cu}]\text{CuCl}_2$ as above. Competition studies of $[^{64}\text{Cu}]\text{CuL}^2$ used the pre-labelled sample prepared above. An aliquot of this sample (20 μL , ~1.4 MBq, before dilution) was added to a solution containing 25 μL sample of competitor and 20 μL DMSO. Competitions were left to stand at room temperature for 60 minutes, before diluting with ethanol, as above, for analysis.

7.3.8 Biodistribution and Imaging

A total of 6 female mice (C57BL/6) were reared. At the appropriate age, the labelled samples of [^{64}Cu]CuL² in 10% DMSO and 8% ethanol in PBS (100 mM, pH 7.4) were administered *via* tail-vein injection.

Biodistribution

Animals were culled *via* CO₂ inhalation after 2 minutes (N = 3) or 60 minutes (N = 3) after imaging. The brain was removed prior to removing the blood through cardiac puncture, followed by the removal of the other organs of interest. Each tissue was weighed and then counted for 30 seconds on a Capintec (Captus 4000e) gamma counter. Data was corrected for radionuclide decay and normalised to %ID/g (tissue) $\pm$ SD.

MicroPET Imaging

Animals were anaesthetised with 1.5–3% isoflurane and placed in the imaging chamber of a PerkinElmer/Sofie Biosciences small animal G8 PET/CT. Images were recorded using the G8 acquisition engine software and reconstructed using a 3D ML-EM algorithm.⁹ Quantification was performed using VivoQuant software (V3.0, inviCRO Imaging Services and Software).

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