

Lipophilic and Cationic Gallium-68 Chelates for Mitochondrial Imaging

By

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degree of

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Declaration of Originality

The work reported in this thesis was completed between October 2015 and September 2018 at the Department of Chemistry, Imperial College London and the Division of Imaging Sciences and Biomedical Engineering, King's College London. The work reported is entirely my own, unless otherwise stated through cited reference or acknowledgement and has never been previously submitted for a degree at this or any other university.

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Abstract

This thesis details the synthesis and radiolabelling of gallium-68-labelled radiotracers for mitochondrial imaging, as well as their uptake in *in vitro* and *ex vivo* biological models. These tracers are designed to be cationic such that they are taken up into mitochondria in a manner dependant on their mitochondrial membrane potential, and lipophilic such that they can pass through lipid bilayer membranes.

The synthesis of novel gallium chelators is described in Chapter 3. Starting with tri-arylphosphonium (TAP)-functionalised DO3A as a base case, DO2A-based variants were synthesised due to their novelty in the literature, using TAP and aryl functionalisation to provide tracers with a range of cationic charges and lipophilicities. Bridged cyclens were also explored with a view to exploiting their preorganisation and complex stability, whilst work into synthesising HBED-based chelators was unsuccessful due to low stability of the chelators.

The radiolabelling of the chelators with generator-produced gallium-68 is described in Chapter 4. DO3A-based ligands were found to radiolabel in near-quantitative RCY as one species after heating at 100 °C for 30 min. After changing the radiolabelling buffer, near-quantitative RCYs were achieved for the DO2A-based compounds, however HPLC showed that these tracers exist as multiple species. Altering reaction conditions revealed that these species exist as kinetic/thermodynamic pairs, although it was not possible to completely isolate a single species. Log *D* values of -1.81, -0.28 and -0.07 were obtained for DO3A-TXP, DO2A-TXP and DO2A-Xy compounds respectively. Quantitative RCY was obtained for diacid-functionalised bridged cyclen, although its log *D* was lower than that of unchelated gallium.

The three candidate radiotracers discussed previously were taken forward to testing in biological models, the results of which are described in Chapter 5. The cardiac uptake of the radiotracers was determined *ex vivo* using an isolated perfused heart model and mitochondrial membrane potential decoupler challenge. Both DO2A-TXP and DO2A-Xy showed a significant change in uptake upon mitochondrial challenge, although it was found that log *D* alone is not an exact predictor of myocardial uptake. *In vitro* tumour cell uptake was also assessed for DO3A-TXP, showing a significant increase in uptake compared to unchelated gallium.

Chapter 6 summarises the overall conclusions of the thesis and suggests further work in this area. Chapter 7 provides experimental procedures.

Publications Arising From This Thesis

Synthesis, Gallium-68 Radiolabelling and Biological Evaluation of a Series of Triarylphosphonium-Functionalized DO3A Chelators; Adam J. Smith, Peter J. Gawne, Michelle T. Ma, Philip J. Blower, Richard Southworth, Nicholas J. Long. *Dalton Transactions*, 2018, **47**, 15448–15457.

DO2A-Based Ligands for Gallium-68 Chelation: Synthesis, Radiochemistry and Ex Vivo Cardiac Uptake; Adam J. Smith, Philip J. Blower, Richard Southworth, Nicholas J. Long. *Chemistry - A European Journal*, 2019, Manuscript In Preparation.

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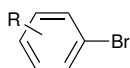
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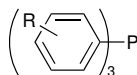
Finally, to Helen, thank you for changing my life. I'd go into more detail here, but frankly anyone who knows us knows how important you are to me, so thank you.

In Loving Memory of Jesse Moses

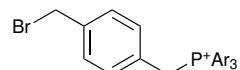
Index of Compounds



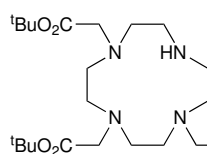
1a: Phenyl
1b: *p*-Me
1c: *m,m'*-Me
1d: *p*-^tBu
1e: *p*-OMe



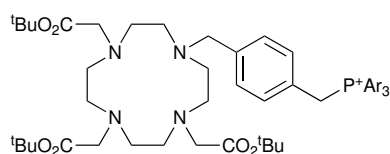
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2b: *p*-Me
2c: *m,m'*-Me
2d: *p*-^tBu
2e: *p*-OMe



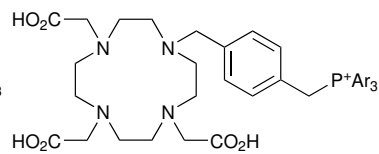
3a: Phenyl
3b: Tolylyl
3c: Xylylyl



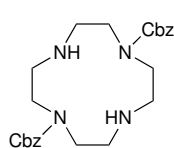
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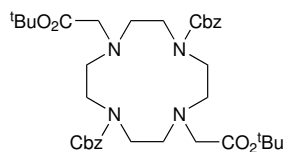
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4c: Xylylyl



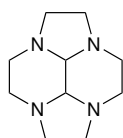
5a: DO3A-xy-TPP
5b: DO3A-xy-TTP
5c: DO3A-xy-TPP



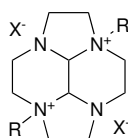
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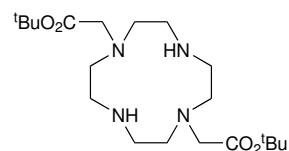
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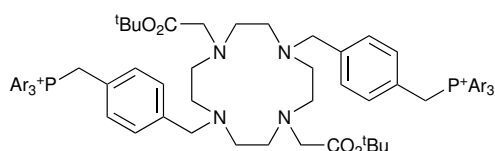
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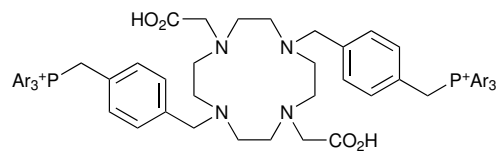
9a: R = Me, X = I
9b: R = Bn, X = Br
9c: R = CH₂CO₂^tBu, X = Br



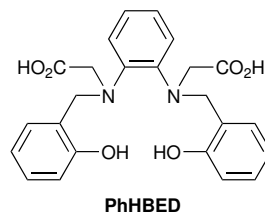
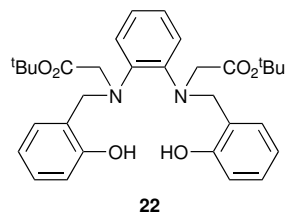
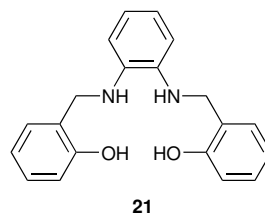
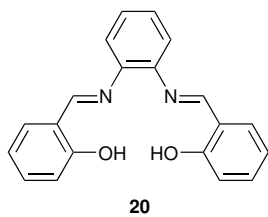
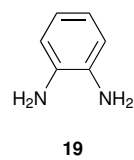
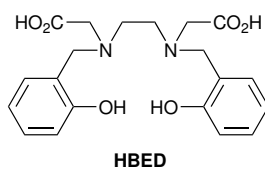
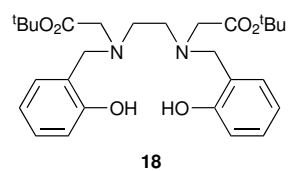
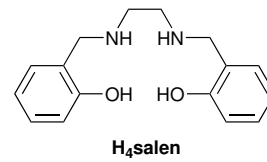
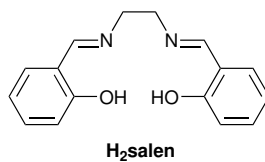
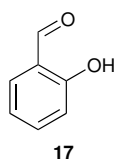
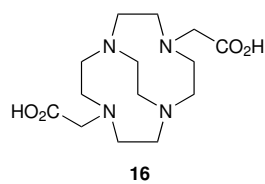
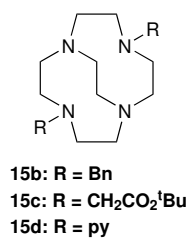
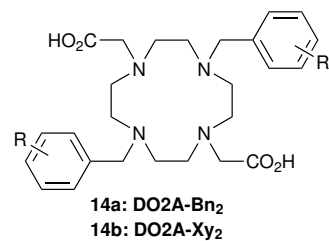
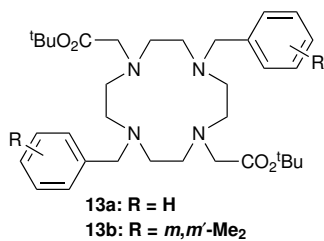
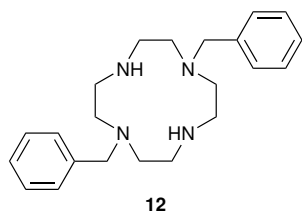
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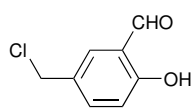


10a: Phenyl
10b: Tolylyl
10c: Xylylyl

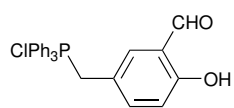


11a: DO2A-xy-TPP₂
11b: DO2A-xy-TTP₂
11c: DO2A-xy-TXP₂

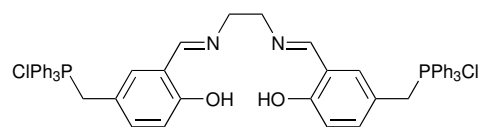




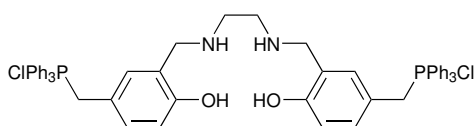
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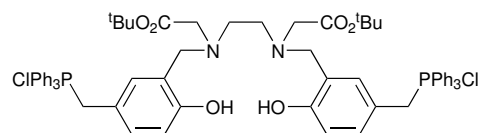
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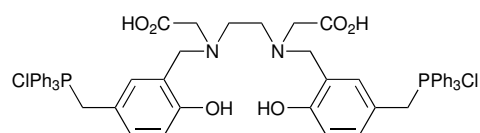
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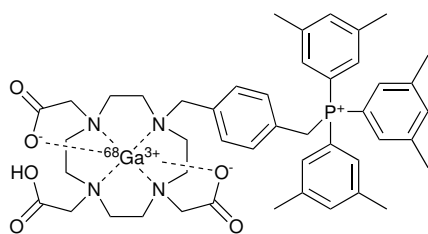
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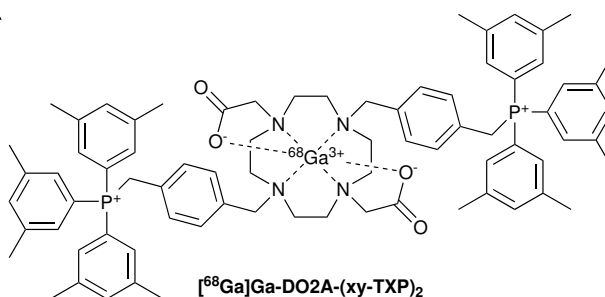
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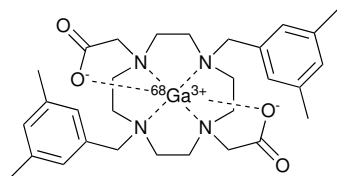
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[⁶⁸Ga]Ga-DO3A-xy-TXP



[⁶⁸Ga]Ga-DO2A-(xy-TXP)₂



[⁶⁸Ga]Ga-DO2A-Xy₂

Abbreviations

[¹⁸F]FDG	[¹⁸ F]fluorodeoxyglucose
ΔΨ_m	Mitochondrial Membrane Potential
ΔΨ_p	Plasma Membrane Potential
ΔG	Change in Gibbs Free Energy
ΔH	Change in Enthalpy
ΔS	Change in Entropy
ε	Dielectric Constant
ε₀	Permittivity of Free Space (8.85 × 10 ⁻¹² F m ⁻¹)
ADP	Adenosine Diphosphate
Ar	Aryl
ATP	Adenosine Triphosphate
bpm	Beats Per Minute
Cbz	Carboxybenzyl (-CO ₂ CH ₂ C ₆ H ₅)
CCCP	Carbonyl Cyanide <i>m</i> -Chlorophenyl Hydrazone
COSY	Homonuclear Correlation Spectroscopy
CT	Computed Tomography
DCM	Dichloromethane (CH ₂ Cl ₂)
cyclen	1,4,7,10-Tetraazacyclododecane
DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO	Dimethyl Sulfoxide ((CH ₃) ₂ SO)
DO2A	1,4,7,10-Tetraazacyclododecane-1,7-diacetic Acid
DO3A	1,4,7,10-Tetraazacyclododecane-1,4,7-triacetic Acid
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic Acid
E_m	Membrane Potential
EC	Electron Capture
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
ES	Electrospray

ETC	Electron Transport Chain
F	Faraday constant ($9.649 \times 10^4 \text{ C mol}^{-1}$)
FAD	Flavin Adenine Dinucleotide
GMP	Good Manufacturing Practice
HBED	N,N'-bis(2-hydroxybenzyl)ethylenediamine diacetic Acid
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High-Performance Liquid Chromatography
HR	Heart Rate
HSQC	Heteronuclear Single Quantum Coherence
i.p.	Intraperitoneal
iTLC	Instant Thin Layer Chromatography
IU	International Unit
KHB	Krebs-Henseleit Buffer
log <i>D</i>	Distribution Coefficient
LVDP	Left Ventricular Developed Pressure
LVEDP	Left Ventricular End Diastolic Pressure
Me	Methyl (-CH ₃)
MIBI	[^{99m} Tc]Tc-Sestamibi
mKHB	Modified Krebs-Henseleit Buffer
MRI	Magnetic Resonance Imaging
NAD	Nicotinamide Adenine Dinucleotide
NMR	Nuclear Magnetic Resonance Spectroscopy
PBS	Phosphate-Buffered Saline
PET	Positron Emission Tomography
P_i	Inorganic Phosphate
PP	Perfusion Pressure
PSMA	Prostate-Specific Membrane Antigen
Q	Ubiquinone
QC	Quality Control
R	Ideal Gas Constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$)

RCY	Radiochemical Yield
R_f	Retention Factor
ROS	Reactive Oxygen Species
rt	Room Temperature
SD	Standard Deviation
SPECT	Single Photon Emission Computed Tomography
SSR	Somatostatin Receptor
T	Temperature
TOF	Time of Flight
TOM	Translocase of the Outer Membrane
TAP	Triarylphosphonium
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran ((CH ₂) ₄ O)
THP	Trishydroxypyridinone
TLC	Thin Layer Chromatography
TPP	Triphenylphosphonium
TTP	Tritolylphosphonium
TXP	Trixylylphosphonium
UCP	Mitochondrial Uncoupling Protein
UV	Ultraviolet
W_B	Born Energy
z	Charge
Z	Atomic number

Chapter 1

Introduction

This thesis describes the development of novel lipophilic and cationic gallium-68 chelators. They are designed to non-invasively assess mitochondrial membrane potential through their mitochondrial uptake as a means of diagnosing mitochondrial disease, injury or dysfunction. This chapter aims to give an introduction to the different research topics involved in the work, starting with an overview of the biology of the mitochondria during health and disease. It will then discuss the nuclear imaging techniques SPECT and PET, with particular emphasis on the coordination chemistry and radiochemistry of gallium-68. Finally, the current uses of lipophilic cationic radiotracers for mitochondrial imaging will be discussed.

1.1 Mitochondria

Mitochondria, as depicted in Figure 1.1, are membrane bound organelles which are found in most eukaryotic cells and are commonly considered fundamental to cell life and death.² Their central role is to provide energy to a variety of biological processes throughout the organism, *via* the synthesis of ATP from ADP and P_i through a series of chemical transfer reactions. Mitochondria are also fundamental to a number of other cellular processes, including cell signalling, cellular growth, cellular differentiation and the induction of cell death.

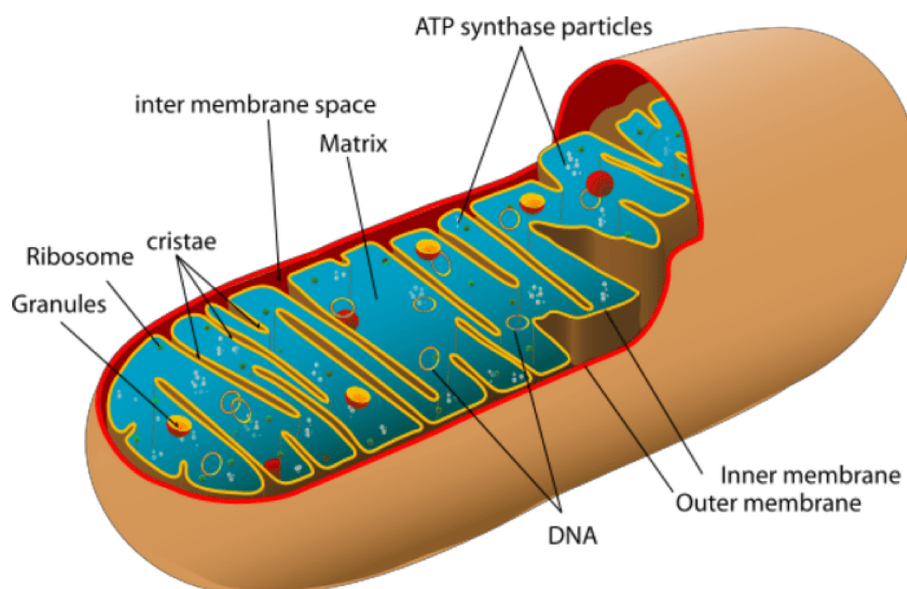


Figure 1.1: Cartoon image of a mitochondrion, highlighting important features. Picture taken from www.umdfr.org.¹

1.1.1 Mitochondrial Structure

Mitochondria consist of two phospholipid bilayers which make up five main organelle regions.³ The outer membrane which encloses the mitochondrion is made up of a phospholipid bilayer. Integrated into the membrane are two different types of transport proteins: porins and translocase of the outer membrane (TOM). Porins allow proteins with a mass of less than 6 kD to enter the organelle by diffusion, whilst larger molecules require the active transport afforded by TOM.⁴ The outer membrane also contains a diverse set of enzymes, and can associate with the endoplasmic reticulum (ER) to aid in the transfer of lipids between the ER and the mitochondrion.

The inner membrane, which separates the intermembrane space and the matrix, is impermeable to all but small molecules such as oxygen, carbon dioxide and water by virtue of its high hydrophobicity. The inner membrane also contains a variety of specialised proteins and phospholipids, including the complexes of the electron transport chain and ATP synthase. It also contains transport proteins which make the membrane selectively permeable to small molecules that are required by enzymes located in the mitochondrial matrix. On the other hand, the inner membrane is highly impermeable to ions, which is likely due to the 'double' phospholipid *cardiolipin* located in the membrane.⁵

Between the outer and inner membranes lies the intermembrane space. Small molecules such as ions and sugars are able to freely enter this region due to the semipermeability of the outer membrane, to the point that the internal concentration of small molecules is the same as that in the cytosol. Larger molecules can enter this space, but active transport by membrane-bound proteins is required. Cristae are compartments formed by the folding of the inner mitochondrial membrane. Due to the large surface area of cristae, they are ideal locations for chemical reactions to occur and as such contain enzymes such as ATP synthase and various cytochromes.

The matrix is the space enclosed by the inner membrane. It contains a highly concentrated mixture of compounds vital to the mitochondria, such as enzymes, special mitochondrial ribosomes, tRNA and several copies of the mitochondrial DNA genome. Perhaps the most important mitochondrial enzymes, those required to carry out the oxidation of pyruvate and fatty acids, and to carry out the citric acid cycle, are located here.

1.1.2 ATP Synthase and the Mitochondrial Electron Transport Chain

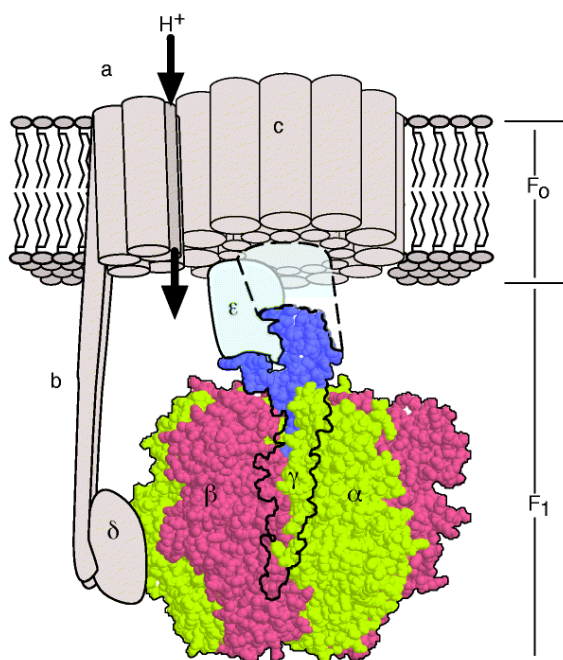


Figure 1.2: Diagram of the ATP Synthase, showing the transmembrane portion (F_0) and the soluble portion (F_1), as well as the direction of proton flow through the enzyme. Picture reproduced with permission from Wang *et al.*⁶

ATP is used as an energy source for most cellular processes, as a large amount of energy is released when it is converted from ATP to ADP and P_i . The corollary of this is that a large amount of energy is required to reform ATP through oxidative phosphorylation such that it can be reused in other cellular processes. The enzyme used to facilitate the oxidative phosphorylation and synthesis of ATP is ATP synthase, shown in Figure 1.2.⁶ The enzyme consists of a static membrane-spanning component (F_0) which comprises a proton channel, and a rotating soluble component (F_1) which contains the catalytic sites. Protons flow through the proton channels contained in F_0 , which transmits a torque to F_1 via the γ -subunit, which functions as an asymmetric shaft. F_1 contains three catalytic sites, which undergo conformational changes as the γ -subunit rotates. This facilitates the uptake of ADP and P_i , the synthesis of ATP and the release of the synthesised ATP from the enzyme. The ATP synthase can make three molecules of ATP for every twelve protons which pass through the enzyme.⁷

In order for the ATP synthase to function effectively, a proton gradient must exist across the inner mitochondrial membrane. To achieve this, ATP synthase is driven by the electron transport chain (ETC), which is a series of complexes that transfer electrons from electron

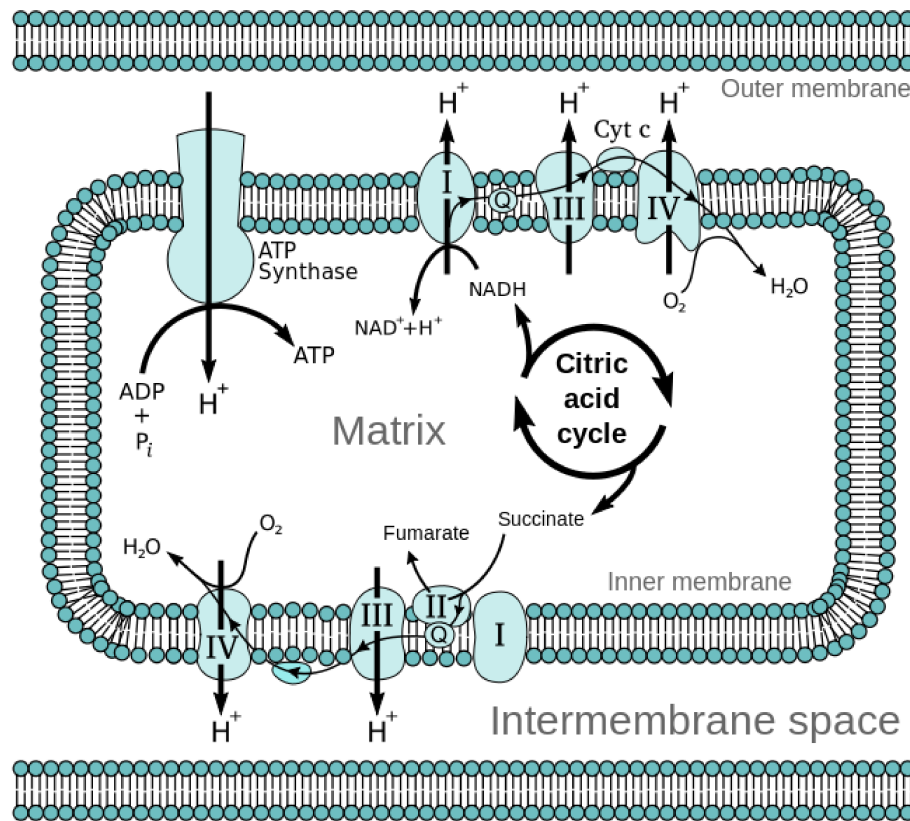


Figure 1.3: Diagram of the electron transport chain, showing the ATP synthase and associated mitochondrial complexes. Picture taken from www.biologydictionary.net.⁸

donors to electron acceptors whilst also pumping protons across the membrane. This process is outlined in Figure 1.3. Electron carriers such as NADH and FADH_2 are generated from the oxidation of carbohydrates and fat by the citric acid cycle. Electrons then enter the ETC either through reduction of NADH to NAD^+ at complex I, or through reduction of FADH_2 to FAD at complex II. Electron donation to complex I is accompanied by a series of redox reactions in the complex, which release energy and drive the pumping of protons from the matrix to the intermembrane space. On the other hand, structures of the two complexes, as well as the lower energy barrier associated with donation of electrons from FADH_2 to complex II, less energy is released and the complex cannot pump protons across the membrane.

Once the electrons have entered the ETC, the electrons are then donated to a molecule of ubiquinone (Q), which transports the electrons through the membrane to complex III in its reduced form, QH_2 . Movement of the electrons through complex III is associated with the pumping of a second proton across the inner membrane, before the electrons are transferred to cytochrome C, a protein-based electron carrier. Cytochrome C carries the electrons to complex

IV, which performs two important functions. Firstly, it pumps a third proton across the inner membrane. Secondly, complex IV uses four electrons to reduce one molecule of O_2 , which then accepts protons from the matrix to form two molecules of water.

1.1.3 Cellular Membrane Potentials

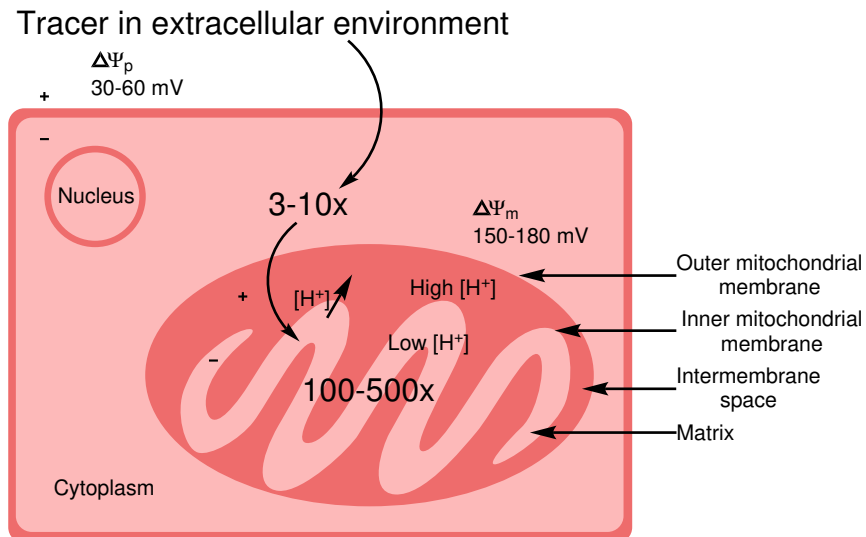


Figure 1.4: Diagram of the various membrane potentials which exist in cells. The plasma membrane potential ($\Delta\Psi_p$) exists across the cell membrane, whilst the mitochondrial membrane potential ($\Delta\Psi_m$) exists across the inner mitochondrial membrane. For monocationic substrates outside the cell, these potentials facilitate uptake into the mitochondrial matrix of up to 500 times that of the extracellular concentration. Diagram adapted from Murphy *et al.*⁹

As a result of oxidative phosphorylation, a proton gradient is established across the inner mitochondrial membrane as shown in Figure 1.4. In order to drive the conformational changes and thus catalytic activity of ATP synthase, there is a higher concentration of protons outside the matrix than within it. In a normally functioning mitochondrion, the mitochondrial membrane potential ($\Delta\Psi_m$) is between 150–180 mV.⁹

In eukaryotic cells, there is also a plasma membrane potential ($\Delta\Psi_p$) across the cell membrane. This arises as a result of ion distribution across the membrane, with Na^+ cations and Cl^- anions being at high concentration outside the cell, and K^+ cations and organic anions at high concentration in the cytosol. The plasma membrane is fully permeable to Na^+ , K^+ and Cl^- ions due to hydrophilic ion channels which span the membrane. The membrane potential is primarily maintained due to the Na^+/K^+ -ATPase, a membrane-bound enzyme which uses ATP to pump both Na^+ and K^+ cations against their respective concentration gradients in or-

der to maintain equilibrium. For every molecule of ATP hydrolysed by the enzyme, three Na^+ cations are transported into the cell whilst two K^+ cations are transported out of it. At its resting state, $\Delta\Psi_p$ is between 30–60 mV.⁹

Cardiac cells show a much wider range of $\Delta\Psi_p$ values, due to cardiac excitation-contraction coupling, the process by which myocytes cause contraction of the heart.¹⁰ This process is dependent on the concentration of Ca^{2+} cations since they are the direct activators of the cardiac myofilaments which cause heart contraction. This means that $[\text{Ca}^{2+}]$ varies throughout the cardiac cycle, leading to a range of $\Delta\Psi_p$ values between -90 and +40 mV. Note that here, convention dictates that negative membrane potentials are more positively charged outside the membrane.

1.1.4 Biophysics of Cation Uptake in Mitochondria

Whilst $\Delta\Psi_p$ is present in all eukaryotic cells, $\Delta\Psi_m$ is substantially larger than any other membrane potential present in cells.¹¹ This means that, as depicted in Figure 1.4, cations in the extracellular environment are attracted into the mitochondrial matrix. They are first attracted through the cell membrane into the cytoplasm by $\Delta\Psi_p$, before being attracted into the mitochondrial matrix by the much higher $\Delta\Psi_m$. The accumulation of cations across cellular membranes is described by the Nernst equation:

$$E_m = \frac{RT}{zF} \ln \left(\frac{[\text{M}^{z+}]_{\text{out}}}{[\text{M}^{z+}]_{\text{in}}} \right) \quad (1.1)$$

where: E_m is the membrane potential being studied, R is the ideal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the temperature, z is the charge on the cation, F is the Faraday constant ($9.649 \times 10^4 \text{ C mol}^{-1}$) and $[\text{M}^{z+}]_{\text{out}}$ and $[\text{M}^{z+}]_{\text{in}}$ are the concentrations of the cation outside and inside the membrane respectively. In this equation, E_m is conventionally negative when the potential is more positive outside the membrane being studied. Substituting known values for monocationic species into Equation 1.1 and subjecting the equation to membrane-binding corrections gives Equation 1.2.^{11–13}

$$E_m = 61.5 \times \log_{10} \left(\frac{[\text{M}^{z+}]_{\text{in}}}{[\text{M}^{z+}]_{\text{out}}} \right) \quad (1.2)$$

In this equation, only the magnitude of E_m is considered, since it is assumed that the

intercellular compartments are negatively charged. Equation 1.2 shows that an increase in E_m also leads to an increase in cation concentration inside the membrane as well as in the mitochondrial matrix. As shown by Figure 1.4, in a normally functioning mitochondrion, membrane potentials cause between a 3–10-fold increase in cation uptake compared to the extracellular environment, and between a 100–500-fold increase in the mitochondrial matrix.⁹

1.1.5 Exploiting Membrane Potentials to Target Mitochondria

Heterogeneity of $\Delta\Psi_m$ can also be linked to different cellular pathologies, and thus uptake of lipophilic cations can be associated to various disease states. One such example is tumour cells, in which $\Delta\Psi_m$ shows an increase when compared to normal healthy cells.^{14–17} Many studies report that cells derived from carcinomas, such as breast cancer, prostate cancer and melanoma cells, show $\Delta\Psi_m$ values at least 60 mV above the corresponding value in normal epithelial cells, approximately a 40% increase. The origin of this hyperpolarisation is disputed, although several explanations have been proposed. One explanation is that it arises from the increased metabolism rates in tumour cells, resulting in an increase in the rate of ATP synthase function. This explanation has some problems however, including that ATP synthase seems to function optimally at a membrane potential of 120 mV and that cancer cells are generally glycolytic and thus are less dependent on mitochondria for ATP production.

Another explanation of mitochondrial hyperpolarisation in tumour mitochondria is the underexpression of mitochondrial uncoupling proteins (UCPs) in cancer stem cells.¹⁴ A study by Derdak *et al.* showed that UCP2-knockout mice developed more colon tumours than their UCP2-expressing littermates.¹⁸ Since the main purpose of a UCP is to dissipate $\Delta\Psi_m$ as heat, underexpression of UCPs in tumour cells leads to higher $\Delta\Psi_m$ values. There is also evidence which suggests that mitochondrial hyperpolarisation also correlates to tumorigenic properties of cancer stem cells, such as their resistance to apoptosis. It is proposed that since dissipation of $\Delta\Psi_m$ is an important step in the apoptosis process, a higher $\Delta\Psi_m$ value could protect against death of the tumour cell.^{19,20}

Due to the constant high workload on the heart as it synthesises approximately 30 kg of ATP a day, cardiomyocytes each contain approximately 5000 mitochondria.²¹ As with all eukaryotic cells, cardiomyocytes require oxygen to function; this is provided by oxygenated blood in the coronary arteries (Figure 1.5) which perfuse the myocardium. If oxygen is no longer being

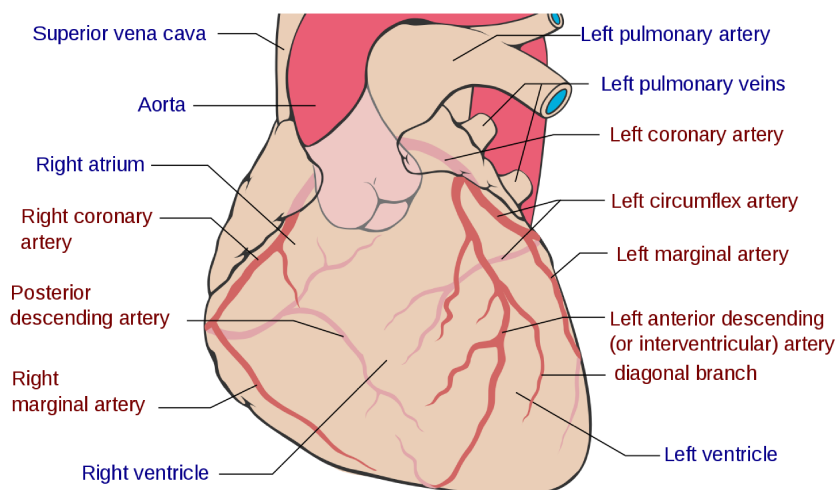


Figure 1.5: Diagram showing the coronary arteries. Picture taken from www.fastbleep.com.²³

provided to the cardiomyocytes, for example due to arterial blockage or rupture, then the ETC cannot function efficiently since there is no oxygen to accept the electron at the end of the chain, as illustrated in Figure 1.3. Healthy cardiac mitochondria generally demonstrate relatively low $\Delta\Psi_m$ values of 100-130 mV, although hearts suffering from ischemia/reperfusion injury demonstrate $\Delta\Psi_m$ values of <100 mV, and these values reach zero as the mitochondria begin to cease function entirely.²² This has the effect that mitochondrial cation uptake is reduced in compromised cardiac tissue.

1.1.5.1 Imaging Anthracycline-Induced Cardiotoxicity

The magnitude of mitochondrial membrane potentials are also affected by cardiotoxic effects from commonly used anticancer agents such as anthracyclines.²⁴ Anthracyclines are highly toxic to the heart and may cause coronary and/or valvular heart disease, cardiomyopathy and heart failure.^{25–27} Clinical focus in oncology is on treating the cancer, with cardiac protection becoming a secondary concern, and as such cardiovascular disease is highly prevalent in cancer survivors.²⁸ Since anthracycline-induced cardiac injury is dose-dependent, and depends heavily on a number of factors such as age, gender and pre- or co-existing disease states, it is difficult to administer an therapeutic drug dose whilst also minimising cardiotoxic effects.^{29–32} These effects are exacerbated by the fact that the development of cardiac damage can occur over a timeline of months or years after treatment has ceased.³³ Whilst the mechanism of the antineoplastic activity of anthracyclines is well characterised, the mechanism and timeline

of cardiotoxicity is still unresolved, although the involvement of mitochondria through iron dysregulation, reactive oxygen species (ROS) generation, metabolic disruption and apoptosis is well established.^{34–36}

Currently, the ability of clinicians to detect cardiotoxicity is poor.³⁷ It is typically identified clinically as a loss of left ventricular ejection fraction by echocardiography or MUGA (nuclear) scanning.^{38,39} Notwithstanding that this functional loss is near the limit of sensitivity of the imaging techniques, loss of contractile function is a late and often irreversible event, and significant injury is observable in biopsies long before a functional change is detectable.⁴⁰ As such, detection of cardiotoxicity at the subcellular level *via* changes in $\Delta\Psi_m$ or in ROS generation before contractile dysfunction occurs would be extremely valuable for cardio-oncology and cardioprotective drug development.

One approach in the Southworth Group to the early detection of anthracycline-induced cardiac toxicity used a fluorine-18-labelled ROS probe, previously used for imaging the effects of ischemia/reperfusion injury. A second approach uses the SPECT imaging agent [^{99m}Tc]Tc-sestamibi (MIBI), the uptake of which is $\Delta\Psi_m$ -dependant, in combination with [^{99m}Tc]TcNOET, which is taken up in cells in a manner independent of $\Delta\Psi_m$ and allows for the correction of regional variations in perfusion in the heart. It can be seen that the synthesis of PET-based tracers would allow for higher resolution imaging of these processes, as well as facilitating dynamic imaging over time.

1.1.6 Lipophilicity and Mitochondrial Uptake

The other main factor governing the uptake of cations into mitochondria is lipophilicity. As stated previously, cellular and mitochondrial membranes are made from phospholipid bilayers, made from a hydrophilic phosphate ‘head’ and a hydrophobic ‘tail’ consisting of two fatty acid chains. This creates an hydrophobic region in the membrane, and as such mitochondrially-targeted compounds must be lipophilic enough to pass into the intermembrane space.

One caveat is that if a compound is too lipophilic, it will preferentially accumulate and remain in the phospholipid bilayer membrane, since hydrophobic interactions will dominate energetically over the potential gradient. Lipophilicity also effects biodistribution, since more lipophilic compounds are less soluble in blood and thus exhibit decreased bioavailability, whilst also showing non-specific tissue uptake due to hydrophobic interactions.

There seems to be no general consensus on the ideal lipophilicity for mitochondrial uptake. [^{99m}Tc]Tc-sestamibi and [^{18}F]FBnTP, successful lipophilic cationic radiotracers discussed *vide infra*, show mean distribution coefficient ($\log D$) values of 1.29 and -0.38 respectively.^{41,42} This requirement for optimal lipophilicity, whilst maintaining a positive charge, is where much of the interest and challenge lies in lipophilic cation synthesis for these types of imaging applications.

1.2 SPECT Imaging

The mitochondrial uptake of lipophilic cations has a wide variety of biological applications, such as in neurodegeneration and cancer therapy.⁴³ There are also a variety of non-invasive medical imaging modalities, summarised in Table 1.1, which can be applied to image mitochondrial function.⁴⁴ In this thesis, the focus will be on the two nuclear imaging modalities: Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET), due to their high clinical translatability and high sensitivity. It should be noted, however, that nuclear medicine has some drawbacks when compared to other imaging techniques, such as its use of potentially harmful ionising radiation and the low resolution of the images produced.

Table 1.1: Qualitative summary of non-invasive molecular imaging modalities.⁴⁴

Modality	Resolution	Depth Penetration	Sensitivity	Ionising	Tracer Quantities
Optical	Variable	Low (cm)	High	No	High
MRI	Moderate (μm)	No Limit	Low	No	High (mM)
Ultrasound	Moderate (μm)	Moderate (mm-cm)	Moderate	No	High
SPECT	Low (mm)	No Limit	High	Yes	Low (pM)
PET	Low (mm)	No Limit	High	Yes	Low (pM)

1.2.1 Principles of SPECT Imaging

SPECT originated from work performed on emission tomography by David Kuhl and Roy Edwards in 1963.⁴⁵ SPECT relies on the detection of gamma photons produced by radioactive nuclei as they relax from excited daughter nuclei, themselves produced by parent nuclei that have undergone α -emission, β -emission or electron capture. These gamma photons can be detected by an apparatus known as a gamma camera, which consists of three basic layers. Gamma photons enter the camera through a collimator, a special lens which only allows for

the entry of photons perpendicular to the surface of the camera. Secondly, the collinear gamma photon hits a scintillator, which produces an electron that travels down a photomultiplier tube, increasing the number of electrons and thus the overall sensitivity of the camera. The signal produced by these electrons generates an image interpretable to a clinician.

In a SPECT imager, the gamma detector is made up of several gamma cameras joined together, which are mounted on a rotating gantry that allows for movement around the patient. The use of multiple camera heads means that the gantry only needs to rotate 120–180 degrees to image the entire body. Through the use of filtered back-projection or direct Fourier transform reconstruction algorithms, 2D and 3D cross-sectional images can be built up, albeit with lower spatial resolution than the raw 2D images.⁴⁴

1.2.2 Technetium-99m

By far the most commonly employed SPECT radioisotope is technetium-99m, which is used in over 40 million imaging procedures a year, accounting for over 80% of all nuclear imaging procedures worldwide.⁴⁶

The decay pathway resulting in the formation of technetium-99m is shown in Figure 1.6. Firstly, molybdenum-99 is produced from uranium-235, primarily by five research nuclear reactors located in Canada, Belgium, The Netherlands, France and South Africa.⁴⁷ Molybdenum-99 undergoes β -decay with a half-life of 66 hours to form technetium-99m. Technetium-99m is

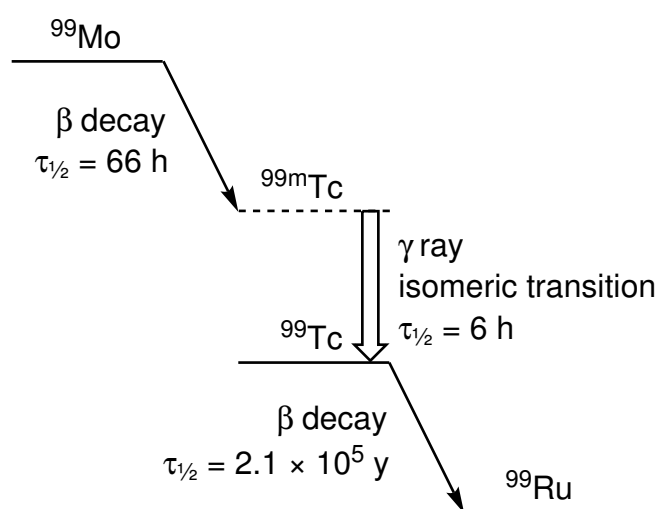


Figure 1.6: A schematic for the decay pathway of molybdenum-99 via technetium-99m. Figure adapted from Long and Wong.⁴⁴

known as a metastable isotope, in that it is energetically excited when compared to technetium-99, with a longer half-life than other comparable excited states. Technetium-99m loses its energy through γ -emission with a half-life of six hours. Depleted technetium-99 decays via β -emission to form ruthenium-99, although the half-life for this process is 2.1×10^5 years.

The long half-life of molybdenum-99, as well as its change in chemical properties as it decays from molybdenum to technetium, facilitates the use of molybdenum in technetium-99m generators as shown in Figure 1.7.⁴⁸ Technetium-99m generators use an alumina column loaded with molybdenum-99m in the form of molybdate, $[^{99}\text{Mo}]\text{MoO}_4^{2-}$. As molybdenum-99 decays, the molybdate turns into pertechnetate ($[^{99\text{m}}\text{Tc}]\text{TcO}_4^-$), which is not as strongly bound to the alumina support by virtue of its monoanionic charge. This means that technetium-99m can be eluted from the column as sodium pertechnetate using a saline solution. One drawback of technetium-99m generators is the relatively short half-life of the molybdenum-99 parent isotope, meaning that the effective lifespan of a clinical technetium-99m generator is a week.

Sodium pertechnetate itself has several medical imaging applications, such as in thyroid imaging and tumour imaging.^{49,50} In addition, the facile redox and coordination chemistry of technetium allows it to be combined with a wide range of ligand systems for a variety of

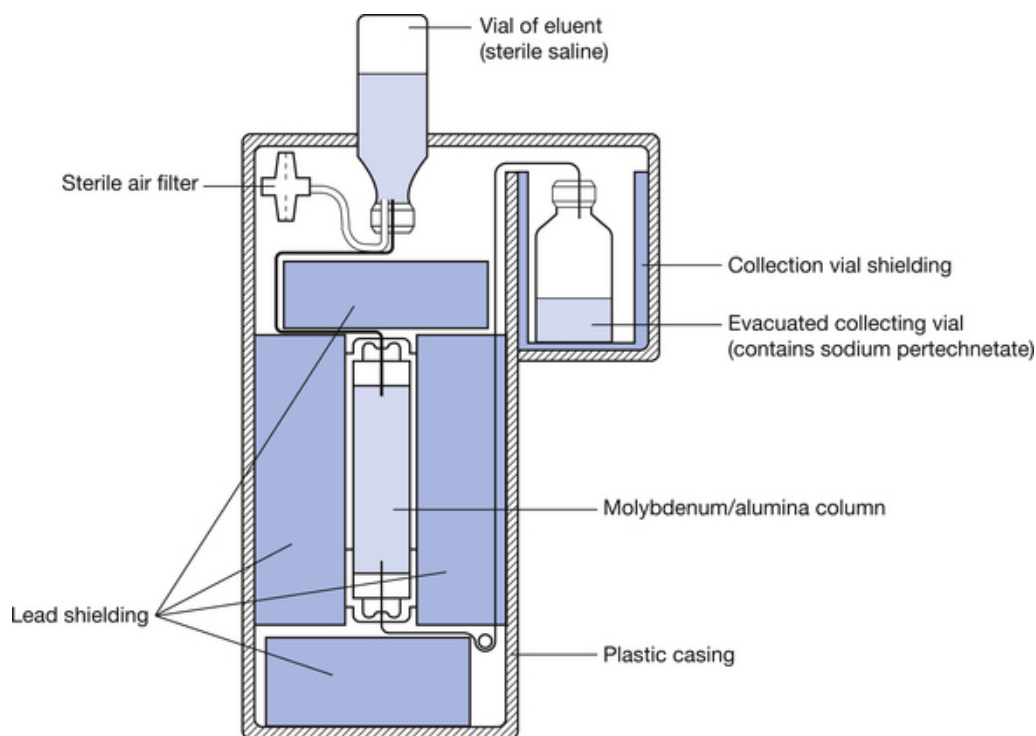


Figure 1.7: Schematic of a technetium-99m generator. Picture taken from www.radiologykey.com.⁴⁸

imaging needs. The utility of technetium can be enhanced further through the use of kits: pre-prepared mixtures of reagents capable of turning generator-produced sodium pertechnetate into a ready-to-use radiopharmaceutical, such as through HMPAo or antibody chelation.^{51,52} Two technetium-99m radiolabelled radiopharmaceuticals relevant to this project are [^{99m}Tc]Tc sestamibi (MIBI) and [^{99m}Tc]Tc tetrofosmin (Figure 1.8), which will be discussed in greater detail *vide infra* in the context of radiolabelled lipophilic cations.^{53,54}

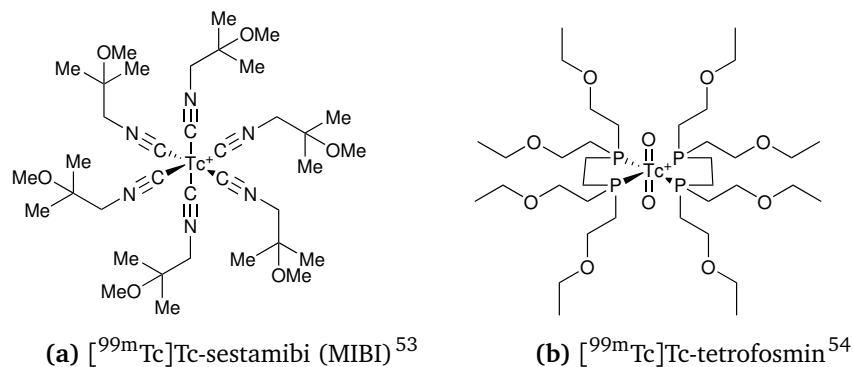


Figure 1.8: Examples of technetium-99m radiolabelled lipophilic cations.

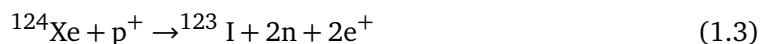
1.2.3 Gallium-67

Gallium-67 is a cyclotron-produced SPECT radioisotope with a half-life of 3.3 days which has historically been used in its citrate or nitrate form for imaging both infections and lymphomas.⁵⁵ Ga(III) is handled *in vivo* in a similar manner to Fe(III) due to their similar ionic radii, meaning that it is bound by biological iron chelators such as transferrin and leukocyte lactoferrin. Once labelled with gallium-67, these proteins locate in areas of inflammation and rapid cell division such as tumours.⁵⁶ More recently, gallium-67 has been superseded for tumour imaging by [¹⁸F]fluorodeoxyglucose ([¹⁸F]FDG) due to the comparatively low specificity of [⁶⁷Ga]Ga-citrate imaging.⁵⁷

1.2.4 Other Commonly Used SPECT Radioisotopes

Whilst technetium-99m has historically dominated the field of SPECT radiochemistry, there are many other SPECT radioisotopes commonly used in clinics today.⁵⁸ Iodine-123 was the predecessor to pertechnetate for thyroid imaging, and in fact shows a marginally higher image quality.⁵⁹ Unfortunately, iodine-123 has a 13 hour half-life, which leads to an increased patient

dose. In addition, iodine-123 is produced by proton irradiation of xenon-124 according to the following equation:



This nuclear reaction requires the use of a cyclotron, a particle accelerator which accelerates small charged particles such as protons, deuterons or α particles onto a chemical target.⁶⁰ A cyclotron uses a magnetic field and an alternating voltage in order to accelerate the charged particles until they have the required kinetic energy to bombard the target and produce the desired isotope. Targets can be in any required physical state: in the case of iodine-123, a gaseous xenon-124 target is used. Cyclotrons take up a large amount of space and infrastructure when compared to radioisotope generators, as well as having far higher operating costs, and as such they are not an option in many hospitals.

Another SPECT radioisotope is indium-111, which has a half-life of 67 hours. This long half-life means that it is useful in conjunction with targeting modalities which take a long time to localise *in vitro*, such as in white blood cell and antibody labelling.^{61,62} Thallium-201 is a SPECT radioisotope with a 73 hour half-life, which was first used as a myocardial perfusion imaging agent in the early 1970's. Thallium-201 behaves like potassium in biological systems, and can be transported into the myocardium by the Na^+/K^+ -ATPase, with an uptake dependant on myocardial blood flow.⁶³⁻⁶⁵

1.3 PET Imaging

Like SPECT imaging, PET imaging relies on the detection of γ -rays produced by nuclear processes, however, the manner in which these γ -rays are produced is different. The first PET scan was performed over 40 years ago, and whilst innovations in all aspects of PET imaging have been abundant in that time, the basic principles remain the same.⁶⁶

1.3.1 Principles of PET Imaging

Radioactive decay by positron emission is found in radioisotopes with too few neutrons, since as can be seen in Equation 1.4, positron emission serves to reduce the atomic number (Z) of

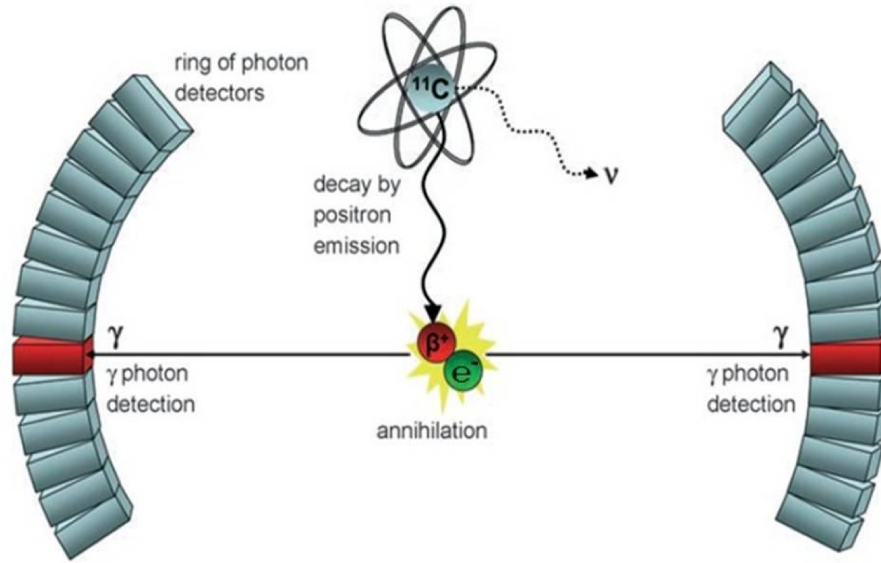


Figure 1.9: Cartoon schematic showing the principles. A PET radionuclide (in this case, carbon-11) decays by positron emission, which annihilates with an electron and emits two antiparallel γ photons, which are then detected by gamma cameras. Picture reproduced with permission from Miller *et al.*⁶⁷

the parent isotope. As the antiparticles of electrons, positrons annihilate with nearby electrons to form a neutrino and two antiparallel γ photons with identical energies of 511 keV, as shown in Figure 1.9.^{67,68} These high-energy γ photons can be detected by a ring of gamma cameras as discussed in Section 1.2.1, and the time delay between the two detection events can be used to locate the decay event in 3D space.^{69,70}



The use of collinear γ photons and the subsequent generation of a 3D image are advantages which PET has over SPECT. In addition, the use of a ring of gamma cameras in PET means that whole-body imaging is far easier to achieve than in SPECT, where the entire gamma camera gantry must rotate around the patient. On the other hand, SPECT does have advantages over PET. Firstly, SPECT is far more clinically widespread and cheaper per scan than PET. Secondly, if a PET isotope emits high-energy positrons, there is a chance it will travel several millimetres before it annihilates, lowering the inherent resolution of the technique. Finally, SPECT can be used with multiple isotopes at the same time, since gamma cameras can distinguish between the different energies of γ photons emitted. On the other hand, due to the fact that

gamma photons produced from electron-positron annihilation have energies of 511 keV, the use of multiple radioisotopes in PET must be performed by deconvoluting the half-lives of their decays. Needless to say, this is a much more complicated process than for SPECT.

1.3.2 Commonly Used PET Radioisotopes

There are a wide variety of PET radioisotopes commonly used for both clinical and research purposes.⁵⁸ Each radioisotope has advantages and disadvantages to its use, both in terms of its chemistry and its radiochemistry; important data about some commonly used radioisotopes are summarised in Tables 1.2 and 1.3.

1.3.2.1 Cyclotron-Produced Radioisotopes

Table 1.2: Summary of the properties of commonly-used cyclotron-produced PET radioisotopes.^{71–75}

PET Nuclide	Half-life	Proportion of β^+ Decay/ %	Max β^+ Energy/ MeV
Carbon-11	20.4 min	100	0.96
Nitrogen-13	9.9 min	100	1.20
Oxygen-15	2.2 min	100	1.72
Fluorine-18	109.8 min	97	0.63
Copper-64	12.7 h	19	0.65
Zirconium-89	3.3 days	23	0.90

Many commonly used PET radioisotopes, such as those listed in Table 1.2, must be produced by a cyclotron. The first radiochemical property to be discussed is half-life, as this governs the amount of time which a radioisotope can be subjected to both chemical synthesis and administration before its activity is too low for effective imaging. On the other hand, radioisotopes with overly long half-lives could give the patient a needlessly high radiation dose, which could cause problems for the patient at a later date.

Radioisotopes such as carbon-11, nitrogen-13 and oxygen-15 have very short half-lives, which means that the time from cyclotron to patient must also be very short. This necessarily limits the complexity of chemical manipulations which can be performed with these radioisotopes. Indeed, the most common use of oxygen-15 is as $[^{15}\text{O}]\text{O}_2$ for imaging blood flow.⁷⁶ The short 2.2 minute half-life of oxygen-15 limits patient dose as well as meaning that multiple imaging sessions can be performed in quick succession. In contrast, whilst radioisotopes with longer half-lives such as copper-64 and zirconium-89 lead to a higher patient dose, they are

useful for imaging protocols which require a longer time frame. Zirconium-89, for example, finds use in antibody radiolabelling as its multi-day half-life matches well with the circulation half-life of antibodies.^{73,77}

PET imaging is also improved when the radioisotope has a high percentage of β^+ -decay, since a lower amount of activity can be administered to achieve the same number of positron-emitted signals. When a radioisotope undergoes other decay pathways such as β^- - or γ -emission, it increases the patient's radioactivity dose and can potentially induce noise in the image. As shown in Table 1.2, carbon-11, nitrogen-13 and oxygen-15 only undergo β^+ -decay, whilst fluorine-18 undergoes decay by electron capture 3% of the time.⁷⁵ Copper-64 shows a far more complicated decay profile, undergoing γ -emission 43% of the time and by β^- -emission 38% of the time.⁷⁴

The third physical property of radioisotopes highlighted in Table 1.2 is the maximum energy of the β^+ particles emitted. A high β^+ -emission energy is always a detriment to effective imaging, since the positron can travel further from its original decay site before an annihilation event occurs. This is tempered somewhat by the fact that below a certain energy threshold, the distance travelled pre-annihilation is less than that of the detector resolution, and the limiting factor of the overall resolution becomes the detector.⁷⁸ For example, the large β^+ -energy of oxygen-15 means that it has an average range of 3 mm and a maximum range of 8.4 mm in water, which induces a large loss of resolution in the final image. On the other hand, the lower β^+ energies of carbon-11 and fluorine-18 mean that they have ranges of 1.2 and 0.6 mm respectively, which is below the resolution limit of the detector and thus any loss of resolution due to β^+ -energy is minimal.⁷⁹

Whilst the physics of various radioisotopes are important to their efficacy as imaging agents, their chemistry must also be considered. The chemistry of differing radioisotopes alters the types of compounds they can be incorporated in, and which pathologies they can effectively image. Due to the ubiquity of carbon in organic and biological molecules, carbon-11 can be used in radiotracers as a direct replacement for atoms already present in the molecule. This is shown by L-[¹¹C]methyl-methionine (Figure 1.10a), in which the thiomethyl group is formed with carbon-11 to form a radiotracer which can be used in oncology.⁸⁰ The ease of methylation reactions, coupled with the relative ease of synthesising radioactive methylation synthons such as [¹¹C]methyl iodide and [¹¹C]methyl tosylate mean that tracers such as [¹¹C]raclopride

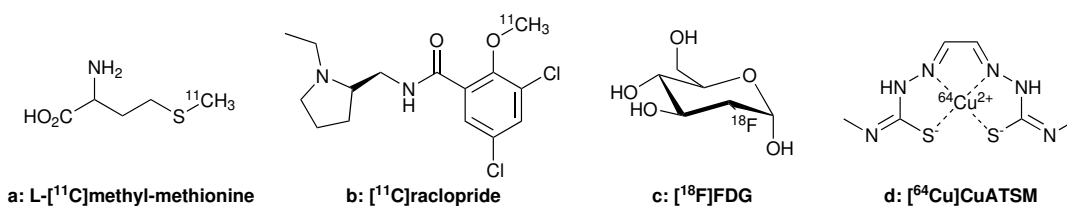


Figure 1.10: Four representative examples of radiotracers utilising the radioisotopes discussed in Table 1.2.^{80,81,84,85}

(Figure 1.10b) for imaging dopamine release can be synthesised with relative ease.^{81,82} Due to its short 10 minute half-life, nitrogen-13 is inherently limited in its applications, and is most notably used in [^{13}N]ammonia imaging for cardiac perfusion.⁸³

It would be remiss to write a discussion of PET imaging without including a mention of [^{18}F]FDG (Figure 1.10c).⁸⁴ [^{18}F]FDG is a glucose mimic with one of the hydroxy groups substituted for a fluorine-18 radiolabel. This means that it is taken into the glycolysis pathway, but the fluorine atom prevents its completion, this causing the tracer to be retained in the affected tissue. As such its localisation in the body can be used to quantify the rate of metabolism. This can be used in a variety of imaging contexts, although most notably in oncology since the elevated rate of metabolism in tumour cells favours their uptake.⁸⁶ [^{18}F]FDG is used in over 80% of clinical PET scans, and as such can be considered ubiquitous.

The vastly differing chemistries of the inorganic elements of the periodic table further diversify the contexts they can be applied in.⁵⁸ For example, the accessible redox states of copper mean that copper-64 can be used to image oxidative processes throughout the body, such as hypoxia. Radiotracers such as [^{64}Cu]CuATSM (Figure 1.10d) exacerbate this effect by using a highly electronically conjugated ligand, such that it can easily pass through lipid bilayer membranes before the copper is reduced in the hypoxic environment, meaning that it becomes trapped in myocardial tissue and allows for the quantification of ROS generation.⁸⁵ Zirconium, on the other hand, is relatively restricted in its accessible oxidation states and as such, its main use in antibody-based imaging techniques derives from the fact that its long radioactive half-life couples with the long blood circulation half-life of antibodies.⁷⁷

1.3.2.2 Generator-Produced Radioisotopes

Compared to the number of cyclotron-produced PET radioisotopes, generator-produced PET radioisotopes such as those listed in Table 1.3 are less numerous. Manganese-52m is made

Table 1.3: Summary of the properties of commonly-used generator-produced PET radioisotopes.^{71,72,87,88}

PET Nuclide	Half-life	Proportion of β Decay/ %	Max β Energy/ MeV
Manganese-52m	21.1 min	98	2.63
Rubidium-82	76 s	100	3.15
Gallium-68	68.3 min	90	1.83

from a generator containing iron-52 ($t_{1/2} = 8.3$ hours) on an anion-exchange column, before being eluted with 8 M hydrochloric acid. The short half-life of manganese-52m limits the amount of chemical transformations which can be performed with it, but it does show high heart and liver uptake, as well as blood clearance.⁸⁷ Rubidium-82, on the other hand, is made from a generator consisting of strontium-82 ($t_{1/2} = 25.55$ days) on an ion-exchange column, and can be eluted every 10 minutes with a 0.9% saline solution. Due to the short 76 second half-life of rubidium-82, the radioactive eluate is infused directly into the patient, where it acts as a potassium mimic and is taken up by cardiomyocytes, and as such it can be used as a myocardial perfusion imaging agent.⁸⁸

Whilst radioisotope generators do have myriad advantages regarding their clinical applicability, they do also have some disadvantages when compared to using a cyclotron. In general, lower amounts of activity are available when using a generator, since if too much activity is loaded onto the generator, it may become unsafe to use without a large amount of shielding. In addition, this eluted activity decreases over time as the loaded activity decays. In the case of technetium-99m as previously discussed, this leads to the generators having a usable time period of a week before they can no longer produce amounts of activity usable for imaging. This also means that generators need constant replacement which can increase their cost when compared to the one-time cost of setting up a clinical cyclotron and associated infrastructure.

1.4 Gallium-68

The last entry in Table 1.2, gallium-68, is the focus of the rest of this thesis. Whilst much of the history of PET imaging has been dominated by fluorine-18 and carbon-11, they must be synthesised using a cyclotron, which limits their use to radiopharmacies with the requisite infrastructure. Gallium-68, on the other hand, can be produced by generators, whilst also having a 68-minute half-life which is useful in a variety of PET imaging contexts. The recent

clinical approval of $^{68}\text{Ge}/^{68}\text{Ga}$ generators has the potential to cause a ‘sea-change’ in the way PET imaging is performed worldwide.⁸⁹

1.4.1 Physics of Gallium-68 Production and Decay

The parent isotope of gallium-68, germanium-68, is produced by high-energy cyclotron from stable gallium-69 as follows:



Germanium-68 is an excellent isotope for use in radioactive generators due to its long 271 day half-life as highlighted in Figure 1.11, meaning that it can generate amounts of activity usable for clinical applications over long periods of time, especially when compared to the week of usable time afforded by technetium-99m generators. Gallium-68 then decays to form zinc-68 with a 68-minute half-life, undergoing positron decay with a 90% probability, with 10% of nuclei undergoing decay by electron capture (EC). It should be noted at this point, however, that approximately 3% of nuclei in a sample of gallium-68 undergo transformation into a higher-energy form of zinc-68, which then undergoes gamma-decay to form the ground-state nucleus.⁹⁰

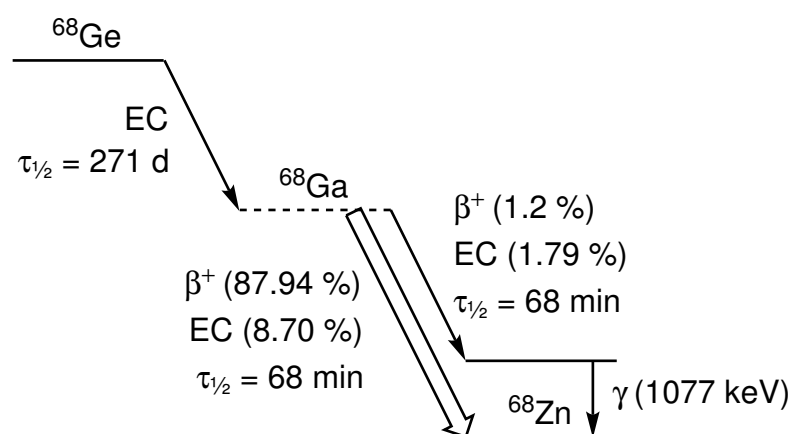


Figure 1.11: A schematic for the decay pathway of germanium-68 via gallium-68.⁹⁰

1.4.2 Germanium-68/Gallium-68 Generators

Surprisingly, initial development of $^{68}\text{Ge}/^{68}\text{Ga}$ generators was performed as early as 1961, merely three years after the first $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generators were produced.⁹¹ Many $^{68}\text{Ge}/^{68}\text{Ga}$ generators function in a similar chromatographic manner to $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$, consisting of a stationary phase impregnated with the parent isotope, which decays to produce a daughter isotope which can be eluted from the column. For $^{68}\text{Ge}/^{68}\text{Ga}$ generators, this is because germanium-68 naturally exists in the +4 oxidation state, and as it undergoes decay by EC it forms tricationic gallium-68. The decrease in charge means that the isotope is less adherent to the stationary phase and thus can be eluted. Over time, a wide selection of generators such as those in Table 1.4 have been tried and tested by altering components such as the solid phase and the elution mixture.

Table 1.4: Brief history of the development of $^{68}\text{Ge}/^{68}\text{Ga}$ generators, including the different stationary and mobile phases used.⁹²

Year	Solid phase	Eluent	Designer
1961	Al_2O_3	0.005 N EDTA	Greene <i>et al.</i> ⁹¹
1964	Al_2O_3	0.005 N EDTA	Yano <i>et al.</i> ⁹³
1975	TiO_2	0.1 N HCl	Malyshev <i>et al.</i>
1980	SnO_2	1.0 N HCl	Loc'h <i>et al.</i> ⁹⁴
1981	Al_2O_3	0.1 N NaOH	Lewis <i>et al.</i> ⁹⁵
1981	Organic Resins	5.5 N HCl	Schuhmacher <i>et al.</i> ⁹⁶

The earliest generators, such as the 1961 generator by Greene *et al.* and the 1964 generator by Yano *et al.* used alumina solid phases; however the alumina was found to bind the gallium-68 daughter isotope too strongly. As such, a solution of EDTA was needed to elute the gallium-68. Unfortunately, gallium-EDTA complexes are highly stable due to their hexadentate nature, thus it is difficult to remove the $[\text{}^{68}\text{Ga}]\text{Ga(III)}$ such that it can be used in further complexation reactions.^{91,93} A decade later, generators which could be eluted using non-chelating mobile phases such as hydrochloric acid were developed, with Malyshev *et al.* and Loc'h *et al.* using titanium dioxide and stannic oxide as stationary phases respectively.⁹⁴ In addition, the 1981 generator designed by Lewis *et al.* using a alumina stationary phase and sodium hydroxide mobile phase show a conscious move towards generators in which the mobile phase can facilitate the radiolabelling coordination chemistry that follows elution.⁹⁵ In order to further improve elution quality and prevent undesired metals leaching from the

generator, Schuhmacher *et al.* experimented with a organic resin stationary phase.⁹⁶

These early generators had a myriad of issues, such as germanium-68 breakthrough which lowers the activity available in the generator and potentially exposes patients to long-lived radioisotopes, as well as undesired metal impurities in the eluate which may compete for chelation and raise issues of toxicity for the patient. In addition, these generators use fairly harsh mobile phases, which require neutralisation before they can be administered to a patient or before complexation chemistry can be performed. Issues such as germanium-68 or zinc-68 breakthrough can be mitigated somewhat by techniques such as pre-elution, in which a generator is eluted two to three hours before use to remove the zinc built up on the column, to post-processing techniques such as the microfluidic-based monolith system developed by He *et al.*, or by fractionation.^{97–99} Further research into generator construction has yielded the four generators listed in Table 1.5, which can produce gallium-68 to pharmacopoeia standards and are thus in use clinically.

Table 1.5: Clinically approved and commercially available $^{68}\text{Ge}/^{68}\text{Ga}$ generators in use today.

Type	Obinsk	IGG100	iThemba	ITG
Supplier	Cyclotron Co, Russia (IEL)	E&Z (IEL)	USA South Africa (IDB)	ITM Munich (DIL)
Column	TiO ₂	TiO ₂	SnO ₂	Organic
Eluant	0.1 N HCl	0.1 N HCl	0.6–1 N HCl	0.05 N HCl

1.4.3 Coordination Chemistry

The most common oxidation state of gallium is +3, as this allows the metal centre to adopt a highly stable $4s^0 3d^{10}$ valence electron configuration. Gallium is sometimes observed in the +1 oxidation state, however this is rare as evidenced by the fact that gallium monochloride disproportionates into Ga(III) and Ga(0). Due to its closed-shell electronic structure, Ga(III) does not experience any stabilisation through gain in ligand field stabilisation energy upon complexation, and instead multidentate ligands are favoured to provide stabilisation to the complex through the chelate and macrocyclic effects. In addition, Ga(III) has a relatively small crystal ionic radius of 62 pm, meaning that it is best chelated with smaller ligands with lower potential coordination numbers.

One factor favouring radiometal coordination is that for a standard aliquot of generator-

produced gallium-68 consisting of 100 MBq of gallium-68 in 500 μL of eluate, the gallium-68 is in a nanomolar concentration. This means that, even in the minimum ligand concentration of 0.05 μM used by Tsionou *et al.* in their radiolabelling studies, the ligand is still in excess by an order of magnitude.⁹⁷ According to Le Chatelier's Principle, the inherent excess of ligand favours complex formation, and as such radioanalytical techniques will show that most of the radiometal forms complexes. In addition, this imbalance in reagent concentrations can make some radiochemical complexations easier to perform than their non-radioactive counterparts.

In radiochemistry, there is a trade-off between the kinetics and thermodynamics of complexation. Fast radiolabelling kinetics are desirable since they allow for short radiolabelling times and gentle radiolabelling conditions, thus allowing for a high amount of activity to be administered to the patient and high-quality images to be obtained. On the other hand, thermodynamically stable radiometal complexes can be difficult to form due to the high activation energy barrier associated with their formation, but they are more stable *in vivo* since the complex stability prevents transchelation of the radiometal to serum. As a rule of thumb, clinical PET scans should be performed within three half-lives of the initial production of the radioisotope, thus gallium-68 complexes only need enough thermodynamic stability to last for approximately three hours *in vivo*. This means that rapid chelation kinetics are generally favoured.

1.4.4 Survey of Chelators

In their 2014 review, Price *et al.* assessed a wide range of chelators for their ability to chelate the myriad of radiometals both used in research and clinically.¹⁰⁰ In Figure 1.12, nine of these chelators are shown, along with their assessment with regards to gallium-68 chelation. Chelators which Price *et al.* deem to be a 'good/best match' for gallium-68 include NOTA, TRAP and H₂dedpa (Figures 1.12a, b and c respectively). When complex stability is the most important factor in chelator design, azamacrocyclic-based chelators such as NOTA and TRAP are favoured, since the triazacyclic ring is a good size match for Ga(III) cations, as well as forming the hexadentate coordination geometry favoured by Ga(III). One problem with NOTA is that it can be difficult to introduce functionalisation motifs without compromising the ligand denticity; carbon-functionalised NOTA derivatives have been designed to circumvent this issue by allowing for the maintenance of hexadentate coordination.¹⁰¹ TRAP is another ligand

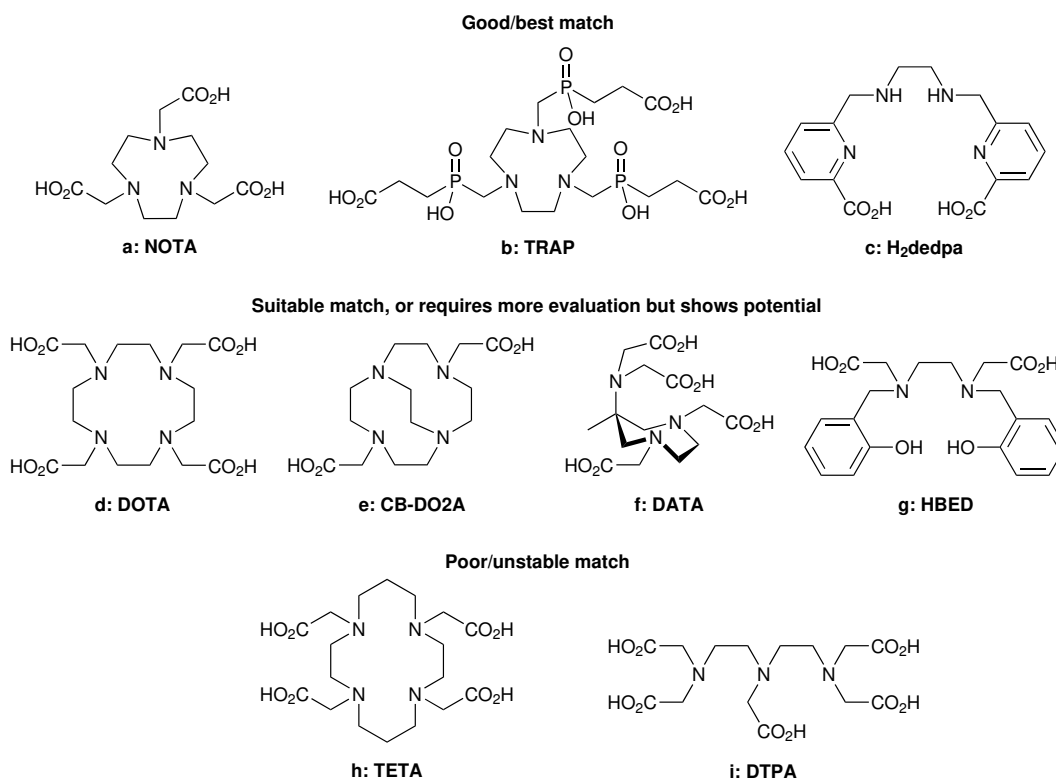


Figure 1.12: Summary of the review by Price *et al.*, outlining the preference of different chelators for gallium-68 chelation.¹⁰⁰

designed on a triazacyclononane scaffold, which allows for functionalisation of the terminal carboxylate groups, since chelation is achieved through the phosphate hydroxyl groups.¹⁰² Whilst macrocyclic chelators generally afford unparalleled complex stability, complexes for *in vivo* gallium-68 imaging are only required to resist metal leaching for three hours to allow for imaging, since the amount of radiometal is low enough to not cause any issues of toxicity. Acyclic chelators are preferred when complexation kinetics are important, since they have a lower activation energy barrier to complexation due to their greater conformational flexibility.⁸⁹ The best-rated example of an acyclic chelator for gallium-68 is H₂dedpa, since it has good size selectivity for Ga(III) whilst also having aromatic groups that allow for additional functionalisation.^{103,104}

The data presented in the review by Price *et al.* can also be compared to the more quantitative study of gallium-68 chelation undertaken by Tsionou *et al.*, who reacted a series of chelators with generator produced gallium-68 for 10 minutes each before calculating their RCY values by iTLC.⁹⁷ A 5 μ M solution of NOTA showed 93% RCY at 25 °C and pH 3.5, which is lower than the corresponding value at 90 °C. This is thought to be a result of the higher

temperature facilitating the thermodynamically favoured chelation of non-radioactive trace metals in the generator eluate. RCY values observed by NOTA are also fairly independent of pH, with <90% RCY observed at pH 6.5. Similar positive results are also observed for TRAP, although H₂dedpa was not included in this study.

The chelators deemed as a ‘poor/unstable match’ for gallium-68 chelation, TETA and DTPA (Figures 1.12h and i respectively) are also not included in the study by Tsionou *et al.* TETA, for example, was shown by Clarke *et al.* to have a very low affinity with Ga(III), whilst the radiolabelling of DTPA with gallium-68 was found by Sun *et al.* to take 60 minutes at 90 °C.^{105,106} These chelators are inappropriate for the chelation of gallium-68 due to the poor size match between the large ligand cavity and Ga(III) centres, especially when compared to the size complementarity with NOTA. This size mismatch decreases the efficacy of ligand-metal interactions, leading to a lower energetic gain upon complexation and a less stable overall complex.

Price *et al.* offer a third tier of chelators for those which are a ‘suitable match, or requires more evaluation but shows potential’ for gallium-68 radiolabelling. Whilst not strictly as appropriate for gallium-68 chelation as NOTA, TRAP or H₂dedpa, these chelators can be useful in other ways which mitigate their slower radiolabelling kinetics. DOTA (Figure 1.12d) is a macrocyclic chelator commonly used for chelating larger metal centres such as Gd(III) due to its large ligand cavity, however it can be also used somewhat effectively for Ga(III) chelation.¹⁰⁷ DOTA-based chelators can be made even more useful by using the triacid derivative DO3A (Figure 1.13), which features an unfunctionalised nitrogen atom, which can be converted to bifunctional chelators that facilitate localisation *in vivo*.¹⁰⁸ This is even more useful when chelating Ga(III), since the small ionic radius of Ga(III) only necessitates hexadentate coordination, meaning that the spare nitrogen atom can be used for functionalisation without affecting the overall complex stability.¹⁰⁹

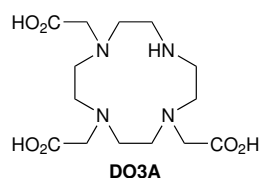


Figure 1.13: DO3A, a macrocyclic chelator derived from DOTA with an unfunctionalised nitrogen atoms which can be functionalised with a variety of motifs such as peptides to facilitate *in vivo* localisation of the radiotracer.

When a higher complex stability is required, bridged chelators such as CB-DO2A (Figure 1.12e) can be used.^{110,111} The cross-bridge improves ligand preorganisation, meaning that the final complex is more kinetically stable since there is a lower entropic loss upon complex formation. In radiolabelling contexts however, this kinetic stability comes at the cost of slower radiolabelling kinetics, as it is more difficult for the ligand to adopt a favourable conformation for chelation. This is a hindrance for the chelation of short half-life radiometals such as gallium-68, however radiometals with longer half-lives such as gallium-67 allow for the use of harsher and longer radiolabelling reactions. In addition, CB-DO2A does not have an easy route to further functionalisation, since all the nitrogen atoms present in the molecule are tertiary. Instead, C-centred functionalisation pathways must be used. HBED (Figure 1.12g), on the other hand, has faster radiolabelling kinetics due to its acyclic nature. Tsionou *et al.* found that an RCY of 86% could be obtained with only 0.5 μM of ligand at pH 6.5. Unfunctionalised HBED, however, forms isomers upon Ga(III) complexation, as shown by Figure 1.14 and as characterised by Tsionou *et al.*⁹⁷ To facilitate the transformation of HBED into a bifunctional chelator, the HBED-CC variant (Figure 1.15) was developed, with two pendant carboxylate arms which can be functionalised with targeting motifs.¹¹²

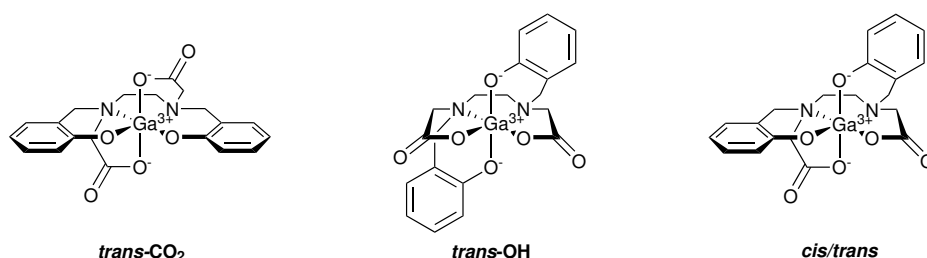


Figure 1.14: The three isomers formed upon the chelation of Ga(III) by HBED.

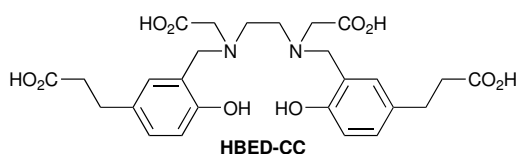


Figure 1.15: HBED-CC, a variant of HBED with two pendant carboxylate arms which facilitate the conversion of HBED into a bifunctional chelator.

The final chelator to be discussed for gallium-68 chelation are the trishydroxypyridinone (THP) ligands, one example of which is shown in Figure 1.16. These chelators were originally synthesised for iron chelation by Zhou *et al.*, however due to the size similarity between Fe(III)

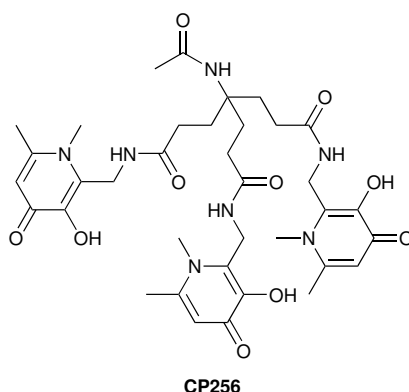


Figure 1.16: CP256, an example of the THP ligands originally synthesised for iron chelation by Zhou *et al.*¹¹³

and Ga(III), they were quickly applied to gallium-68 radiochemistry.¹¹³ THP can be radiolabelled in 5 minutes at room temperature at ligand concentrations as low as 0.5 μM , and has even been shown to sequester Ga(III) from serum *in vitro*.¹¹⁴ The amide group present on the central quaternary carbon atom also facilitates the conversion of CP256 and other THP ligands into bifunctional chelators with fast radiolabelling kinetics and high complex stability.

1.4.5 Current Clinical Applications

1.4.5.1 Gallium Citrate

Before the advent of [^{18}F]FDG imaging, citrate radiolabelled with either gallium-67 or gallium-68 was considered the gold-standard in lymphoma imaging, and to this day is still used in the imaging of inflammation and infection. Gallium salts are treated by the body as though it is Fe(III), and as such it is bound by transferrin and lactoferrin proteins in the plasma.¹¹⁵ These labelled proteins are then transported to sites of inflammation, where they can be imaged using standard nuclear medicine techniques. In many cases of inflammation imaging the bacteria themselves become directly labelled with gallium, either by consumption or through initial uptake by siderophores before in both cases the bacteria localise in the inflammation site. It should be noted that the identity of the counteranion is of little relevance to the imaging, as the gallium acts as unchelated Ga(III). Instead, biologically tolerable anions such as citrate or nitrate are used to prevent side effects to the patient.

1.4.5.2 [^{68}Ga]Ga-DOTATATE

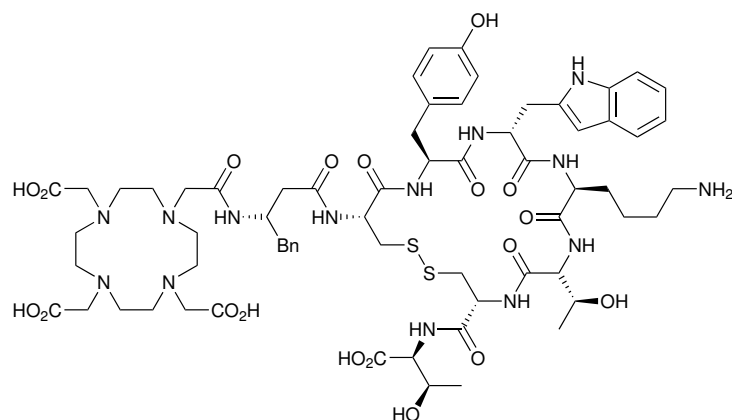


Figure 1.17: DOTATATE, a bifunctional chelator with a DO3A chelating moiety attached to an octreotate peptide, designed for tumour imaging.

Another branch of gallium-based imaging is using the bifunctional chelator [^{68}Ga]Ga-DOTATATE (Figure 1.17), which consists of a DO3A chelating moiety attached to an octreotate peptide. Octreotate acts as a somatostatin receptor (SSR) antagonist, which means that DOTATATE locates in regions where SSRs are overexpressed, such as in malignant tumours found in breast, lung and lymphatic tissue. Neuroendocrine tumours also have a high overexpression of SSRs, but cannot be imaged effectively by [^{18}F]FDG as they do not consume glucose. As such, the use of DOTATATE to image these tumours is well established in the literature.^{116,117}

Once bound to the surface transmembrane receptors of the tumour cells, the radiolabelled ligand then undergoes active transport into the cell by endocytosis. This trapping of the radio-tracer serves to increase the contrast of gallium-based imaging, although it proves even more useful when therapeutic nuclei such as lutetium-177 or yttrium-90 are considered. This is because the isotope is held in close proximity to the tumour the probability of double strand DNA breakage due to the short range of the emitted beta particles *in vivo* and subsequent tumour treatment is increased.¹¹⁸ Diagnosis of neuroendocrine tumours using [^{68}Ga]Ga-DOTATATE was approved by the FDA in 2016.

1.4.5.3 PSMA Imaging

The main current use of gallium in nuclear medicine is likely prostate-specific membrane antigen (PSMA) imaging. PSMA is a transmembrane protein located in non-cancerous prostate

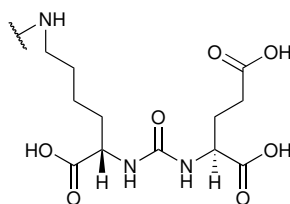


Figure 1.18: Glu-urea-Lys, a small-molecule PSMA inhibitor which can be used as a targeting vector for imaging as part of a bifunctional chelator.¹²⁰

tissue, where it has been shown to have several functions, including nutrient uptake and cell migration. PSMA is overexpressed in malignant prostate tumour cells, meaning that PSMA-targeting groups can be used to efficiently image prostate cancer. Small molecule PSMA inhibitors, such as the Glu-urea-Lys motif shown in Figure 1.18, are used to image PSMA expression in preference to antibody-based imaging techniques, since antibodies show poor tumour penetration and long blood circulation half-lives. Once the inhibitor ligand has bound to the PSMA, it is internalised into the tumour cytoplasm, meaning that once excess activity has been washed out, high contrast images of the prostate cancer can be obtained.¹¹⁹

In 2010, Banerjee *et al.* published the first PSMA targeting gallium-based radiotracer, following on from their work on technetium-99m based tracers, using DO3A as a chelating moiety. Gallium-68 radiolabelling occurred in 10 minutes at 95 °C, and was found to delineate *in vivo* tumours clearly, likely a result of the higher resolution afforded by PET over SPECT.¹²¹ By 2014, Eder *et al.* had started work on HBED-CC based PSMA imaging agents, which allowed for radiolabelling at room temperature in under 10 minutes, favourable results especially when compared to the NOTA derivative studied in the same paper.¹²² The efficacy of gallium-labelled HBED-PSMA was clinically found to be a more effective imaging agent than an alternative fluorine-18 labelled choline imaging agent, showing significantly improved contrast and the ability to detect relapses and metastases.¹²³ The DOTA, NOTA and HBED-CC PSMA imaging agents were compared in 2016; the study showed that the HBED-based radiotracer showed the highest uptake and retention in normal tissues, whilst the NOTA-based compound showed the highest retention in tumour cells as well as the fastest washout from normal tissues, and is thus the most effective PSMA imaging agent of the three studied.¹²⁰

More recently, Young *et al.* have made a PSMA imaging agent using THP chelators as shown in Figure 1.16. The main advantage of their radiotracer is the facile radiolabelling, which occurs in over 95% RCY in 5 minutes at room temperature and is then stable in human serum

for over 6 hours. Indeed, it is proposed by the authors that this facile radiolabelling could herald the advent of kit-based gallium radiotracers. *In vivo* experiments show that the THP radiotracer shows similar biological characteristics to HBED tracers.¹²⁴ It will be interesting to see in the coming years which radiotracer will become clinically prevalent, be it the ease of synthesis of the HBED tracer, the high contrast of the NOTA tracer or the facile radiolabelling of the THP tracer. DO3A should also not be discounted, since its flexibility and ubiquity make it useful in other imaging contexts, such as the growing field of theranostics. It is also possible that fluorine-18 based PSMA tracers could also become clinically prevalent due to their scalable production and higher image resolution.¹²⁵

1.5 Lipophilic and Cationic Radiotracers

So far in this introduction, the uptake of lipophilic cations by mitochondria has been discussed; it has also been shown that the degree of cation uptake is related to mitochondrial function by $\Delta\Psi_m$ (Figure 1.4, Equations 1.1 and 1.2). Various nuclear imaging methods have been detailed, which provide methods to non-invasively image molecular processes *in vivo*. The final section of this introduction will discuss how SPECT and PET can be applied to image mitochondrial function and thus a wide array of mitochondrial pathologies through their use in lipophilic cations.

1.5.1 Thallium-201 Chloride

Whilst not strictly a lipophilic cation, the history of myocardial perfusion imaging starts with the use of thallium-201 chloride in 1979. Thallium-201 is a gamma-emitting radioisotope with a 73 hour half-life, and can be made from a generator containing a lead-201 parent isotope. Thallium-201 chloride is transported into viable cells through the potassium pumps; the rate of uptake is dependant on both the rate of perfusion of the myocardium and the flow rate through the coronary vasculature. Bodenheimer *et al.* were the first to assess the use of thallium-201 chloride for myocardial perfusion imaging, finding it provided improved sensitivity and good specificity in the diagnosis of coronary heart disease when compared to electrocardiography.¹²⁶

1.5.2 [^{99m}Tc]Tc-Sestamibi and [^{99m}Tc]Tc-Tetrofosmin

Technetium-99m took over from thallium-201 as the gold-standard radioisotope for mitochondrial imaging and myocardial perfusion imaging in the 1990s, since its 6 hour half-life provides a lower radiation dose to the patient, whilst the longer half-life of the molybdenum-99 parent isotope means that the generators remain usable for longer than their lead-201 analogue. The two gold-standard technetium-99m lipophilic cations are [^{99m}Tc]Tc-sestamibi (MIBI) and [^{99m}Tc]Tc-tetrofosmin, shown in Figure 1.8. Both of these radiotracers consist of a cationic technetium core; Tc(I) in the case of MIBI and Tc(V) in tetrofosmin. These metal cores are surrounded by alkoxy ligands which shield the metal centre from hydrophilic interactions and thus improve the overall lipophilicity of the radiotracers.

MIBI was developed in 1984 by Jones *et al.* as part of a larger study of hexakis(alkylisonitrile)technetium(I) complexes, in which the *tert*-butyl isonitrile variant was found to be the most effective at myocardial imaging by virtue of its high lipophilicity, especially when compared to the previous thallium-201 compounds.⁵³ By 1990, MIBI had evolved to the familiar alkoxy ligand system shown in Figure 1.8a, and was found to be able to image defects in individual coronary arteries.¹²⁷ [^{99m}Tc]Tc-tetrofosmin (Figure 1.8b) was later developed, consisting of a [^{99m}Tc]TcO₂⁺ core bound to two phosphine alkoxy ligands. [^{99m}Tc]Tc-tetrofosmin shows an increased clearance rate from nontarget tissue, especially blood and liver, and is stable for over 8 hours after constitution.^{54,128} It is notable that these early SPECT tracers are still the clinical gold-standard myocardial perfusion imaging agents, and are available in kit form for ease of reconstitution and administration to the patient without the need for complex radiosynthesis techniques.¹²⁹

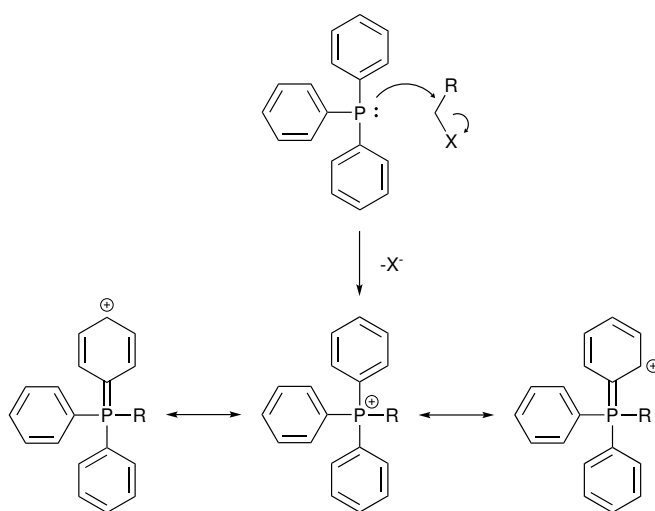
The imaging utility of MIBI does not stop at myocardial perfusion imaging however, as the elevated $\Delta\Psi_m$ in tumour cells assists uptake of lipophilic cations. MIBI is the most commonly used SPECT radiopharmaceutical for imaging breast cancer, with only [^{18}F]FDG eclipsing it in the PET arena, and there is also interest in the use of MIBI in imaging laryngeal and hypopharyngeal cancers.^{130,131} Bridging the gap between the uses of MIBI in myocardial and cancer imaging, MIBI has recently found a use in imaging heart complications as a result of cancer treatment. Zhang *et al.* have studied the onset of radiation-induced heart disease as a result of radiotherapy for oesophageal cancer.¹³² Work in the Southworth Group is being performed

regarding the preclinical study of the effects of anthracycline-based anti-cancer drugs, such as doxorubicin on heart function, with a view to the detection of cardiac dysfunction through depression of $\Delta\Psi_m$ before the effects manifest themselves as a drop in myocardial function detectable by echocardiography.

1.5.3 PET-Labelled Triarylphosphonium Cations

Whilst the wide availability of technetium-99m generators and their associated kits mean that SPECT-based techniques are the most commonly used in the clinic, research has also focussed on PET-based lipophilic cations in order to take advantage of the higher resolution and dynamic imaging afforded by PET imaging over SPECT imaging. The most common PET radioisotopes, carbon-11 and fluorine-18, are organic in nature, and thus cannot rely on a lipophilic coordination sphere in the same way technetium-99m can. The most common method of achieving mitochondria targeting for organic molecules is to add a triphenylphosphonium (TPP) group, which can easily be achieved through an S_N2 reaction between the phosphorus-centred lone pair of triphenylphosphine as shown in Scheme 1.1. The high lipophilicity of this motif is due to electronic resonance, allowing for the delocalisation of the positive charge across the large surface area of the three aromatic rings.

The tendency of TPP-functionalised compounds to target the mitochondria has been exhaustively reviewed by Zielonka *et al.*, who put forward a generic scheme for the three parts



Scheme 1.1: Generic reaction scheme showing the formation of a quaternary TPP cation through an S_N2 reaction, before resonance forms allow for the delocalisation of the positive charge throughout the aromatic rings.

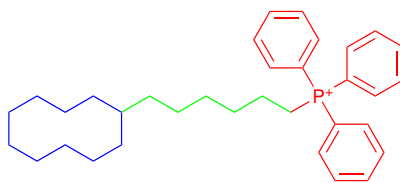


Figure 1.19: Cartoon of a generic mitochondrial targeting molecule as described by Zielonka *et al.*, consisting of: a functional moiety/cargo (blue), a linker (green) and a targeting moiety (TPP).⁴³

of mitochondrial-targeted molecules as shown in Figure 1.19. These molecules start with a functional moiety or cargo, which can consist of drugs such as metformin or doxorubicin, antioxidants such as vitamin C and imaging agents such as Gd-DOTA. These are then connected to the TPP targeting moiety by a linking group. The linking group is important, as it can distance hydrophilic cargoes from the lipophilic TPP group and permits their mitochondrial uptake. For example, Mito-Gd-DOTA has a ten-carbon long chain separating the Gd-DOTA complex from the TPP, whilst MitoParaquat has a ten-carbon linker to assist the uptake of its dicationic cargo.¹³³ This concept is applied to the SPECT arena through the compound [^{99m}Tc]Tc-Mito-MAG₃, in which a TPP motif is bound to a chelated Tc=O core by a nine-carbon linker.¹³⁴

1.5.3.1 [¹¹C]C-TPMP

The first TPP-based PET radiotracer was synthesised by Fukuda *et al.* in 1986, and consisted of TPP methylated with [¹¹C]methyl iodide. [¹¹C]C-TPMP was synthesised in 30 minutes, before intravenous injection in mice, rats and dogs showed that the tracer preferentially locates in the myocardium, where it is retained for 60 minutes. This experiment gave an estimate for $\Delta\Psi_m$ in canine myocardial tissue of -148.1 ± 6.0 mV, which has since been confirmed by other experiments.¹³⁵

1.5.3.2 Fluorine-18 Labelled TAP

The utility of carbon-11 based PET imaging is limited by its short 20 minute half-life, which limits the length and complexity of the chemistry that can be performed with it. The focus then moved to fluorine-18, most notably in the radiotracer [¹⁸F]FBnTP (Figure 1.20) designed by Ravert *et al.* in 2004.¹³⁶ Initial tests of [¹⁸F]FBnTP were promising, showing rapid clearance from the blood pool and lungs, and after 60 minutes showed a high contrast between the myocardium and the blood, lungs and liver *in vivo*.¹³⁷ Since then, [¹⁸F]FBnTP has been used

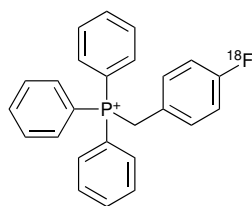


Figure 1.20: [^{18}F]FBnTP, a major example of a radiofluorinated TPP radiotracer for mitochondrial imaging.¹³⁶

in a variety of biological settings, including clinical trials for breast cancer, models of cardiac infarcts, and in the imaging of brown adipose tissue.^{138–141} The main issue hindering the widespread use of [^{18}F]FBnTP is its lengthy and complex radiosynthesis, which originally took 82 minutes over four steps to give an RCY of 6%. In 2016 however, Zhang *et al.* devised a one-step synthesis using a pinacolyl arylboronate precursor in over 60% RCY, before an automated route was discovered in 2018 by Waldmann *et al.*^{142,143}

In line with other studies involving TPP motifs, Kim *et al.* produced [^{18}F]FBnTP derivatives with ethoxyethyl and hexyl linking groups between the fluorine-18 radiolabel and the TPP cation.^{146,147} Haslop *et al.* in the Long Group went even further in their study of radiofluorinated TPP cations, developing the MitoPhos series of compounds shown in Figure 1.21.^{144,145} These compounds eschewed the lengthy synthetic methodology of [^{18}F]FBnTP instead employing a two-step reaction scheme starting with the synthesis of the [^{18}F]fluoroethylazide synthon which is then coupled to the TPP cation by a copper-catalysed click reaction. The second innovation presented by the MitoPhos compounds is the alkyl functionalisation motifs on the triarylphosphonium (TAP) moieties. These alkyl groups change the lipophilicities of the compounds, and thus change how readily the compounds pass through the lipid bilayer membranes of the mitochondria. The differing lipophilicities of the MitoPhos compounds also change their retention times by reverse-phase HPLC, meaning that the three MitoPhos tracers

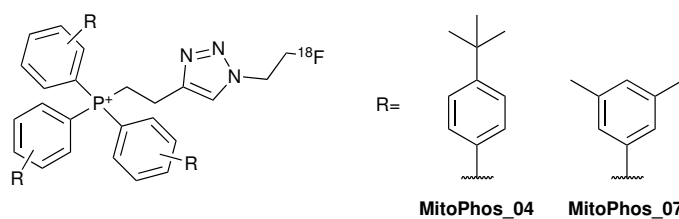


Figure 1.21: [^{18}F]MitoPhos_04 and _07 compounds developed by Haslop *et al.*^{144,145} These compounds consist of a click chemistry motif to facilitate radiosynthesis, whilst they have differing alkyl functionalisation motifs to alter their mitochondrial uptake properties.

studied by Haslop *et al.* can be synthesised in ‘one pot’ and then isolated after the reaction has finished.

1.5.3.3 Copper-64 Labelled TAP

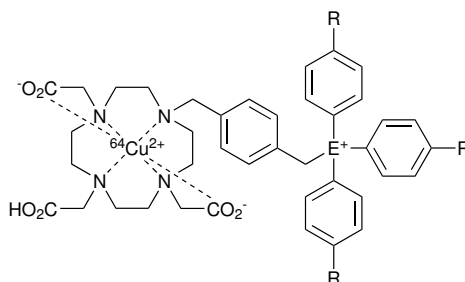


Figure 1.22: General form of the copper-64 labelled DO3A compounds functionalised with triphosphines designed by Wang *et al.*. R = H, OMe; E = P, As.¹⁴⁸

TAP motifs have also been used to make lipophilic cations with a copper-64 radiolabel, as shown by the complexes outlined in Figure 1.22 and originally designed by Wang *et al.* in 2007.¹⁴⁸ Unlike the carbon-11 and fluorine-18 examples discussed previously, copper-64 and other metal radioisotopes cannot label a TAP motif directly; instead a chelator such as DO3A must be used to connect the radiolabel to the TAP. In Figure 1.22, the functionalisation of the triarylphosphine can be altered to include methoxy groups, and perhaps uniquely in this work the phosphorus cation can be altered for an arsenic cation. Despite seeming to be neutrally charged and having low log *D* values, the TAP motifs allow for these tracers to effectively target tumour mitochondria, with the methoxy variant demonstrating a tumour/heart uptake ratio 40 times greater than MIBI at 120 minutes post-injection. The arsenic variant, however, did not provide sufficient improvement over the phosphorus versions, and as such does not seem to be prevalent in the literature after this point.

The fact that these chelators show tumour uptake preferentially over heart uptake is likely a result of the low lipophilicity of the compounds, meaning that the elevated $\Delta\Psi_m$ in tumours can overcome their increased hydrophilicity. These TAP-functionalised DO3A ligands for copper-64 chelation were reviewed in 2011 by Zhou *et al.*¹⁴⁹ This paper presents a wide range of phosphine cations, including an asymmetric diphenylphosphine oxide variant, as well as mono-TPP and bis-TPP functionalised DO2A chelators. The review also takes into account different linking groups, such as propyl and butyl, alongside the pre-established xylyl linker.

Finally, different synthetic methodologies are explored, most notably functionalising a DO3A or NOTA chelator with a thiocyanate group, then using an amine group in the TPP motif to couple the two parts of the bifunctional chelator together.

1.5.4 Gallium-Radiolabelled Lipophilic Cations

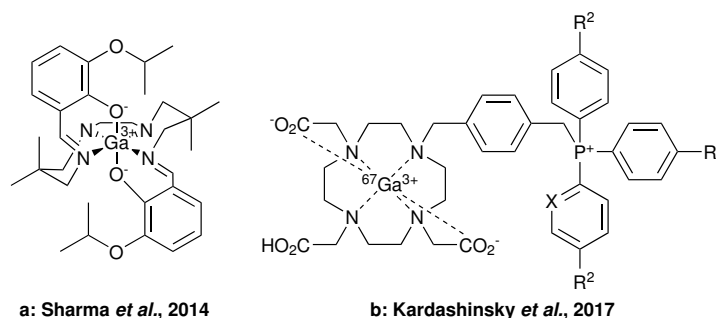


Figure 1.23: The two examples of gallium-radiolabelled lipophilic cations in the literature. **a:** Developed by Sharma *et al.* in 2014.¹⁵⁰ **b:** Developed by Kardashinsky *et al.* in 2017. $R^1 = \text{I, H, Me, OMe}$; $R^2 = \text{H, Me, OMe}$; $X = \text{CH, N}$.¹⁵¹

At the time of writing, there are two examples of gallium-labelled lipophilic cations in the literature; these are shown in Figure 1.23. The first compound (Figure 1.23a) was developed in 2014 by Sharma *et al.*, and consists of a gallium-68 metal core surrounded by aromatic and isopropoxide groups, which serve to improve the lipophilicity of the complex.¹⁵⁰ It should be noted that the biological analyses of the complex was performed using gallium-67 to take advantage of its longer half-life. Unfortunately, the authors neglected to report a log *D* value for the complex. In addition, the $[^{68}\text{Ga}]\text{GaCl}_3$ generator eluate must be converted into $[^{68}\text{Ga}]\text{Ga}(\text{acetylacetonate})_3$ before radiolabelling can occur, since a low affinity to the initial ligand is needed to counteract the energy needed to remove the protecting group from the chelator upon radiolabelling. Despite these negative aspects, the compound shows good uptake in *in vitro* assays, with *in vivo* experiments showing a heart/blood activity ratio of 147 ± 25 after 120 minutes, although after the same time a heart/liver activity ratio of only 3.5 ± 0.88 was observed, demonstrating significant liver uptake of the complex. Finally, metabolite analysis showed that the complex was stable over 60 minutes, no matter whether the activity was located in the heart, liver or urine.

The second series of compounds shown in Figure 1.23 are the TAP-functionalised DO3A compounds developed by Kardashinsky *et al.* in 2017.¹⁵¹ This series of compounds includes

methyl and methoxy aryl functionalisation, as well as monoiodo and pyridyl asymmetric variants, although full synthetic data is only supplied for the pyridyl and methyl variants. The radiochemistry of these compounds again focusses on gallium-67 to take advantage of its longer half-life; the radiosynthetic procedure involves pH adjustment steps followed by two purification steps, which would not be feasible for the shorter half-life gallium-68. The paper makes no attempt to elucidate the effects of the aryl functionalisation on lipophilicity, instead seeming to focus on the long-term stability of the complexes. The four complexes studied are stable over 24 hours in dPBS, whilst they seem to decompose over a number of hours in human serum. HPLC metabolite analysis seems to suggest that these compounds decompose at the xylyl bridge, with [^{67}Ga] Ga(III) , [^{67}Ga] Ga(DO3A) and [^{67}Ga] Ga(DO3A)-xy metabolites visible, whilst the pyridyl variant seems to be immune to this degradation process.

Despite this degradation, TAP-functionalised DO3A, as also studied by Wang *et al.* in 2007, seemed like a promising starting point to begin research into gallium-68 labelled lipophilic cations as it was thought that the degradation would not be an issue on the timescale afforded by the half-life of gallium-68.¹⁴⁸ The research outlined in this thesis begins by combining the DO3A chelation motif to the modular alkyl functionalisation afforded by the MitoPhos compounds.¹⁴⁵ Radiochemical and biological experiments performed on these compounds will inform the design of future iterations of gallium-68 lipophilic cations contained in this thesis.

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Chapter 2

Thesis Aims and Overview

This chapter outlines the aims and objectives of the research carried out in this thesis, as well as giving an overview of the content of each chapter.

2.1 Thesis Aims

Lipophilic and cationic radiotracers are clinically used for measuring changes in mitochondrial membrane potential in a variety of diagnostic contexts from oncology to cardiovascular imaging. Despite the ubiquity of currently used lipophilic cations such as MIBI, there is a need to develop improvements upon these tracers. This could be to take advantage of the higher resolution afforded by PET when compared to SPECT in order to identify smaller regions of aberrant $\Delta\Psi_m$, or to optimise radiotracer half-lives or emission energies to reduce patient dose. Gallium-68 is a radioisotope that answers some of these problems, with its PET capabilities and relatively short half-life. The fact that it is generator-produced, and said generator has achieved recent clinical approval, make it poised to become a driving force in nuclear medicine, pending the design of appropriate chelators.

The primary aim of this thesis was to develop functionalisation motifs for chelators in order to form lipophilic and cationic complexes with gallium-68, with a view to assessing their mitochondrial uptake and retention in pre-clinical biological studies. To achieve this, initially literature chelators for gallium-68 were functionalised in order to improve their lipophilicity whilst also maintaining a cationic charge. Their radiolabelling was assessed both in terms of yield and speciation in order to determine ease of clinical translation through potential regulatory compliance. As an index for lipophilicity, $\log D$ values for the tracers were obtained. This work then led into less well-known chelators for gallium-68, with a view to increased lipophilicity and stability.

This work then took the most promising radiotracers as determined by their $\log D$ values, then used them to perform pre-clinical assessments of their mitochondrial uptake and retention in a variety of biological settings. These tests included the *ex vivo* Langendorff isolated perfused heart model to assess the uptake and retention of the tracers in both healthy and mitochondrially-compromised hearts, whilst *in vitro* cultured cancer preparations were used to assess the potential tumour uptake of the radiotracers. These experiments were performed not only to demonstrate the versatility of lipophilic and cationic radiotracers for mitochondrial

imaging, but also to determine whether any of the novel radiotracers produced in this thesis are suitable for *in vivo* assessment and potential clinical translation. These biological experiments also influence the design of further generations of tracers, both within this thesis and for the future.

2.2 Thesis Overview

The chapters of this thesis follow the initial steps on the path to designing a clinically viable radiotracer, starting with ligand design through to pre-clinical biological testing in heart and cancer models.

The opening results chapter considers the chemical synthesis of analogous chelator series for gallium-68, starting with the known chelator DO3A before moving to less well-known gallium-68 chelators such as DO2A and bridged cyclens, as well as acyclic chelators such as HBED. There was a focus on synthesising analogous series of tracers with tunable lipophilicities through functionalisation of aryl motifs within the molecule, which could be combined with the chelators in a modular approach.

The chelators were then radiolabelled with gallium-68 in the next chapter. Initial radiolabelling experiments to ensure a high RCY value, such that a high specific activity of radiotracer could be obtained for both pre-clinical biological experiments in this thesis and for potential future *in vitro* translation. Following this, the main experiments performed were log *D* measurements to assess the lipophilicities of the radiotracers and their likely mitochondrial uptake, as well as HPLC in order to assess the degree of speciation in the radiolabelled compound and their potential future regulatory compliance. When speciation occurred, such as for the DO2A-based compounds, additional experiments were performed both to determine the nature of the speciation, and whether a single isomer could be obtained.

The third and final results chapter discusses the pre-clinical biological assessment of the most promising radiotracers from the first two chapters as measured by their log *D* values. The initial focus of this project was on using the *ex vivo* Langendorff isolated perfused heart model to assess the suitability of the tracers to myocardial perfusion imaging. Langendorff perfusion was chosen as it mimics the manner in which radiotracers flow through coronary vasculature and are taken up into myocardial tissue, thus providing greater clinical translatability to *in vivo*

myocardial perfusion imaging. It was also possible to determine whether $\log D$ calculations provide an accurate indicator of myocardial uptake. For the compounds that did not show appreciable myocardial uptake due to low lipophilicity, a separate experiment was performed using an *in vitro* preparation of breast cancer cells to assess their uptake, again with a view to further *in vivo* experiments if any tracers showed promise.

Chapter 3

Synthesis of Lipophilic and Cationic Chelators for Gallium-68

The first step in the development of gallium-68 based lipophilic and cationic radiotracers is the design and synthesis of chelators that not only bind [^{68}Ga]Ga(III) effectively, but also have an overall positive charge and demonstrate an appropriate degree of lipophilicity. This chapter outlines the design of these chelators, as well as their synthesis and characterisation. This project was performed in an iterative manner, since data from later radiolabelling and biological experiments were used to inform the design of further chelators.

3.1 DO3A-Triphosphines

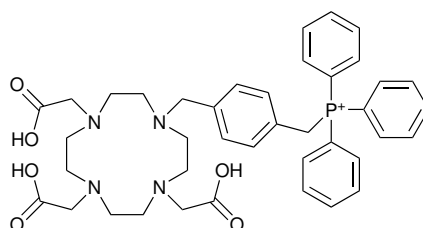


Figure 3.1: DO3A-xy-TPP, which has been used for both copper-64 and gallium-67 chelation by Wang *et al.* and Kardashinsky *et al.* respectively.^{1,2}

In their 2014 review, Price and Orvig describe DOTA as a ‘suitable match’ for the chelation of Ga(III).³ Triphenylphosphonium (TPP) functionalised variants, such as that shown in Figure 3.1, were originally used as copper-64-labelled PET tracers by Wang *et al.* in 2007, and were found to image *in vivo* tumours as effectively as MIBI.¹ This, coupled with the expertise in the group regarding DO3A-based syntheses, made DO3A-xy-TPP an ideal starting point for this project. An innovation for these compounds was inspired by Haslop *et al.*, who in their synthesis of the MitoPhos series of compounds functionalised TPP moieties with *tert*-butyl and *m,m'*-dimethyl groups to provide tracers with tunable lipophilicities.⁴

The analogous series of target compounds designed according to this paradigm are shown in Figure 3.2. These consist of a DO3A chelating moiety, a *p*-xylyl linker, and a triphenylphosphonium, tritolylphosphonium (TTP) or a trixylylphosphonium (TXP) group, with a view to altering the lipophilicities of the final radiolabelled compounds. The overall charge of these compounds is expected to be +1, since the trianionic charge provided by the three carboxylic acid arms is cancelled out by the tricationic Ga(III) metal centre, leaving a monocationic charge from the triarylphosphonium (TAP) group. Haslop *et al.* showed in their MitoPhos series of compounds that increasing the degree of alkyl functionalisation increased the log *D* values of

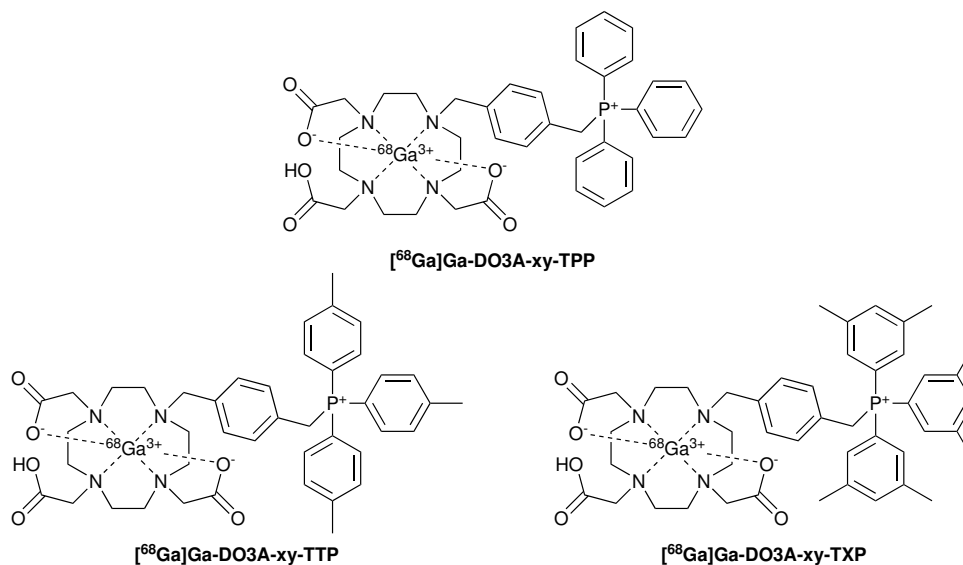
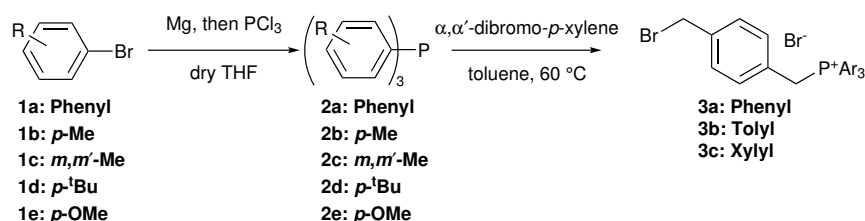


Figure 3.2: The three target DO3A compounds investigated in this thesis. These consist of a DO3A moiety capable of chelating $^{68}\text{Ga}]Ga(\text{III})$, a xylyl linking group, and a TPP, TTP or a TXP cation designed to provide a cationic charge as well as lipophilicities. The synthesis of other variants was explored, but were discovered to be unsuccessful for a variety of reasons.

their compounds, an effect expected to be observable in these DO3A-based compounds.⁴

3.1.1 Synthesis of Triarylphosphines

The first two synthetic steps towards the synthesis of DO3A-xy-TAP are outlined in Scheme 3.1. The first step, originally used by Haslop *et al.*, began with aryl bromides (**1**), which were converted into Grignard reagents *in situ* through reaction with magnesium turnings, before phosphorus trichloride was added to form bright yellow triarylphosphines (**2**).⁴ Any excess Grignard reagents were quenched with water, before the phosphines were isolated. Triphenylphosphine (**2a**) is an inexpensive commercially available reagent, whilst the xylyl and *tert*-butyl variants (**2c** and **2d**) were originally synthesised by Haslop *et al.*



Scheme 3.1: The first two steps towards the synthesis of DO3A-xy-TAP compounds. Aryl bromides were converted into triarylphosphines, before coupling to α, α' -dibromo-*p*-xylene to form the triarylphosphonium cations attached to the xylyl linking group. Of the five aryl bromides studied, only three formed TAPs.^{1,4}

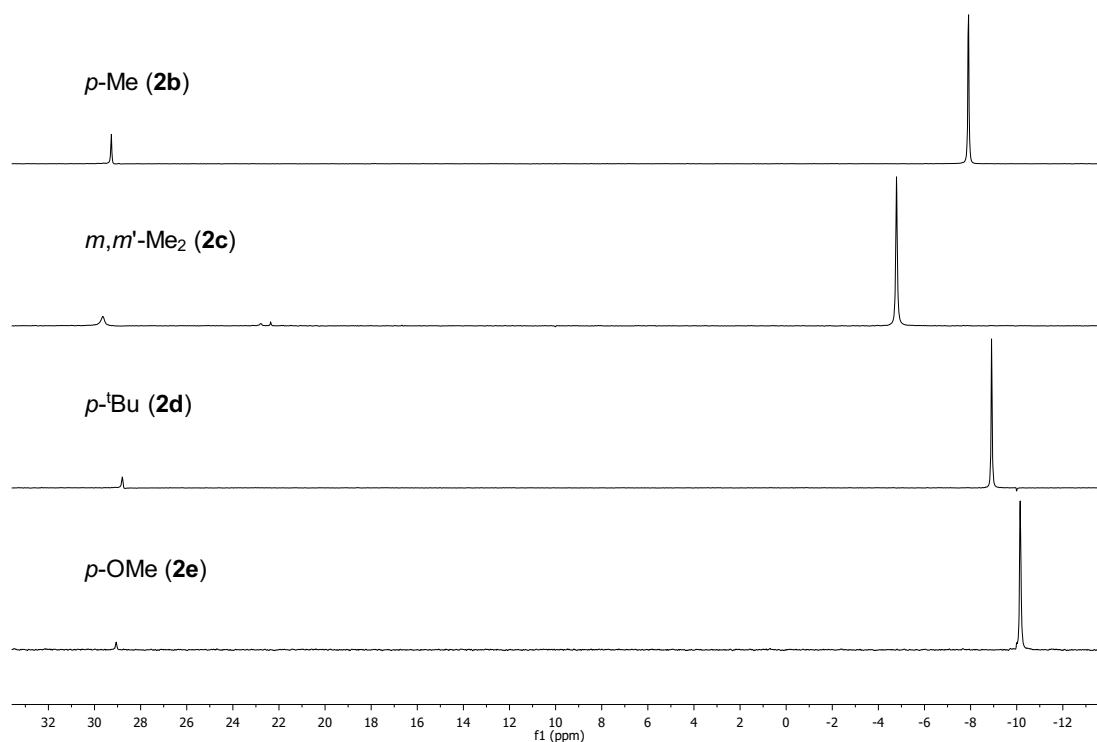


Figure 3.3: $^{31}\text{P}\{^1\text{H}\}$ NMR spectra of the triarylphosphines **2b-e** (162 MHz, 298 K, CDCl_3). The peaks with negative chemical shifts refer to the intended triarylphosphine product, whilst the smaller peaks around 30 ppm refer to the oxidised side product. This allows for a rough estimate of the purity of the phosphorus-containing species in the final product.

Proton-decoupled phosphorus-31 NMR spectra of the triarylphosphonium compounds, including the tolyl and methoxy variants (**2b** and **2e** respectively) are shown in Figure 3.3. Each compound shows two peaks: one with a negative chemical shift corresponding to the product, and one with a chemical shift at approximately 30 ppm which refers to the product with an oxidised phosphorus atom. The desired product can be assigned in comparison to the literature ^{31}P NMR shift of triphenylphosphine of -4.7 ppm.⁵ Despite the maintenance of anaerobic conditions, some product was oxidised, a likely result of quenching of the excess Grignard reagent with water. Integration of the peaks in Figure 3.3 shows that the products were isolated in over 90% purity in all cases, and as will be shown in the following step in Scheme 3.1, oxidised impurities can be carried forward without further purification. Comparison of the differing chemical shifts of the phosphorus atoms cannot be made at this point, since an internal phosphorus-containing standard was not included in the NMR sample.

3.1.2 Formation of Triarylphosphonium Cations

3.1.2.1 Functionalisation of Dibromoxylene

As shown in Scheme 3.1, the next step was the synthesis of the triarylphosphonium cations. α,α' -Dibromo-*p*-xylene was used as a substrate, as this provides the TAP cation with a xyl-yl linking group and an alkyl bromide which will allow the cation to couple to the DO3A chelating moiety. This reaction was originally designed by Wang *et al.*, who synthesised the TPP-functionalised bromoxylene **3a** in toluene at 100 °C, with the product precipitating out of solution at low temperatures due to the low solubility of cationic substrates in toluene.¹ Since this reaction proceeds through nucleophilic substitution using the phosphorus-centred lone pair, the oxidised impurity present in the triarylphosphines is inert and can be carried through without concern.

Synthesis of **3a** occurred as described in the literature, however the use of more electron donating triarylphosphonium substrates such as **2b** and **2c** led to impurities as typified by

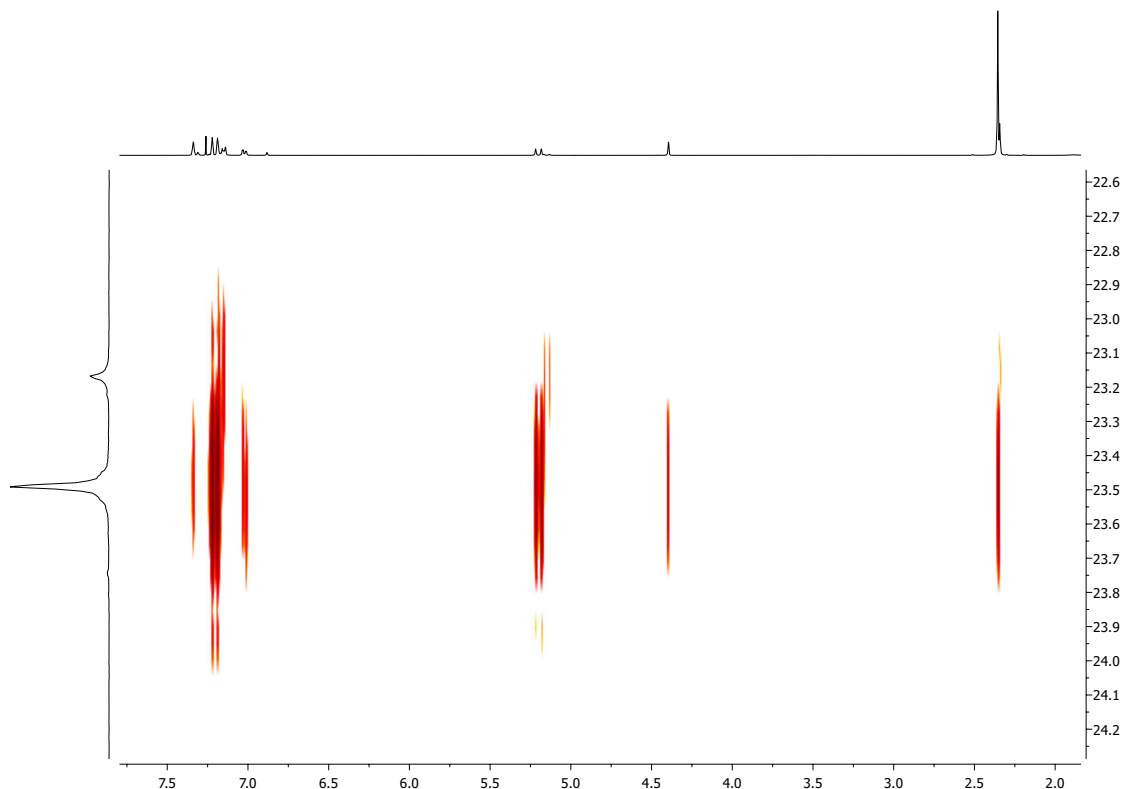


Figure 3.4: $^1\text{H}\text{-}^{31}\text{P}\{^1\text{H}\}$ HMBC NMR spectrum of the result of the reaction between TXP (**2c**) and α,α' -dibromo-*p*-xylene (400 MHz, 298 K, CDCl_3). The vertical axis ($^{31}\text{P}\{^1\text{H}\}$ NMR) displays two peaks which refer to the desired monosubstituted product and the disubstituted impurity. These could be characterised by the associated ^1H NMR spectrum on the horizontal axis as well as mass spectrometry.

the ^1H - $^{31}\text{P}\{^1\text{H}\}$ Heteronuclear Multiple Bond Correlation (HMBC) NMR spectrum of **2c** in Figure 3.4. The $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum on the vertical axis of Figure 3.4 shows two peaks at 23.5 and 23.1 ppm. Unlike in Figure 3.3, the peaks have similar chemical shifts, indicating that the impurities are not the result of phosphorus-centred oxidation; indeed, the downfield shift of the two peaks compared to the starting trixylylphosphine implies that the phosphorus atoms have become more deshielded, and that substitution has occurred.

In order to try to characterise the impurity, the ^1H - $^{31}\text{P}\{^1\text{H}\}$ HMBC NMR spectrum in Figure 3.4 was used. HMBC was chosen over Heteronuclear Single Quantum Coherence (HSQC) spectroscopy since HSQC only allows for single bond coupling, and there are no phosphorus-proton bonds in the molecule. Both phosphorus peaks show cross-peaks with the aromatic region, as well as with the proton-phosphorus coupled doublets at approximately 5.2 ppm. Only the phosphorus peak at 23.5 ppm shows a cross-peak to the singlet at approximately 4.4 ppm, indicating that this is the desired monosubstituted product since it displays proton environments adjacent to both the phosphorus and bromine atoms. On the other hand, the peak at 23.1 ppm likely refers to the disubstituted impurity shown in Figure 3.5, since the only cross-peaks seen arise from phosphorus-coupled protons. In addition, mass spectrometry showed peaks referring to the monosubstituted product and the disubstituted impurity. Even though it is not characterised by the ^1H - $^{31}\text{P}\{^1\text{H}\}$ HMBC NMR spectrum, it seems reasonable that the product mixture contains the α,α' -dibromo-*p*-xylene starting material, as it is insoluble in toluene at room temperature.

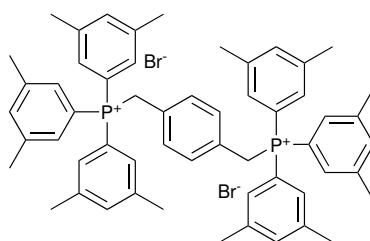


Figure 3.5: Structure of the disubstituted impurity as referred to by the phosphorus peak at 23.1 ppm in Figure 3.4. The structure of this impurity was also elucidated by mass spectrometry.

Due to the cationic nature of the substrates, column chromatography could not be used as a purification technique since the compounds adhere to the silica stationary phase. Instead, it was decided to exploit the decrease in solubility in toluene upon cation formation by performing the reaction at the minimum temperature required to dissolve the α,α' -dibromo-*p*-xylene

starting material, especially since the triarylphosphines were fully soluble in toluene at room temperature. Performing the reaction at 60 °C meant that as monosubstitution occurred, the cation precipitated out of solution and did not react further. Isolation of the product was performed by vacuum filtration, and the only purification needed was the washing of the product by diethyl ether, which caused further monosubstituted product to precipitate from the toluene filtrate, and thus further increasing the yield.

Unfortunately, the *tert*-butyl- and methoxy-substituted triarylphosphines (**2d** and **2e** respectively) did not form the desired products. The monosubstitution of **2d** was hindered since the *tert*-butyl functionalisation lies outside the plane of the aromatic rings, hindering π - π interactions with the toluene solvent and preventing solubility. As such, no reactivity was seen, even at elevated temperatures. Alternative solvents such as xylenes and acetonitrile were also used, but with limited success. The methoxy-substituted variant synthesised using **2e** was also found via the $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum shown in Figure 3.6, in which three peaks at 20.5 ppm are observed, amongst other impurities. This lack of control and regioselectivity over this reac-

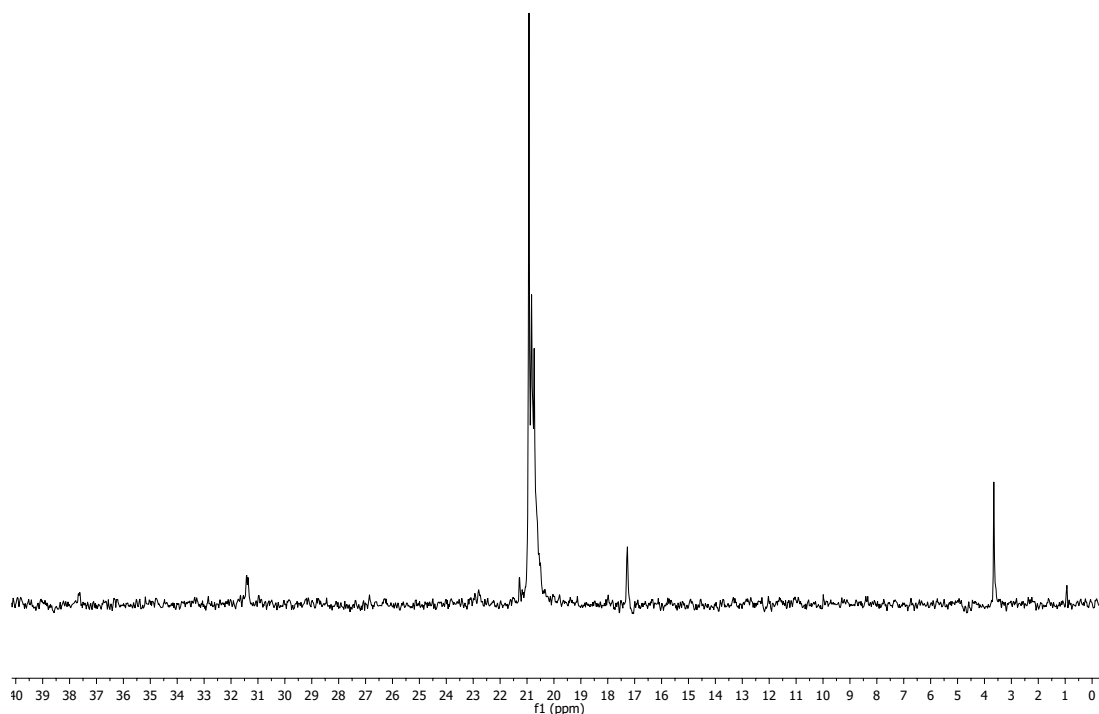


Figure 3.6: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum (162 MHz, 298 K, CDCl_3) of the result of the reaction between **2e** and α,α' -dibromo-*p*-xylene. The large group of peaks at 20.5 ppm consists of three individual peaks.

tion is likely attributable to the increased nucleophilicity of the phosphorus-centred lone pair arising from mesomeric electron donation from the oxygen atoms. As such, only the phenyl, tolyl and xylyl phosphines **3a-c** were used for macrocycle functionalisation.

3.1.2.2 Other Attempted Syntheses: Aliphatic Triphosphines and Linkers

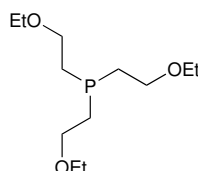


Figure 3.7: Tris(2-ethoxyethyl)phosphine, one of two failed attempts at synthesising trialkylphosphines.

Triarylphosphines are commonly used to form lipophilic cations, since the positive charge localised on the phosphorus atom can be delocalised across the aromatic rings, lowering the overall polarity. Some brief experiments, however, were devoted to the ultimately unsuccessful synthesis of aliphatic triphosphines. The main target was tris(2-ethoxyethyl)phosphine (Figure 3.7), inspired by the alkoxy groups in the clinically used lipophilic cationic radio-tracers MIBI and [^{99m}Tc]Tc tetrofosmin.^{6,7} Performing this synthesis in an analogous manner to that used for triarylphosphines using 1-bromo-2-ethoxyethane as a starting material did not lead to product formation, and $^{31}\text{P}\{^1\text{H}\}$ NMR showed a multitude of peaks. In order to ascertain that the Grignard step was not hindering the reaction, the analogous synthesis of tributylphosphine was attempted using commercially available butylmagnesium chloride as a starting material, however this process yielded a similar result. This lack of selectivity and increased air-sensitivity arises from the increased nucleophilicity of the phosphorus-centred lone pair due to inductive electron donation from the alkyl groups. As such, the synthesis of these trialkylphosphines was not pursued further.

In their studies of gallium-67 labelled DO3A-xy-TAP analogues, Kardashinsky *et al.* use HPLC to suggest that serum causes the xylyl linker to become metabolised over a period of 24 hours.² As such, the synthesis of the alternate linker shown in Figure 3.8 was explored.

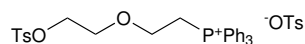
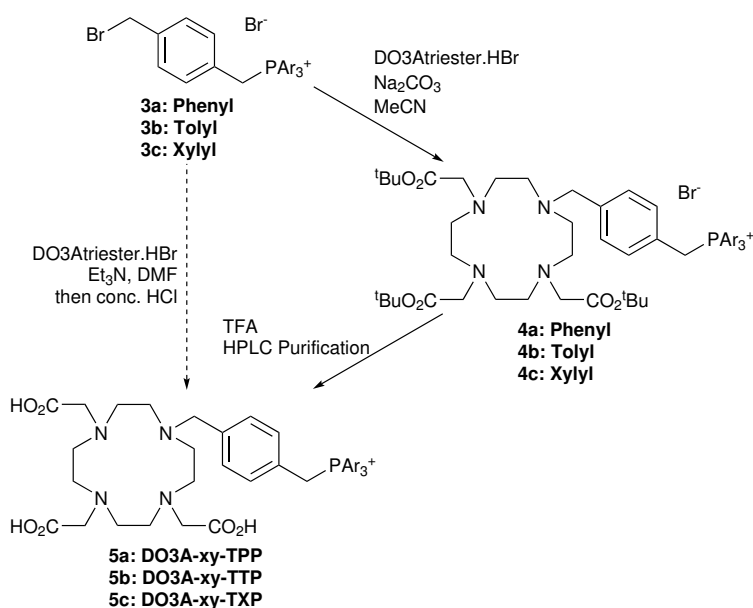


Figure 3.8: Example of a non-xylyl linked triarylphosphonium cation, using tosylate as a leaving group.

Diethylene glycol was used as a starting material, and was converted to its ditosylate according to Kim *et al.*⁸ Functionalisation with triphenylphosphine was also found to be successful, however the reaction had to be performed at 100 °C, since performing the reaction at the minimum temperature to dissolve the ditosylate starting material was not observed to facilitate product formation. The elevated temperature meant that the product had to be purified by column chromatography despite its cationic nature, and as such the reaction was low-yielding. The coupling of this product to DO3Atriestester was attempted, but no product was observed. The bromide analogue was also synthesised according to Barrán-Berdón *et al.* in order to determine whether changing the leaving group would aid the reaction, but again no reaction was observed.⁹ This aspect of the project was not pursued further on the basis that the timescale of the metabolism of the xylyl group in serum is shorter than the timescale permitted by gallium-68 imaging.

3.1.3 Coupling of Triarylphosphonium Cations to DO3A



Scheme 3.2: Synthesis of the DO3A-xy-TAP series of compounds from the triarylphosphonium functionalised bromoxylenes **3a-c**. The dashed arrow shows the synthesis according to Wang *et al.*, whilst the solid arrows show the synthesis according to Kardashinsky *et al.* which was published during this work.^{1,2} For compounds **5a-c**, the counterion is likely a chloride if Wang *et al.* is followed, whilst ¹⁹F{¹H} NMR shows that it is a trifluoroacetate if Kardashinsky *et al.* is followed.

The two main methods used to synthesise DO3A-xy-TAP compounds are summarised in Scheme 3.2. DO3Atriestester.HBr was synthesised in high purity and yield on a 4 gram scale

according to Jagadish *et al.*¹⁰ At the start of this project, the synthesis was based upon the pseudo-one pot reaction methodology designed by Wang *et al.*, who initially performed a coupling reaction between **3a** and DO3Atriesters using triethylamine as a base. The solvent was then removed *in vacuo*, before the residue was taken up in concentrated hydrochloric acid in order to deprotect the carboxylate arms. Finally, the desired product was purified by reverse-phase semi-preparative HPLC.¹ A representative trace for the purification can be found in Figure 3.9a, which shows a large peak between approximately 40–55 minutes, as well as a series of other impurities. The lack of a defined peak is due to the relatively low detection limit of the UV detector, which is capped at approximately 3000 mAU. In this case, mass spectrometry

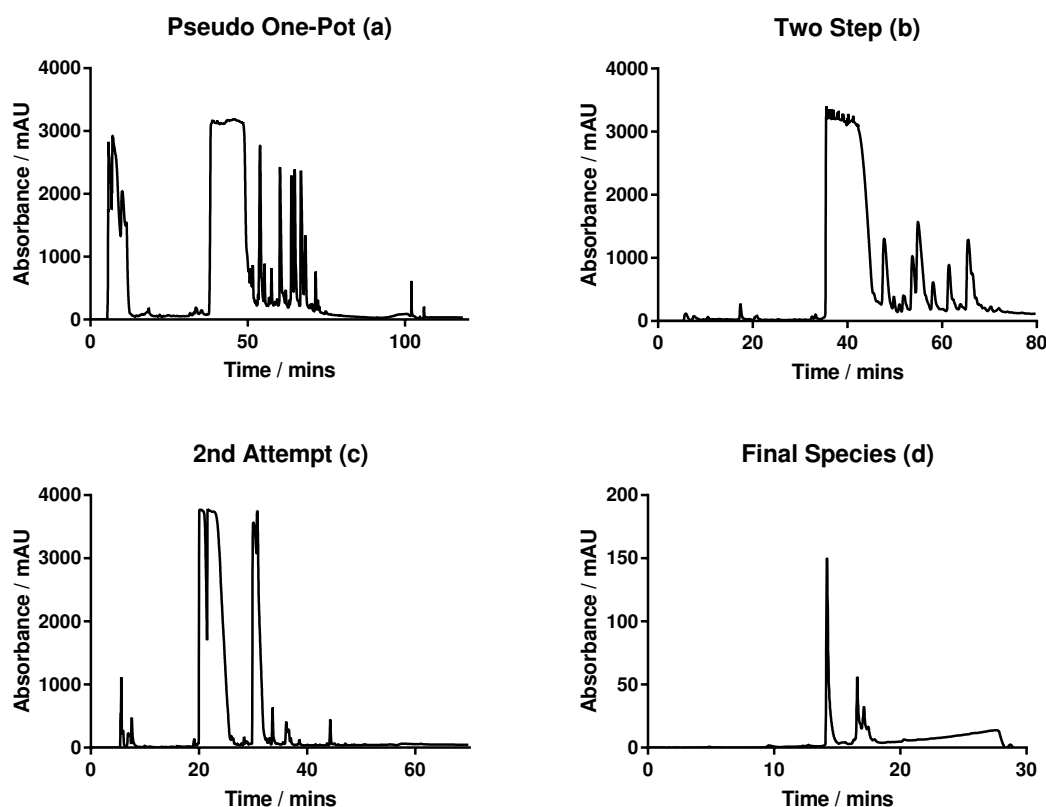


Figure 3.9: Representative semi-prep and analytical HPLC traces obtained from the purification of DO3A-xy-TAP compounds after the acid-based removal of the *tert*-butyl protecting groups. In each trace, the detector becomes saturated just over 3000 mAU. **a:** Pseudo-one pot purification of DO3A-xy-TTP according to Wang *et al.*¹ Gradient: 0–100% B in A for 100 min, isocratic 100% B for 20 min. **b:** Two step deprotection of DO3A-xy-TXP according to Kardashinsky *et al.*² Gradient: 0–100% B in A for 65 min, isocratic 100% B for 5 min. **c:** Second attempt at purifying the compound in Figure 3.9b. The split of the main peak into two species is clearly visible. Gradient as for **b**. **d:** Final analytical HPLC of DO3A-xy-TTP, taken from a radiolabelling experiment. The smaller peak refers to a radiolabelled compound (radiotracer not pictured). Gradient: Gradient: 0–50% B in A for 25 min, isocratic 100% B for 5 min.

was used on each fraction obtained from this peak in order to determine the location of the product, which in this case was found to be at the start of the peak. This detector saturation could be alleviated by trying to purify a smaller quantity of ligand, or by using a less sensitive UV detector as will be seen for the DO2A-based tracers in the next section.

During this work, Kardashinsky *et al.* published a series of gallium-67 labelled DO3A-xy-TAP ligands functionalised with asymmetric triarylphosphonium groups.² This paper proposed an alternative reaction methodology, also outlined in Scheme 3.2. This reaction is performed in two steps; firstly, the functionalised bromoxylene is coupled to DO3Atriesther in the presence of sodium carbonate, before this intermediate is purified by recrystallisation in acetone and diethyl ether. This purified intermediate is then deprotected in trifluoroacetic acid to yield the DO3A-xy-TAP compounds **5a-c** as above. One perceived advantage of this two step synthesis is that it would remove some impurities from the HPLC traces and make the product easier to purify. As can be seen from the trace in Figure 3.9b, this proved to be the case, with the large impurity peak at the start of the trace removed and the following impurities showing lower intensities. The large peak corresponding to the product and an almost co-eluting impurity, however, still saturates the detector.

A second, more dilute purification attempt (Figure 3.9c) clearly shows the main peak splitting into two isolable species. The efficacy of this technique is shown not only by NMR and mass spectrometry, but also by the analytical HPLC UV trace of the later radiolabelling experiments shown in Figure 3.9d, in which a clear single peak is formed, along with a smaller peak referring to radiolabelled product. The overloading of semi-prep HPLC columns with product ceased to be an issue with a move towards reverse-phase flash chromatography later in this thesis.

After the HPLC purification, the products were lyophilised such that they could be stored at -18 °C until they were required to be used. It was discovered that the DO3A-xy-TAP ligands, along with many others in this thesis, decompose if left in solution at room temperature, and as such, if a known amount was needed for a stock solution of radiotracer, the ligands were taken into solution immediately before they were used. Nevertheless, methods from the synthesis of the DO3A-xy-TAP compounds were found to be useful for the synthesis of other ligand series.

3.1.4 Synthesis and Characterisation of Non-Radioactive Reference Compounds

It is often important in radiochemistry to synthesise the non-radioactive analogue of a radio-tracer as a reference compound. This is due to the fact that radiolabelled compounds cannot be analysed by standard analytical chemistry techniques such as NMR and mass spectrometry, since they are in low concentration and are constantly undergoing radioactive decay. As such the non-radiolabelled analogue is normally synthesised, and once fully characterised is analysed by the same HPLC method as the radiolabelled analogue. If the two products show identical retention times, it can be confirmed that the radiolabelled compound is similar in structure to that of the characterised non-radioactive compound. This is easy to do with fluorine-labelled compounds, as organofluorination techniques are commonplace; whilst technetium reference compounds are synthesised from its fellow group VII metal rhenium.

3.1.4.1 Synthesis of Ga-DO3A-xy-TAP

Synthesis of non-radioactive Ga-DO3A-xy-TPP is outlined by both Yang *et al.* and Kardashinsky *et al.*^{2,11} Studying the procedure described by Kardashinsky *et al.* in greater detail however, reveals that it uses gallium trichloride in aqueous solvent. The main problem with this reaction was that gallium trichloride is highly unstable, to the point that it fumes in anaerobic environments. Upon addition to water, gallium trichloride immediately forms insoluble gallium hydroxide, which is so stable that it does not allow the gallium to bind to the chelator.

Instead, it was decided to follow the methodology outlined by Yang *et al.*, who used the far more stable gallium nitrate in 0.5 M ammonium acetate buffer.¹¹ Whilst using a nitrate counterion does not perfectly reflect the radiolabelling conditions used with gallium-68 generator eluate, the fact that there are other counteranions such as acetate and TFA in the HPLC mean that the identity of the complex counterion is unknown in the final experiments. In order to purify the complex, Yang *et al.* filter the reaction mixture to remove any insoluble impurities, before adding acetone to precipitate out the complex. This process was found to be effective for Ga-DO3A-xy-TTP, with the minor alteration that the insoluble precipitate was removed by centrifugation followed by isolation of the supernatant liquid, since the small 200 μL reaction volume did not lend itself to effective filtration. This process produced the ^1H NMR spectrum shown in Figure 3.10, whilst the presence of gallium was confirmed by mass spectrometry.

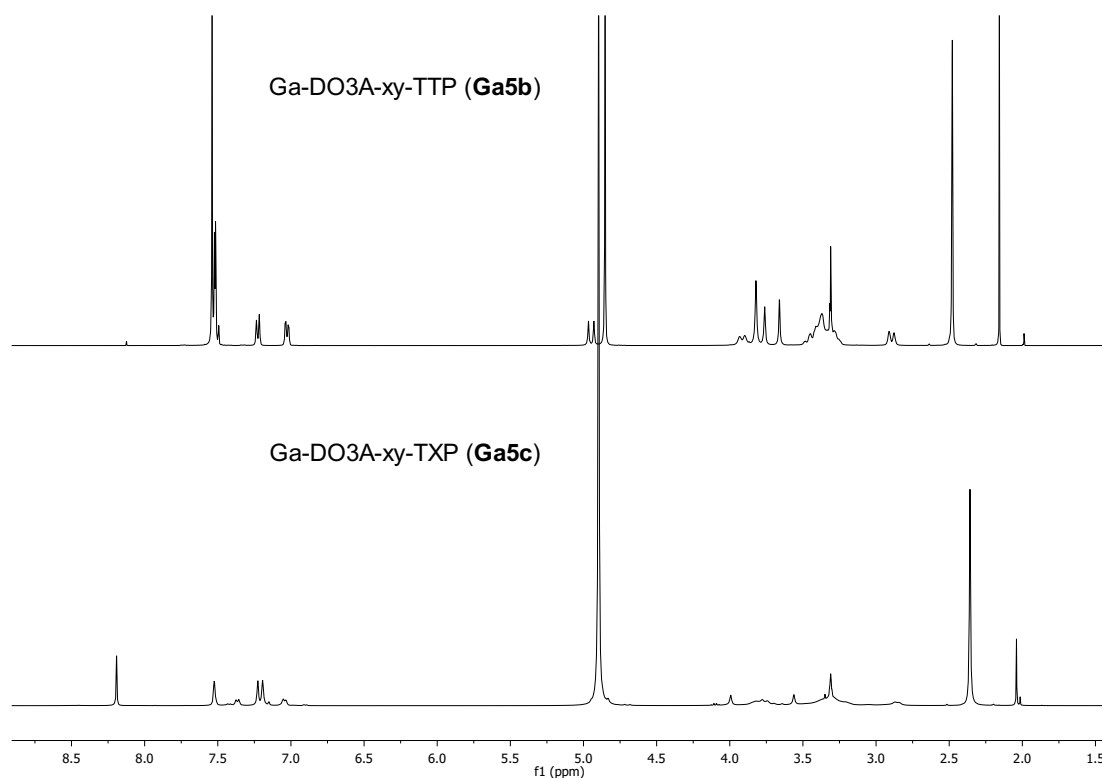


Figure 3.10: ^1H NMR spectra of the non-radioactive complexes Ga-DO3A-xy-TTP (**Ga5b**) and Ga-DO3A-xy-TXP (**Ga5c**) (400 HMz, 298 K, MeOD). The water peak has been truncated for clarity.

Upon attempting this process with Ga-DO3A-xy-TXP however, no precipitate was observed even with cooling of the recrystallisation mixture to $-78\text{ }^\circ\text{C}$; allowing the acetone counter-solvent to slowly diffuse into the filtered reaction mixture merely caused the aqueous product to decompose and turn brown. Since recrystallisation was not an option for the TXP-functionalised variant, the reaction mixture was instead purified by reverse-phase flash chromatography. The ^1H NMR spectrum is shown in Figure 3.10, with the presence of gallium being confirmed by mass spectrometry.

3.1.4.2 HPLC Analysis of Ga-DO3A-xy-TAP

The HPLC traces of the Ga-DO3A-xy-TTP and TXP complexes can be found in Figure 3.11a. Each compound shows one peak, which not only highlights the purity of the gallium-chelated reference compounds but also highlights a lack of isomerisation and speciation. The difference between the retention times of the two compounds (shown in Table 3.1) indicates a change in lipophilicity of the two compounds, with the TXP-functionalised variant showing a longer re-

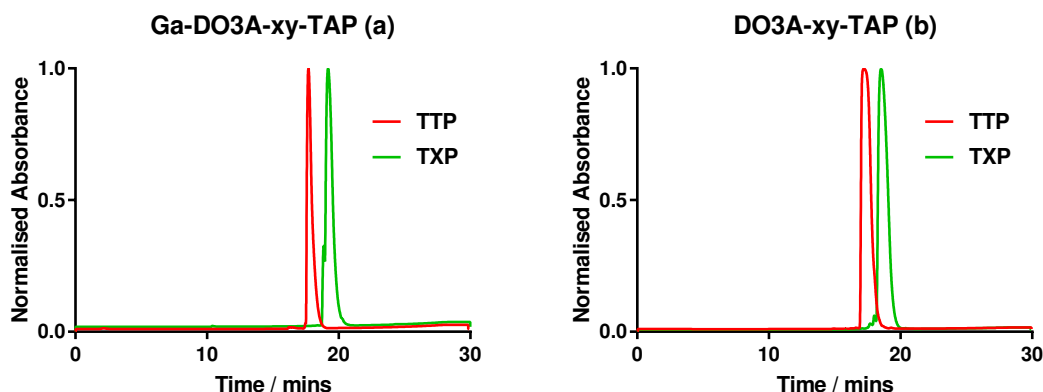


Figure 3.11: HPLC traces of **a:** the non-radioactive reference Ga-DO3A-xy-TTP and Ga-DO3A-xy-TXP compounds and **b:** the corresponding HPLC traces for the unchelated ligand, included for reference. Eluent gradient: 100% A for 5 min; 0–80% B in A for 20 min; 100% A for 5 min at a flow rate of 1 mL min^{−1} ($\lambda_{\text{ex}} = 250$ nm). All traces have been normalised such that the maximum absorbance measured for each compound is equal to 1.

tention time on reverse-phase stationary phases and thus a likely higher degree of lipophilicity. In order to ensure that that these peaks referred to the free ligand without any gallium complexation, the HPLC traces of the unchelated ligands were obtained and are shown in Figure 3.11b. As shown by the retention times in Table 3.1, gallium complexation causes a notable decrease in the retention time, indicating that the HPLC traces in Figure 3.11a do in fact refer to the gallium complexes, but also that the binding of the pendant carboxylate arms to the gallium centre reduces the hydrophilicity of the compounds. These non-radioactive complexes are now ready to be compared to their gallium-68 radiolabelled analogues in Chapter 4.

Table 3.1: Summary of the data presented in the HPLC traces in Figure 3.11.

	Retention Time / mins	
	DO3A-xy-TTP	DO3A-xy-TXP
Ga-Chelated	17.7	19.2
Unchelated	17.2	18.5

3.2 DO2A-Based Chelators

One problem with using DO3A as a chelator for Ga(III) is the size mismatch between the metal centre and the ligand cavity. As shown by the crystal structure of Ga-DO3A-xy-TTP obtained by Yang *et al.* (Figure 3.12), Ga(III) centres display a preference for hexadentate coordination in the DO3A cavity.¹¹ This results in an unbound carboxylate arm (shown by

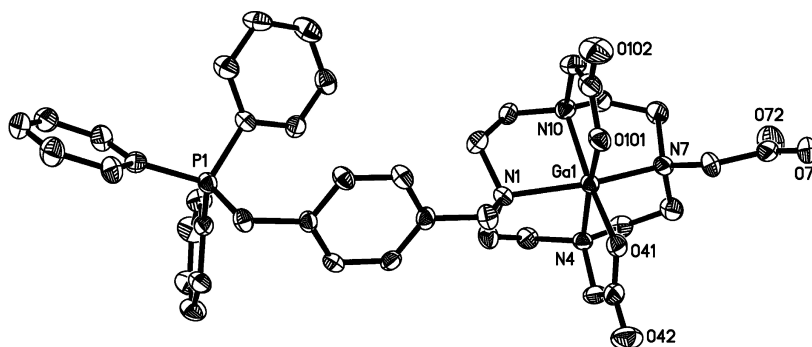


Figure 3.12: Crystal structure of Ga-DO3A-xy-TPP obtained by Yang *et al.*, demonstrating the unbound carboxylate arm.¹¹ Reprinted with permission from *Inorg. Chem.*, 46, 21, 8988-97. Copyright 2007 American Chemical Society.

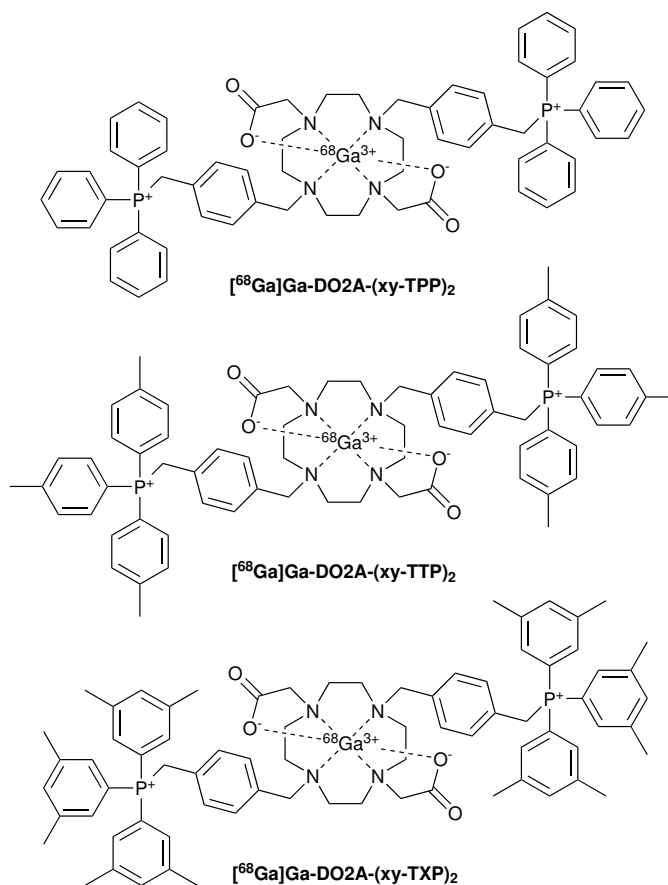


Figure 3.13: The three target DO2A-(xy-TAP)₂ compounds investigated in this project. These consist of a DO2A moiety capable of chelating [⁶⁸Ga]Ga(III) and two xylyl linking groups each functionalised with a TPP, TTP or TXP cation respectively.

O71 and O72 in Figure 3.12) which are likely to serve to lower the overall lipophilicity of the radiotracer. It seemed natural for the next set of compounds to be synthesised to be based on 1,4,7,10-tetraazacyclododecane-1,7-diacetic acid (DO2A), since it is functionally identical to DO3A apart from the fact that it lacks the extra carboxylate arm.

The first three DO2A-based compounds to be synthesised, the DO2A-(xy-TAP)₂ series, are shown in Figure 3.13. These can be considered analogous to the DO3A-based shown in Figure 3.2, although one of the carboxylate arms has been replaced with a TAP-functionalised xylyl group (**3a-c**). In addition, increasing the overall charge of the molecule should increase the mitochondrial uptake of the radiotracer, as shown by the following equation derived from the Nernst Equation (Equation 1.1):

$$E_m = \frac{2.303 \times RT}{zF} \log_{10} \left(\frac{[M^{z+}]_{in}}{[M^{z+}]_{out}} \right) \quad (3.1)$$

For a fixed value of E_m , an increase in the cationic charge z leads to an increase in the intracellular concentration of the radiotracer. It should be noted at this point, however, that an increase in cationic charge leads to an increase in the Born energy (W_B):

$$W_B = \frac{q^2}{8\pi\epsilon_0 r} \left(\frac{1}{\epsilon_1} - \frac{1}{\epsilon_2} \right) \quad (3.2)$$

The Born energy is the main electrostatic component of the activation energy of a positively charged species crossing a lipid bilayer membrane, arising from the desolvation of water molecules from the cationic tracer as it passes into the lipid bilayer membrane.¹² If known values for the constants in Equation 3.2 are set: q is the electronic charge per mole of cationic tracer, ϵ_0 is the permittivity of free space ($8.85 \times 10^{-12} \text{ F m}^{-1}$), ϵ_1 and ϵ_2 are the dielectric constants within the core of the membrane and of water (approximately 2 and 80 respectively) and r is the ionic radius, the equation can be simplified thus:

$$W_B = \frac{339 \times z^2}{r} \quad (3.3)$$

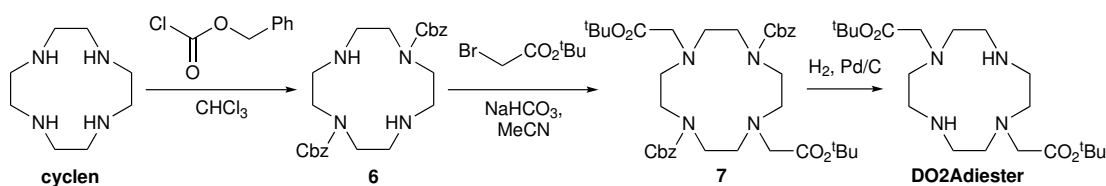
where z is the cationic charge.¹² From Equation 3.3, it can be deduced that if the charge of a cation doubles, the Born energy associated with moving from an aqueous phase into a lipid bilayer membrane quadruples. In this case however, the tracers in Figure 3.13 are tricationic,

and therefore will suffer an eight-fold increase in Born energy. It is hoped that by removing the unbound carboxylate arm, as well as providing an extra lipophilic group to shield the central polar chelating motif from unfavourable hydrophilic interactions, the increase in Born energy should be overcome.

3.2.1 Synthesis of DO2Adiester

DO2A is non-trivial to synthesise, since the selectivity of the reaction is far harder to control than in the equivalent synthesis of DO3A, which can be synthesised by simply adding cyclen and *tert*-butyl bromoacetate together in the presence of inorganic base.¹⁰ Instead, protection/deprotection routes are required for DO2A.

3.2.1.1 Synthesis of DO2A by Cbz-Protection



Scheme 3.3: Synthesis of DO2Adiester as designed by De León-Rodríguez *et al.* (1st step) and Hirayama *et al.* (2nd and 3rd steps)^{13,14}

The most commonly used method for the synthesis of DO2Adiester is shown in Scheme 3.3. Unlike many other syntheses in this thesis, this was performed according to the literature without major modifications. The first step was the carboxybenzyl (Cbz) protection of cyclen to form **6**, according to De León-Rodríguez *et al.*¹³ **6** was formed in 85% yield without the need for further purification techniques. The addition of the *tert*-butyl bromoacetate groups to form **7** was performed according to Hirayama *et al.*, in the presence of sodium hydrogen carbonate.¹⁴ The paper also included details on the chromatographic purification of **7** using a mobile phase of 2% MeOH in DCM, which gave the product in 34% yield. This could likely be improved through the use of a gradient to facilitate the removal of the initial impurities without co-eluting the desired product.

The final step of the reaction scheme shown in Scheme 3.3 was the removal of the Cbz protecting groups to form DO2Adiester, which was performed using hydrogen gas in the presence of a palladium on carbon catalyst in accordance with Hirayama *et al.*¹⁴ After the hydrogen gas

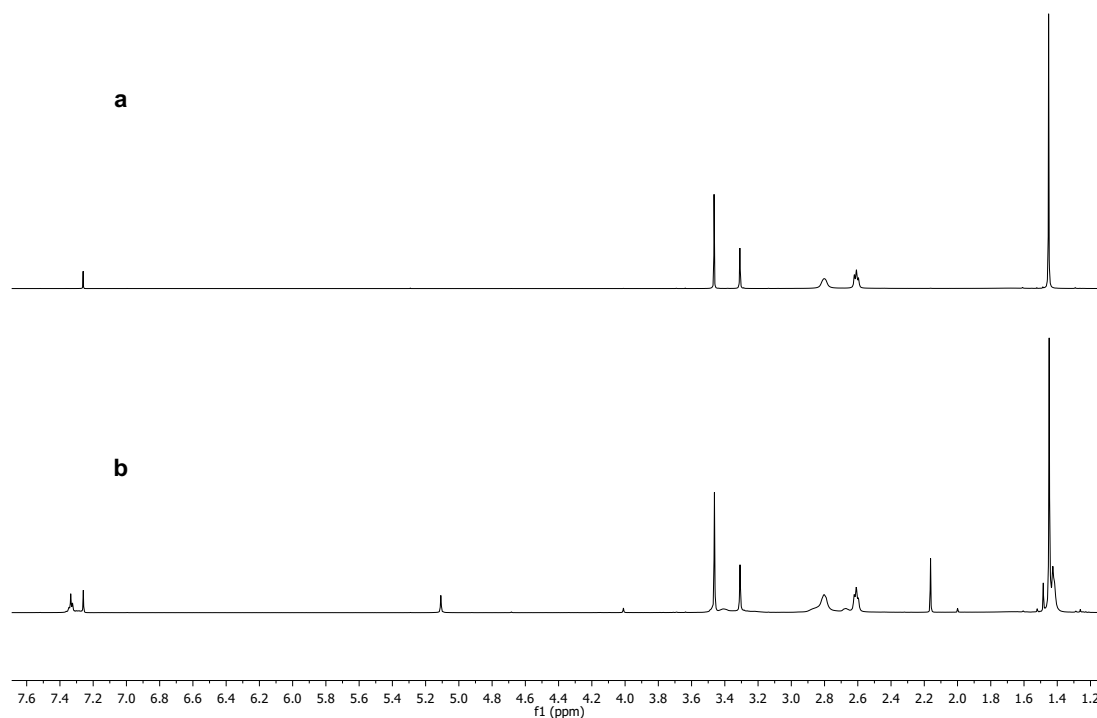
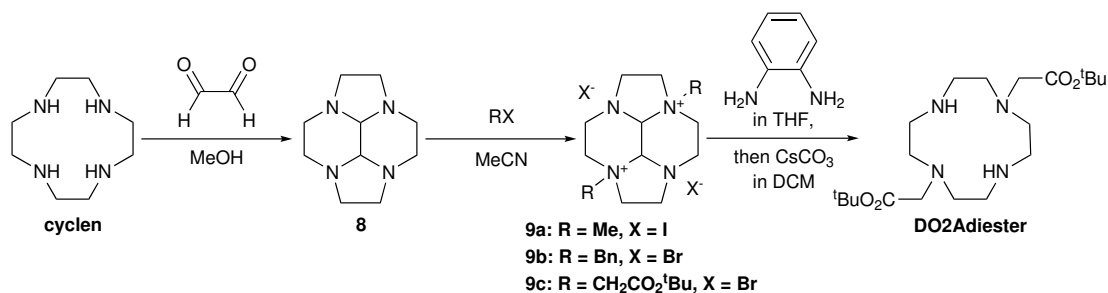


Figure 3.14: Two ^1H NMR spectra (400 MHz, 298 K, CDCl_3) of different preparations of DO2Adiester according to Scheme 3.3. Spectrum **a** shows pure DO2Adiester albeit with some residual methanol at 3.46 ppm, whilst spectrum **b** shows DO2A diester containing a series of aromatic peaks around 7.34 ppm which likely refer to the Cbz groups in the unreacted **7**.

was consumed, the reaction mixture was filtered through Celite to remove the Pd/C catalyst as well as to remove some of the impurities formed during the reaction. The ^1H NMR of the product of this reaction is shown in Figure 3.14a; despite some residual methanol as shown by the peak at 3.46 ppm this sample of the product was pure. Some samples of DO2Adiester prepared according to this synthesis still contained starting material, as shown by the series of peaks at 7.34 ppm in Figure 3.14b, which correspond to Cbz groups still present in the reaction mixture. Nevertheless, this slightly impure DO2Adiester was found to be usable in the next step of the reaction, as functionalisation of the nitrogen atoms facilitates purification.

3.2.1.2 Synthesis of DO2A by Glyoxal Protection

In addition to the Cbz protected route to DO2Adiester shown in Scheme 3.3, the route shown in Scheme 3.4 was also explored. This synthetic methodology uses glyoxal as a protecting reagent to form the bisaminal intermediate cyclen glyoxal (**8**). This facilitates the *trans*-disubstitution



Scheme 3.4: Synthesis of DO2Adiester by using glyoxal protection. Steps in this reaction are adapted from Jorge *et al.*, Rohovec *et al.*, Niu *et al.* and Oukhatar *et al.*^{15–18}

of the bisaminal, before the glyoxal bridge can be removed through reaction with a diamine such as ethylenediamine or *o*-phenylenediamine. The perceived advantage of this synthesis was that it does not require the use of chromatographic purification techniques or hydrogen gas for the final deprotection step.

The first step, formation of the bisaminal cyclen glyoxal (**9**), was performed according to Jorge *et al.*¹⁵ This reaction began with a slow addition using a syringe pump of the glyoxal in methanol at 0 °C over 1 h, which is intended to promote intramolecular reaction of both ends of the glyoxal with the cyclen ring over intermolecular reactions and polymerisation. This was followed by heating at 55 °C for 2 h then room temperature overnight, both of which were likely to facilitate the bisaminal formation. It is easy to observe the extent of polymerisation, since the polymeric side products are orange in colour. The polymers are insoluble in diethyl ether, meaning that the desired products can be extracted easily and in high yield.

Since the initial aim of exploring this synthetic regime was to synthesise DO2Adiester, the first substrate reacted with cyclen glyoxal was *tert*-butyl bromoacetate, in order to synthesise **9c**. Oukhatar *et al.*, as well as providing the methodology for the deprotection step, also provided a method for the disubstitution which used two equivalents of alkylating agent in 15 mL of acetonitrile.¹⁸ According to the paper, the diammonium product should have formed as a precipitate, but after 24 h no precipitate was observed, even after cooling to -18 °C.

In order to elucidate a series of reaction conditions for the formation of diammonium bisaminals, it was decided to simplify the reaction procedure. Firstly, the synthesis of the dimethyl variant **9a** using methyl iodide was explored, instead of the more complex dicarboxylate variant. Secondly, an older paper on the use of various substrates for bisaminal substitution by Rohovec *et al.* was used, who recommended using three equivalents of alkylating

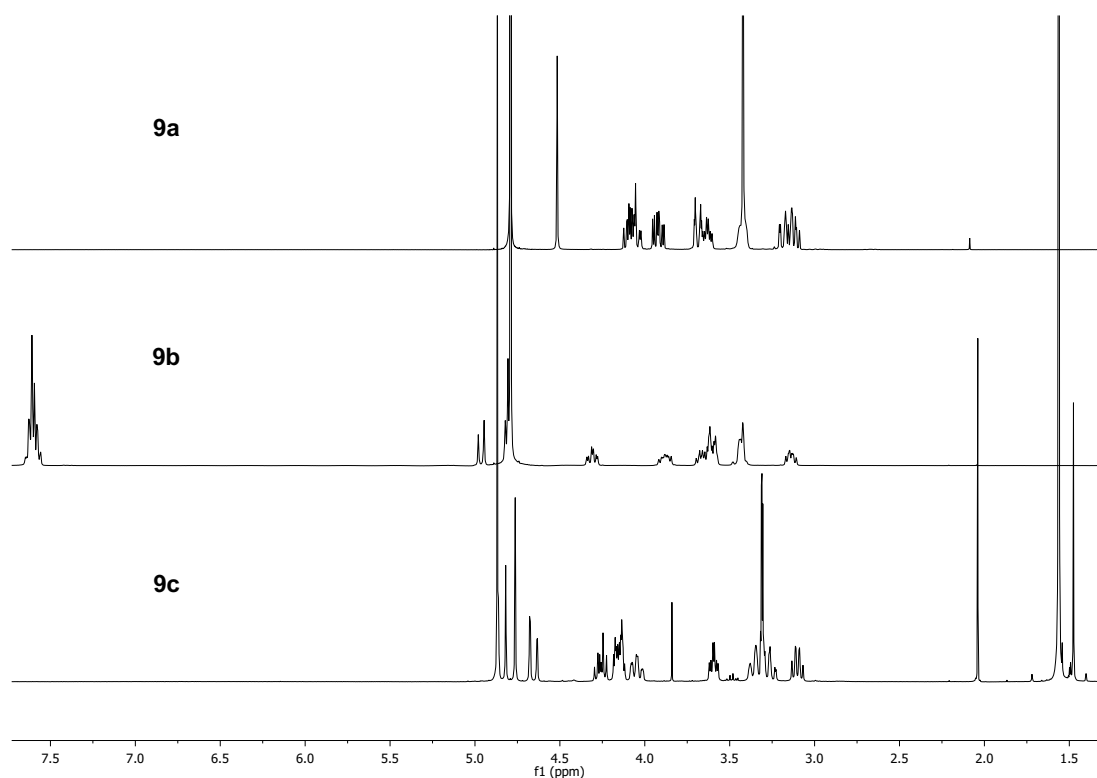
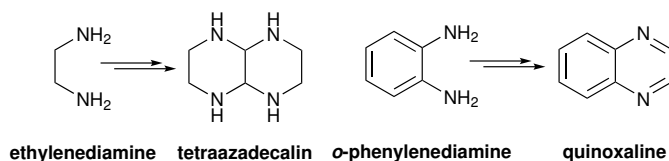


Figure 3.15: ^1H NMR of the disubstituted bisaminals **9a**, **9b**, **9c** (400 MHz, 298 K, $\text{D}_2\text{O}/\text{MeOD}$). Some peaks have been truncated for clarity.

agent in a more concentrated reaction mixture.¹⁷ This method formed the dimethyl bisaminal **9a** as a white precipitate in seconds, which could then be isolated by vacuum filtration before being characterised as pure. This method was applicable to the dicarboxylate bisaminal **9c**, although with the caveat from the literature that as the degree of functionalisation of the alkylating agent increases, the amount of time for the precipitate to appear also increases. The synthesis of the dibenzyl bisaminal **9b** was also explored through this method, but with no success; instead a synthesis specifically for the synthesis of **9b** designed by Niu *et al.* was used, which used six equivalents of benzyl bromide instead of three. ^1H NMR spectra of the three compounds are shown in Figure 3.15.

The final step in this synthesis of DO2Adiester is the removal of the glyoxal bridge according to Oukhatar *et al.*¹⁸ As shown in Scheme 3.5, this process utilises a 1,2-diamine such as ethylenediamine or *o*-phenylenediamine, which removes the bridge as the bicyclic side products tetraazadecalin or quinoxaline respectively. The success of this reaction depends on the solubility of both the leaving group and the macrocycle. The initial attempt at this reaction

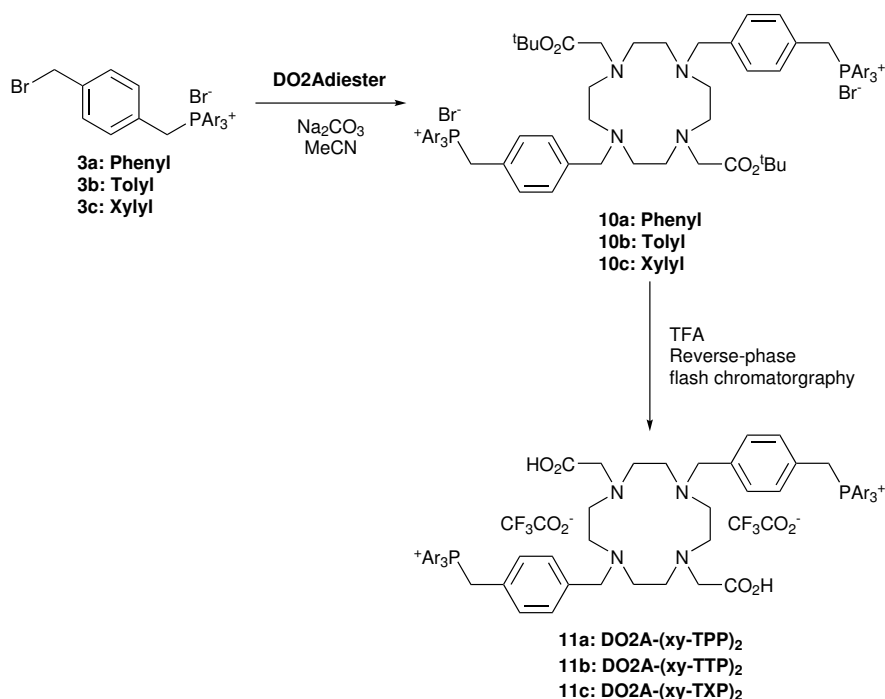


Scheme 3.5: Diimine products of the deprotection of the disubstituted bisaminal intermediates **9a-c**. These reactions, designed by Oukhatar *et al.*, use 1,2-diamine reagents such as ethylenediamine and o-phenylenediamine.¹⁸

used ethylenediamine in DCM, since DO2Adiester is soluble in DCM whilst tetraazadecalin is not, thus it can be removed from the reaction mixture by filtration. Unfortunately, no precipitate was observed despite overnight stirring, and so it was assumed that this reaction did not work.

Instead, the other method proposed by Oukhatar *et al.* was attempted, which used o-phenylenediamine in THF since quinoxaline is soluble in THF whilst DO2Adiester is insoluble. It was vital to maintain anoxic conditions during this process due to the high air-sensitivity of diamines such as quinoxaline; initial attempts at removing the THF used cannula filtration, although the high viscosity of the reaction mixture rendered this technique ineffective. To remove the THF and exclude as much oxygen as possible, the solid was isolated by vacuum filtration under a nitrogen funnel, and washed with further aliquots of THF to remove any residual quinoxaline. Deprotonation of the product was achieved using caesium carbonate since the DO2Adiester cannot chelate the large caesium counteranion.

The two syntheses of DO2Adiester reported in this section vary greatly in protection methods, purification of intermediates and in final deprotection step. Initially, it was thought that the cyclen glyoxal protected synthesis (Scheme 3.4) was the better of the two syntheses, as it did not rely on purification by column chromatography or the use of hazardous hydrogen gas for deprotection. This opinion changed as the reaction was scaled up, as the requirement to maintain anaerobic conditions meant that removing the quinoxaline side product through washing with THF aliquots was ineffective. Instead, the Cbz-protected route (Scheme 3.3) was found to be far more amenable to scale up, and so became the main method of synthesising DO2Adiester in this project. The syntheses of the disubstituted bisaminal intermediates **9a-c** were found to be useful in the synthesis of other chelators for gallium-68; synthesis of these chelators are discussed in later sections of this thesis.

3.2.2 Synthesis of DO2A-(xy-TAP)₂

Scheme 3.6: Synthesis of DO2A-(xy-TAP)₂ compounds. The synthesis is adapted from the DO3A-xy-TAP synthesis designed by Kardashinsky *et al.* outlined in Scheme 3.2.²

In order to synthesise the DO2A-(xy-TAP)₂ compounds shown in Figure 3.13, the synthesis outlined in Scheme 3.6 was used. This synthesis is adapted from the one shown in Scheme 3.2 and based on Kardashinsky *et al.*, in that it starts by using the *tert*-butyl protected macrocycle and reacts it with two equivalents of the TAP-functionalised bromoxylene **3a-c** in the presence of the inorganic base sodium carbonate to form **10a-c** respectively.² Due to the cationic nature of these intermediates, they could be easily purified by recrystallisation in acetone and excess diethyl ether. The final step was deprotection of the carboxylate arms in TFA, although the main difference this time was the use of reverse phase flash chromatography for ligand purification instead of semi-preparative HPLC, which meant that the two products alluded to in Figure 3.9 could be identified and isolated with greater precision on a higher scale. The work performed using HPLC did provide a basis for this purification; the flash chromatography gradient used was from 0–100% MeCN in H₂O (0.1% TFA) in an analogous manner to the HPLC gradient.

Characterisation of these novel compounds began with the ¹H NMR spectra shown in Figure 3.16, which show several common features such as the doublet at 5.02 ppm arising from

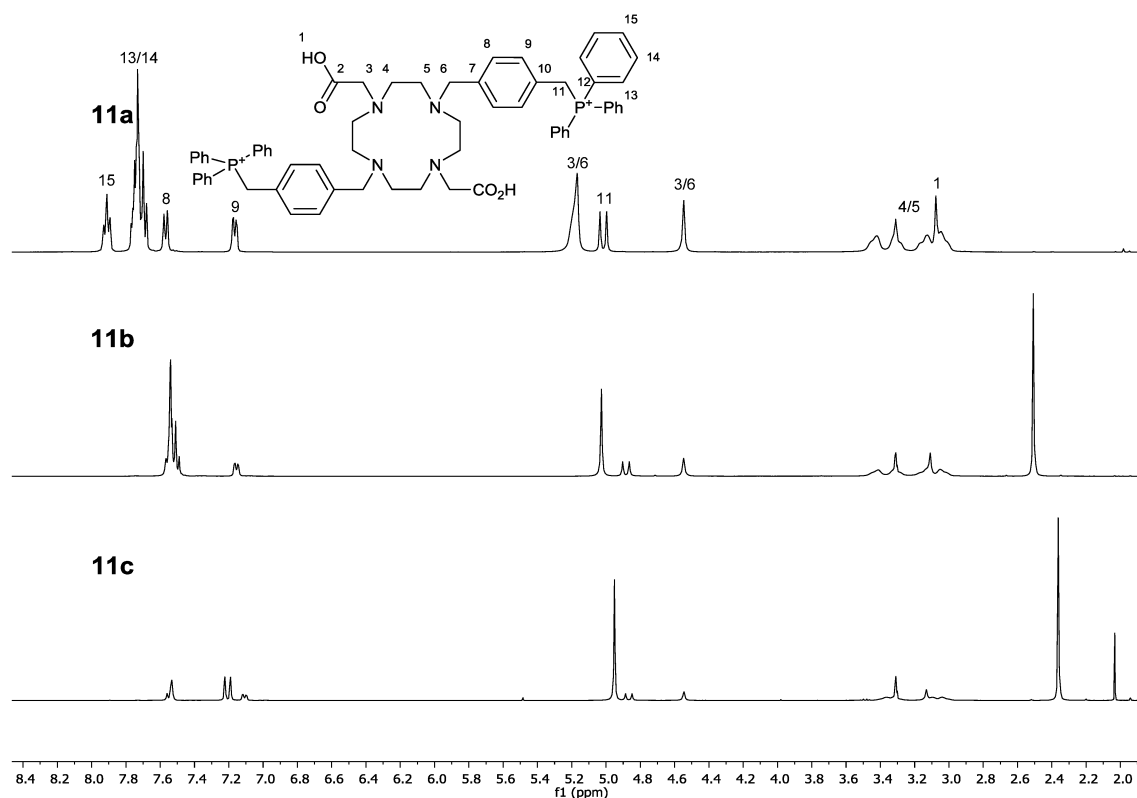


Figure 3.16: ^1H NMR spectra of the deprotected DO2A-(xy-TAP) $_2$ compounds (MeOD, 400 MHz, 298 K). From top to bottom: DO2A-(xy-TPP) $_2$ (**11a**), DO2A-(xy-TTP) $_2$ (**11b**), DO2A-(xy-TXP) $_2$ (**11c**). The labelled structure of **11a** and NMR characterisation is also included.

geminal proton phosphorus coupling, as well as the broad peaks referring to the macrocyclic protons. As the degree of aryl functionalisation increases, the aromatic region simplifies due to the removal of opportunities for vicinal coupling; additionally the methyl peaks increase in intensity as they become more numerous in the compounds. $^{31}\text{P}\{^1\text{H}\}$ NMR of all three compounds confirmed that only one phosphorus-containing species was present in each product, whilst $^{19}\text{F}\{^1\text{H}\}$ NMR of each compound gave a single peak at -77 ppm, which implies that trifluoroacetate is present in each compound as the counteranion. The $^{13}\text{C}\{^1\text{H}\}$ NMR spectra could also be assigned; due to coupling between the carbon and phosphorus atoms most peaks appear as doublets in the spectrum. DEPT-135 and HSQC NMR were used to assign several peaks through comparison to the proton NMR, whilst the remaining peaks were assigned by using the tendency of coupling constants to decrease as the number of bonds increases. One-bond coupling constants of approximately 87 and 48 Hz were observed, whilst five-bond couplings were also observed. Mass spectrometry was also used to characterise these com-

pounds, however elemental analysis was not used due to the large amount of product needed as well as the unknown nature of the counterions.

3.2.3 Attempted Synthesis of Non-Radioactive Reference Compounds

As discussed in Section 3.1.4, it is advantageous to synthesise non-radioactive reference complexes of ligands in order to aid the characterisation of their radiolabelled analogues. It was decided to attempt the synthesis of Ga-DO2A-(xy-TPP)₂ using analogous conditions to those for Ga-DO3A-xy-TAP i.e. gallium nitrate hydrate as a Ga(III) source and 0.5 M ammonium acetate as a buffer. Upon addition of acetone however, no precipitate was observed as occurred for the DO3A analogue, a likely result of the increased lipophilicity of the complex aiding its solubility in lower-polarity solvents such as acetone. Instead, reverse-phase flash chromatography was employed as a purification technique due to the analogous HPLC technique proving ideal for the assessment of their radiolabelled counterparts.

¹H and ³¹P{¹H} NMR spectra were inconclusive as to whether the complex had formed; the chelation of Ga(III) does little to affect the chemical shift of the phosphorus centre, whilst no obvious broadening of the proton peaks was observed. Additionally, mass spectrometry was also inconclusive as it is difficult to find *m/z* values for tricationic species. Instead, reverse-phase analytical HPLC was used, the result of which is shown in Figure 3.17a. Upon comparison to the HPLC trace of the unchelated DO2A-(xy-TPP)₂ ligand under identical conditions

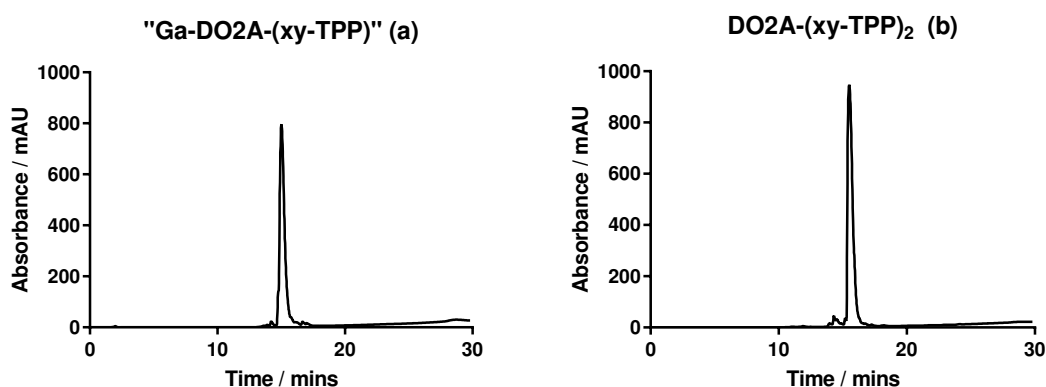


Figure 3.17: HPLC traces of **a:** the product of the synthesis of Ga-DO2A-(xy-TPP)₂ and **b:** the unchelated DO2A-(xy-TPP)₂ ligand, taken from Figure 4.14 and included for reference. Eluent gradient: 100% A for 5 min; 0–100% B in A for 20 min; 100% B for 5 min at a flow rate of 1 mL min⁻¹ (λ_{ex} = 250 nm).

Table 3.2: Summary of the data presented in the HPLC traces shown in Figure 3.17.

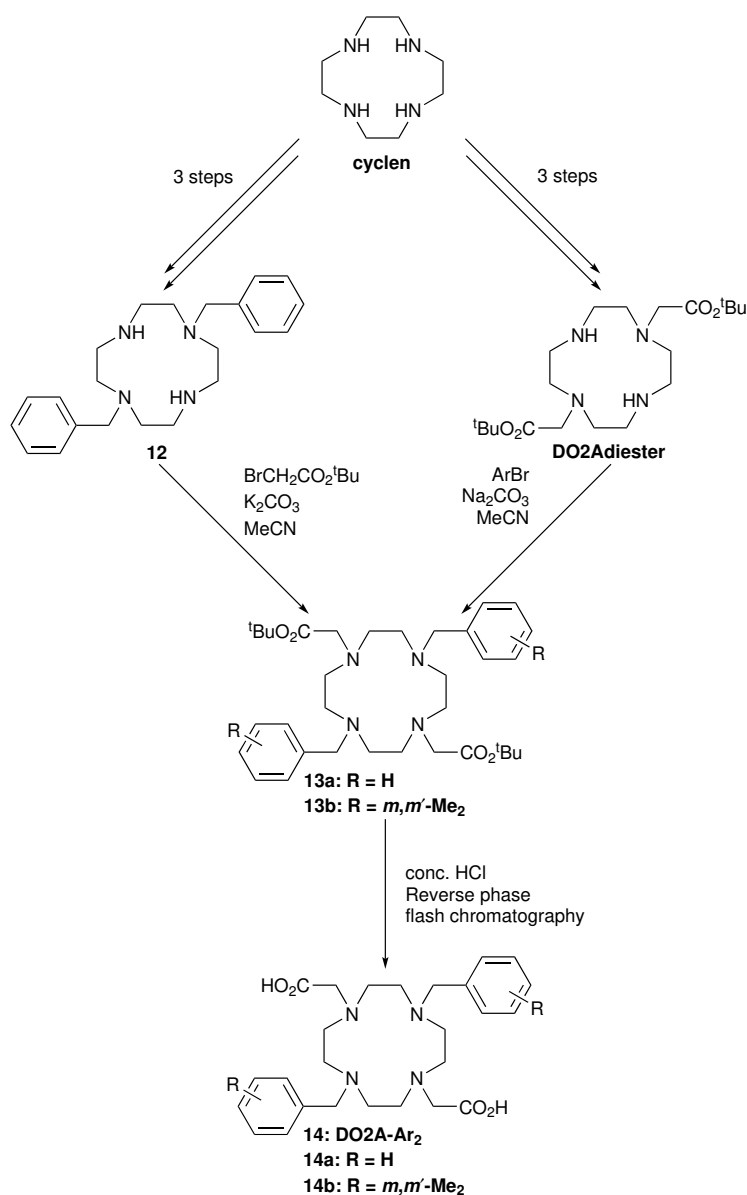
Species	Retention Time / mins
'Ga-DO2A-(xy-TPP) ₂ '	15.0
DO2A-(xy-TPP) ₂	15.5

(Figure 3.17b, summary in Table 3.2) however, it can be seen that the two traces show similar retention times, with gallium chelation resulting in a decrease in retention time. This is in opposition to the result shown for the DO3A chelators (Figure 3.11 and Table 3.1) as the binding of the carboxylate arms to a metal centre lowers the hydrophilicity of the overall complex. This result, coupled with the fact that radioHPLC trace of [⁶⁸Ga]Ga-DO2A-(xy-TPP)₂ shown in Figure 4.13 shows peaks at 14.3 and 16.0 minutes indicates that the non-radioactive reference compound was not synthesised in this case.

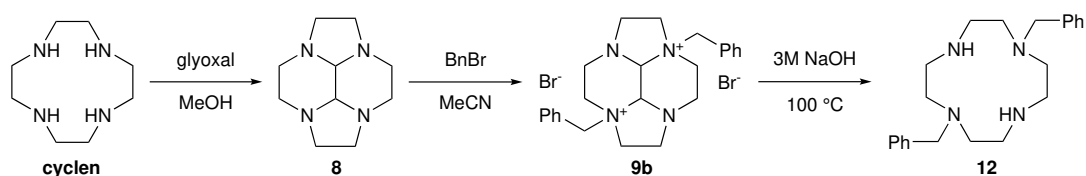
3.2.4 Synthesis of DO2A-Ar₂ Derivatives

Another variant of the DO2A chelators explored in this thesis are DO2A-Ar₂ derivatives, compounds **14a** and **b** in Scheme 3.7. The theory behind these compound is that the gallium complex will only exhibit monopositive charges, compared to the 3+ charge exhibited by the gallium-chelated DO2A-(xy-TAP)₂, which in turn will lower the Born Energy (Equation 3.3) and decrease the degree of hydrophilic interactions displayed by the molecule. For the aromatic groups, unfunctionalised phenyl groups were chosen due to their lipophilicity, as well an inexpensive base case with which to optimise the synthesis of further DO2A-Ar₂ derivatives. Due to the effect xylyl functionalisation has on overall complex lipophilicity (see Chapter 4), a dixylyl variant (**14b**) was also synthesised. Unfortunately, due to time constraints the tolyl variant explored for other chelator systems was not synthesised here, as focus was instead placed on the synthetic base case and the likely most lipophilic compound.

The synthetic strategies used to synthesise DO2A-Ar₂ derivatives are shown in Scheme 3.7. Both routes explored started with cyclen, before addition of either the two *tert*-butyl protected carboxylate groups or the two aromatic groups trans to one another. Whichever route was chosen, the following step was to add the other two functional groups; at this point the two synthetic pathways converge. The final step in this reaction scheme was the deprotection of the two carboxylate groups in concentrated acid. Previous deprotections used TFA, however in these experiments concentrated hydrochloric acid was used instead in line with the depro-



Scheme 3.7: The two different synthetic routes explored to the synthesis of DO2A-Ar₂ derivatives (**14a** and **b**). These synthetic routes involve two different starting materials: DO2Adiester is synthesised according to Scheme 3.3, whilst dibenzyl cyclen (**12**) is synthesised according to Scheme 3.8. Ar = phenyl, *m,m'*-xylyl



Scheme 3.8: Route to the synthesis of dibenzyl cyclen (**12a**). The synthesis of **9b** was performed according to Niu *et al.* as shown in Scheme 3.4, whilst the deprotection was performed according to Rodríguez-Rodríguez *et al.*^{16,19}

tections performed by Wang *et al.*¹ Concentrated HCl also had the advantage of being far less expensive than TFA.

3.2.4.1 Synthesis by Initial Addition of Aromatic Groups

Dibenzyl cyclen **12** was synthesised in three steps according to Scheme 3.8. The first two steps, synthesis of cyclen glyoxal (**8**) and the dibenzyl intermediate **9b**, were synthesised as discussed previously as according to Scheme 3.4.^{15,16} This reaction was simple to perform, as the dicationic product readily precipitates out of solution and the unreacted starting materials can be removed by washing with diethyl ether. Unlike in Scheme 3.4, the glyoxal bridge here can be removed through boiling the dicationic intermediate **9b** in 3 M sodium hydroxide solution for three days before extracting the pure product with chloroform in 96% yield according to Rodríguez-Rodríguez *et al.*¹⁹ The ¹H NMR spectrum of this compound is shown in Figure 3.18. It should be noted at this point that this deprotection methodology was also applied to the synthesis of DO2Adiester through deprotection of **9c**, but without success.

The next step was the addition of two equivalents of *tert*-butyl bromoacetate in the presence of inorganic base, a fairly standard procedure for adding carboxylic acid groups. After 48 hours of reflux, removal of the inorganic base and solvent yielded an orange oil with the expected ¹H NMR spectrum shown in Figure 3.19, seemingly without the need for additional purification steps. Unfortunately, this result was not found to be replicable, with other attempts at this reaction leading to a large number of species in the reaction mixture. It was thought that alkylation of the secondary nitrogen atoms would lead to facile purification by column chromatography, but no appropriate conditions for separation could be found.

3.2.4.2 Synthesis using DO2A

Instead, the alternative synthetic methodology shown in the right-hand side of Scheme 3.7 was explored. This synthesis is achieved using DO2Adiester, the synthesis of which is discussed in Section 3.2.1. DO2Adiester can then be reacted with two equivalents of aryl bromide in the presence of inorganic base to form **13** as was attempted by the other synthetic route, before the *tert*-butyl protected carboxylate groups can be deprotected with strong acid to form the desired compounds **14**. ¹H NMR spectroscopy seemed to suggest that the products of the initial reaction between DO2Adiester and aryl bromides was relatively pure, and as such it could be

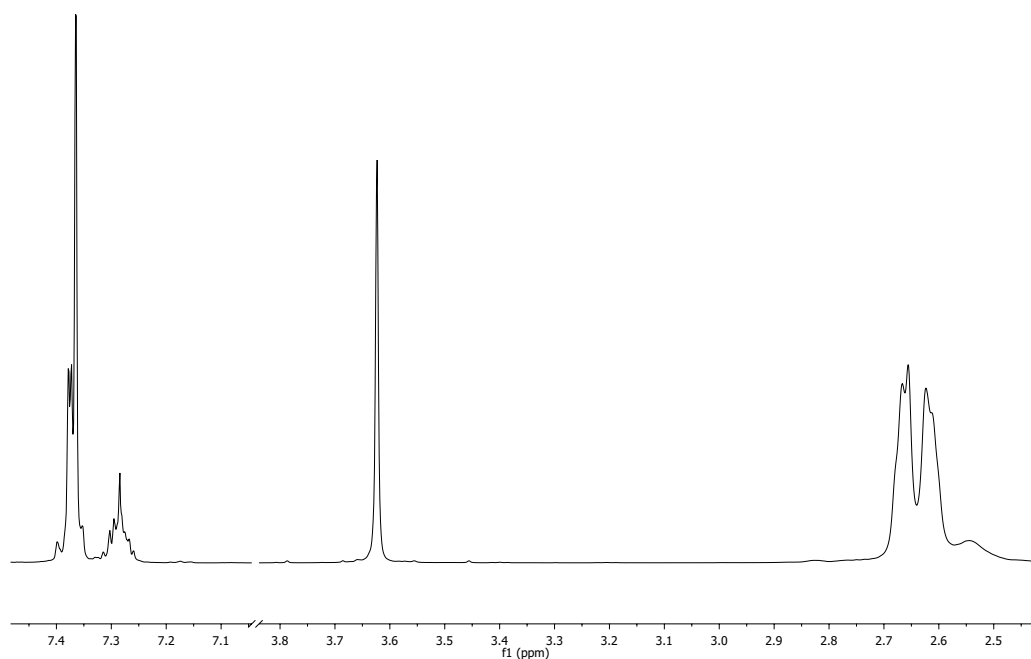


Figure 3.18: ^1H NMR spectrum of the dibenzyl functionalised cyclen **12** synthesised according to Rodríguez-Rodríguez *et al.* (CDCl_3 , 400 MHz, 298 K)¹⁹ The region between 3.8–7.1 ppm has been truncated for clarity.

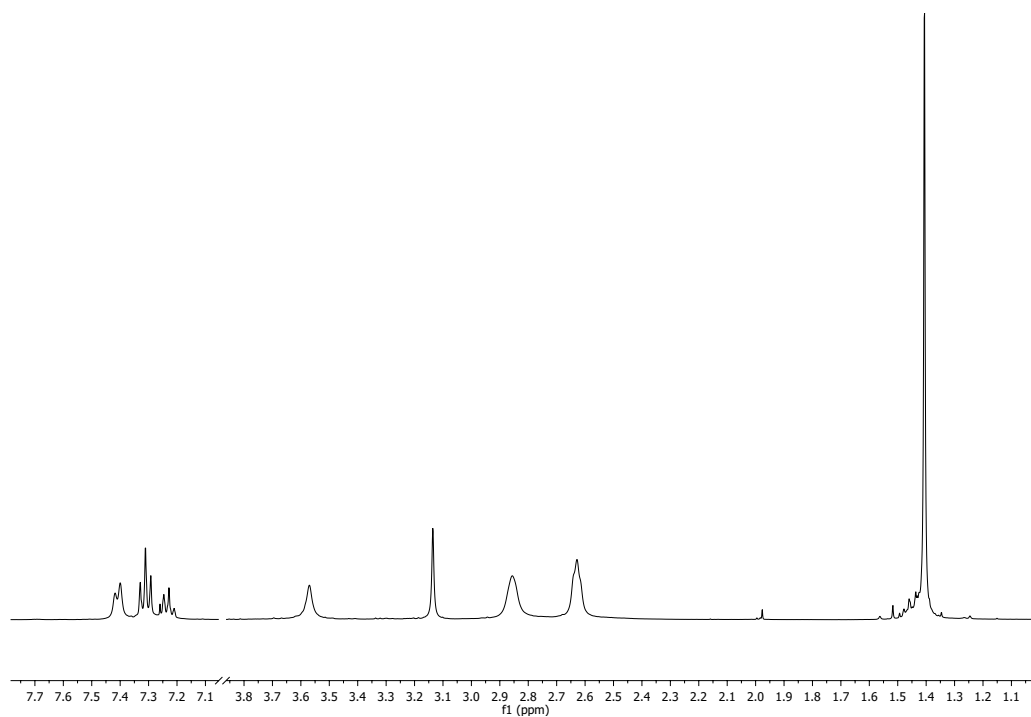


Figure 3.19: ^1H NMR spectrum of the *tert*-butyl protected dibenzyl cyclen (**13a**) produced from **12** by reaction with *tert*-butyl bromoacetate (CDCl_3 , 400 MHz, 298 K). The region between 3.8–7.1 ppm has been truncated for clarity.

carried through to the deprotection step with minimal purification or characterisation.

The deprotection of the carboxylate groups was performed using a strong acid, in this case concentrated HCl, as has been seen multiple times in this thesis. The formation of carboxylic acid groups, coupled with the UV activity afforded by the aromatic rings, make these compounds prime candidates for purification by reverse-phase flash chromatography, which allowed for the isolation of the product in high yield. This process was found to also produce the bis-xylyl DO2A variant in an identical manner, using 1-(bromomethyl)-3,5-dimethylbenzene as a starting material instead of benzyl bromide. The ^1H NMR spectra of these two compounds are shown in Figure 3.20. As this point, these two species were ready for subsequent radiolabelling experiments.

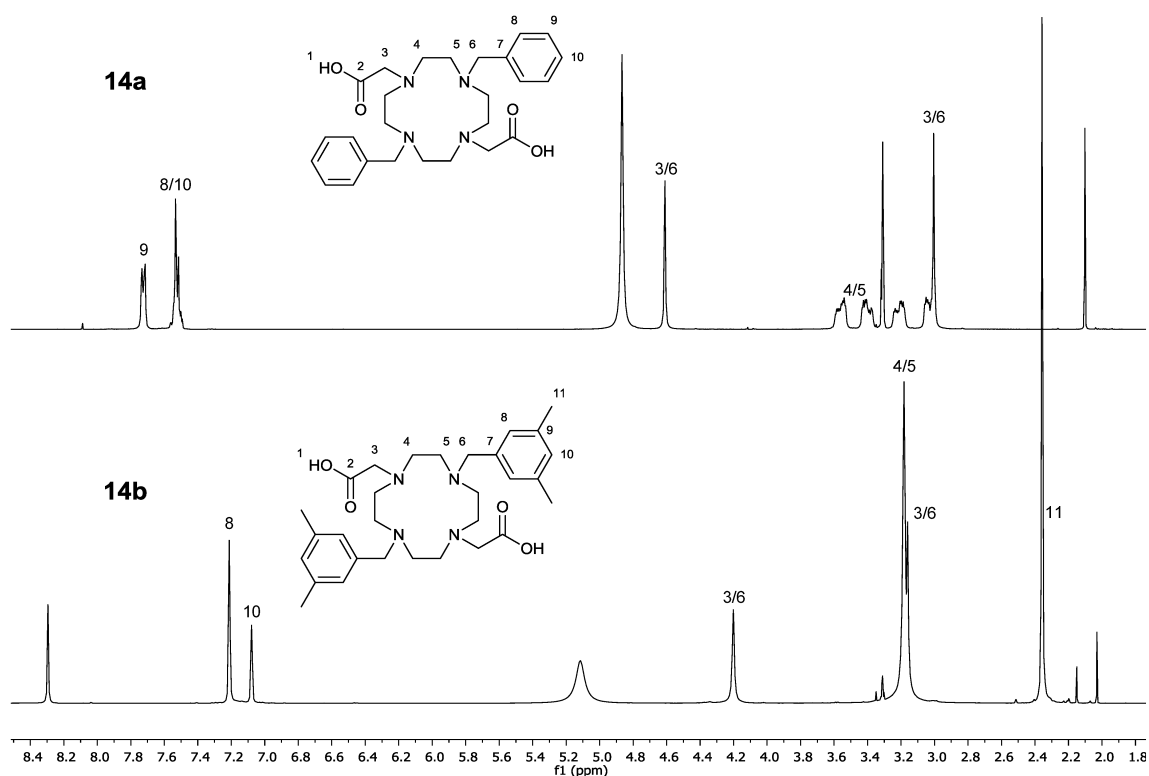


Figure 3.20: ^1H NMR spectra of the deprotected aryl-functionalised DO2A compounds **14a** and **14b** (MeOD, 400 MHz, 298 K).

3.3 Bridged Cyclens

The introduction of bridged cyclen ligands into this project was inspired by the formation of the disubstituted bisaminal intermediates **9**, which are commonly used as precursors to bridged macrocycle ligands. Bridged macrocycles such as sarcophagines form extremely stable complexes with cationic metal centres, since ligand preorganisation means that there is a smaller loss of entropy upon complexation when compared to non-bridged macrocycles. According to the equation for Gibbs free energy (ΔG):

$$\Delta G = \Delta H - T\Delta S \quad (3.4)$$

Reactions with a smaller loss of entropy (ΔS) lead to more favourable Gibbs free energy values and thus a higher degree of favourability. This comes with the drawback that the complexation kinetics are much slower than in their non-macrocyclic equivalents, since the ligand orientation is not optimised to facilitate metal complexation. This means that there is a high activation energy associated with the requirement of the ligand to adopt an appropriate conformation for complexation, and it is likely that harsh radiolabelling conditions will be required to facilitate radiolabelling. Once formed however, these complexes are highly stable, since the high activation energy of complexation works in reverse to keep the metal bound.

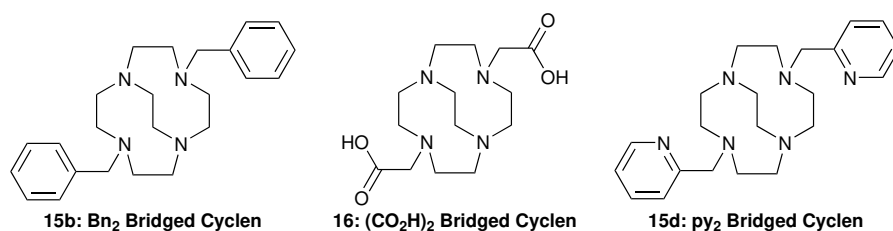
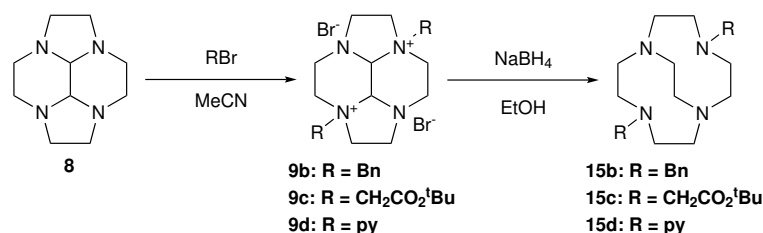


Figure 3.21: The three examples of bridged cyclens, the synthesis and radiolabelling of which is explored in this thesis.

The three target compounds are shown in Figure 3.21. The dibenzyl variant (**14b**) was selected as an initial test case due to its simplicity in chemical terms; benzyl groups are generally chemically inert and also provide a UV-active handle should chromatographic purification be required in its synthesis. In terms of radiochemistry, the benzyl groups should provide a higher degree of lipophilicity to the final gallium complex when compared to carboxylate arms. Benzyl groups, however, do not provide lone pairs of electrons to bind gallium metal centres,

and as such a dicarboxylate variant (**14c**) was another target should the bridged cyclen alone prove insufficient to bind gallium. Finally, the dipyridyl variant **14d** was also proposed as a ligand with intermediate binding properties between the potential lipophilicity of **14b** and the chelating ability of **14c**.

3.3.1 Synthesis of Disubstituted Bisaminals Revisited



Scheme 3.9: Synthesis of bridged cyclens **15** from the disubstituted bisaminals **9**, the synthesis of which was discussed in Section 3.2.1.2.¹⁶

The synthesis of the dibenzyl bridged cyclen was originally described by Niu *et al.*, and a general version of this synthesis is shown in Scheme 3.9.¹⁶ The paper describes the use of **15b** as an intermediate towards unfunctionalised bridged cyclen, since the benzyl groups can be cleaved through reaction with hydrogen gas and a palladium on carbon catalyst. Starting from cyclen glyoxal **8**, the disubstituted bisaminals **9b** and **9c** were synthesised as discussed in Section 3.2.1.2.

The dipyridyl variant **9d** however, had not been synthesised before. To achieve this, cyclen glyoxal **8** was reacted with 2-(bromomethyl)pyridine hydrobromide. The presence of the hydrobromide salt seemed to hinder the solubility of the pyridine in acetonitrile, however the salt was soluble enough to allow the reaction to proceed. The insoluble precipitate formed after 24 h gave the ¹³C{¹H} NMR spectrum shown in Figure 3.22. The five aromatic carbon atoms are clearly visible between 154–128 ppm, whilst the bridge and the benzyl carbon atoms seem to appear at 73.8 and 25.8 ppm respectively. The macrocyclic carbon atoms are likely to be represented by the broad peaks around the MeOD solvent, with the broadening arising from the flexibility of the molecule on the NMR timescale. On the other hand, whilst the ¹H NMR shows peaks with the expected chemical shifts and multiplicities, the integrals of the macrocyclic proton peaks is lower than that predicted from the aromatic peaks, which potentially suggests that the reaction has not gone to completion. In addition, mass spectrometry was

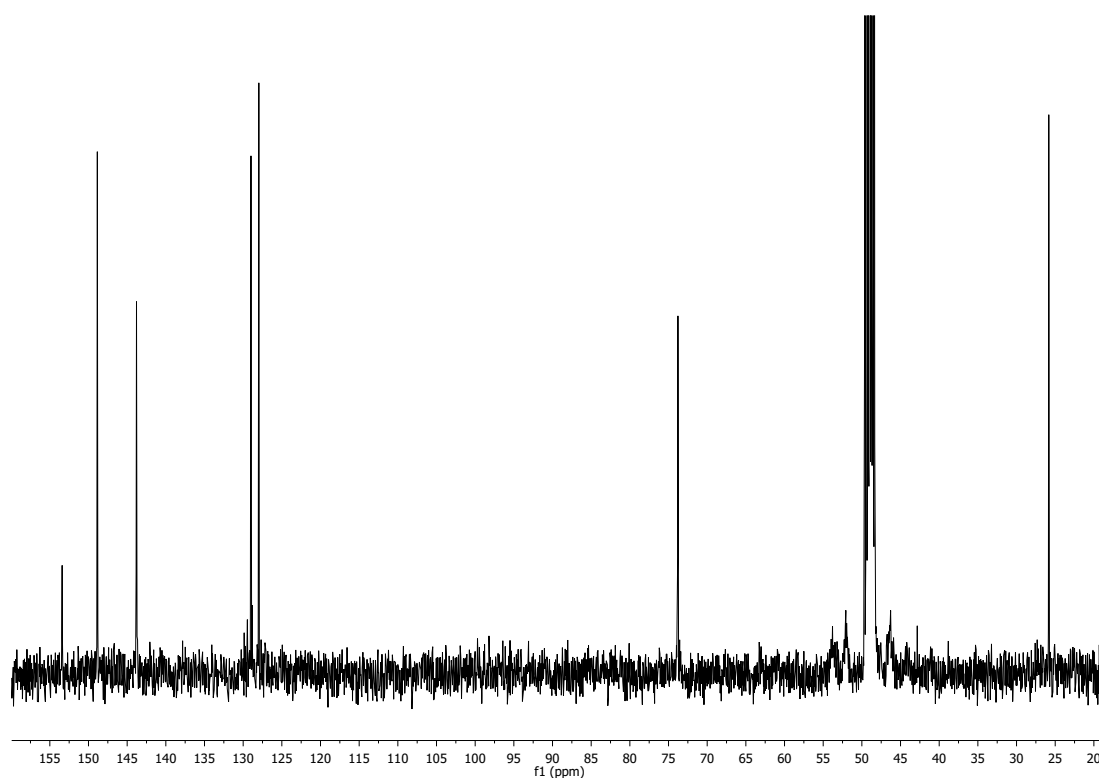


Figure 3.22: $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of the dipyrldyl bisaminal **9d** (100 MHz, 298 K, MeOD). The solvent peak has been truncated for clarity. The macrocyclic carbon atoms are likely represented by the broad peaks surrounding the MeOD solvent peak.

not able to locate the m/z values that may refer to the product. Nevertheless, it was decided to carry this product through to the next step, on the basis that the carbon NMR spectrum suggests the presence of the expected product, and that it would be easier to isolate the final bridged cyclen product by reverse phase chromatography.

3.3.2 Formation of Bridged Cyclens Through Sodium Borohydride Reduction

The next step, as shown in Scheme 3.9, was reaction of the bisaminals **9b-d** with sodium borohydride in order to partially reduce the bisaminal bridge in order to form the bridged cyclens **15b-d**.¹⁶ In order to form **15b**, the literature procedure uses approximately 37 equivalents of sodium borohydride. At the start of the addition, the ethanolic bisaminal solution turns from yellow to colourless and clear whilst increasing in temperature, indicating the exothermic reaction progress. As following equivalents are added, the unreacted and insoluble sodium borohydride begins to seize up the reaction mixture and hinder stirring. The excess sodium borohydride was decomposed using hydrochloric acid, before the the excess acid was removed

through the addition of sodium hydroxide. Niu *et al.* suggest working up the product through extraction into benzene, although this was substituted for toluene in this project for safety reasons.

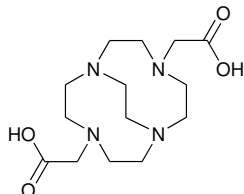


Figure 3.23: Diacid bridged cyclen **16**, produced by the TFA-based deprotection of the *tert*-butyl protected intermediate **15c**.

Both the dibenzyl (**15b**) and di-*tert*-butyl carboxylate (**15c**) variants were found to be easy to synthesise through this method, requiring no further chromatographic purification. On the other hand, the pyridyl variant **9d** did not obviously show product formation, and even the use of reverse-phase flash chromatography did not allow for the isolation of the product. As such, further work on the dipyridyl variant was halted here. **15c** acts as a *tert*-butyl protected intermediate for the diacid bridged cyclen **16** (Figure 3.23), as it must be deprotected in TFA then purified by reverse-phase chromatography, as has been seen numerous times in this project. ^1H NMR spectra of **15b** and **16** are shown in Figure 3.24. The dibenzyl variant **15b** is fairly pure by NMR, although it retained a large amount of toluene from the extraction step which was difficult to remove due to its high boiling point.

The dicarboxylate **15b** was deprotected by TFA, with an initial attempt using washing with chloroform as was seen for the triarylphosphonium-functionalised compounds seen previously; however no UV-active species were present in the chromatograph obtained by reverse-phase flash chromatography. This is likely a result of the lack of cationic TAP groups favouring solubility into the non-polar chloroform fraction rather than the aqueous fraction as expected. Instead, flash chromatography was performed on the crude mixture, which provided the NMR spectrum shown in Figure 3.24. HPLC analysis of these ligands is shown in Figure 3.25, which confirms that whilst impurities are present in the products, one major peak dominates in each case. As such, these compounds are ready for initial radiolabelling assessment which is detailed in the following chapter.

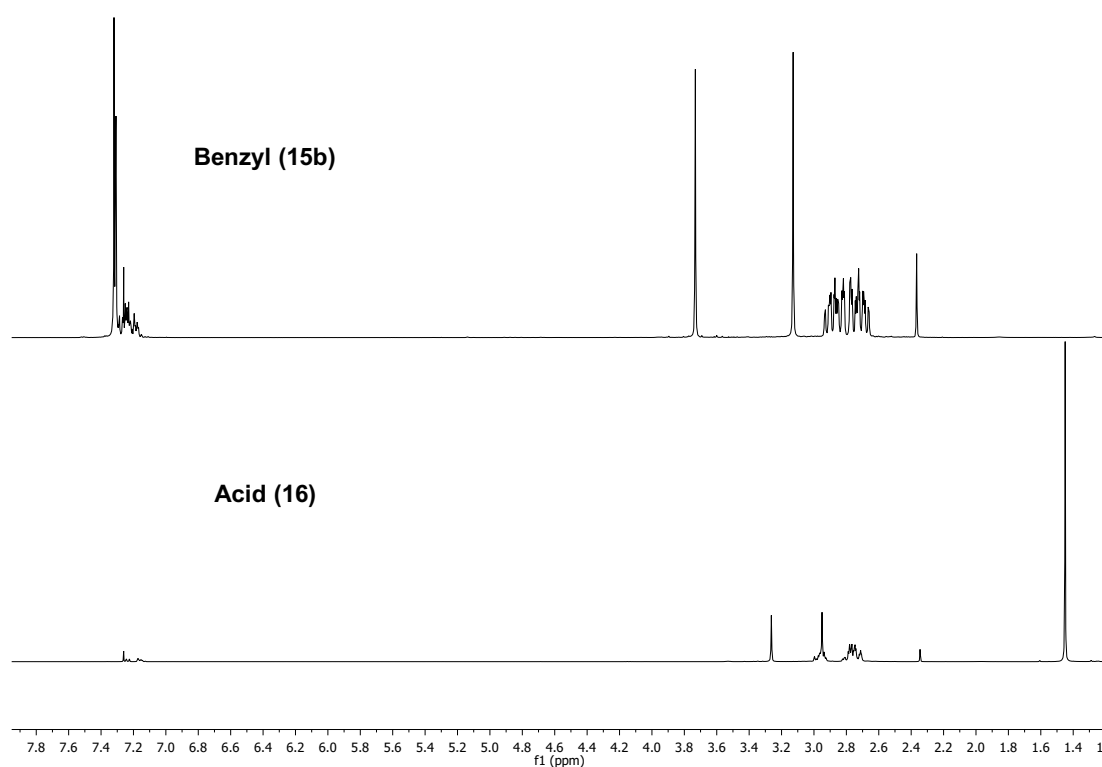


Figure 3.24: ^1H NMR spectra of the disubstituted bridged cyclens shown in Figure 3.21 (400 MHz, 298 K). Top: Dibenzyl bridged cyclen **14b** (CDCl_3). Bottom: Diacid bridged cyclen **15** (MeOD).

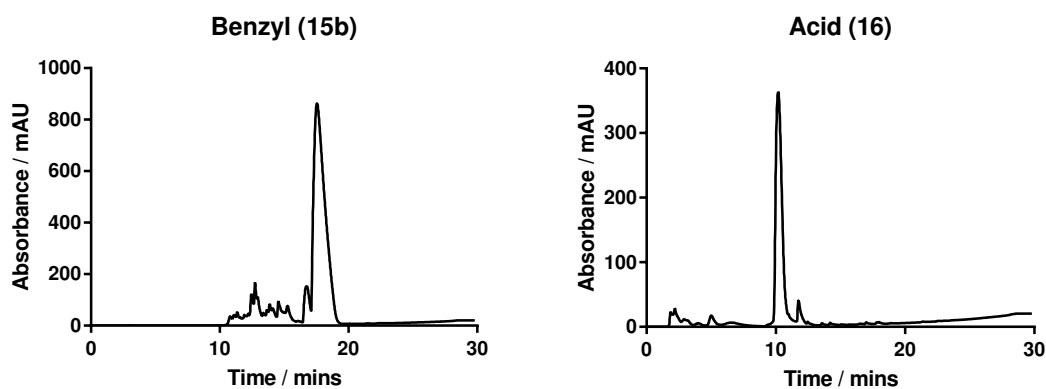
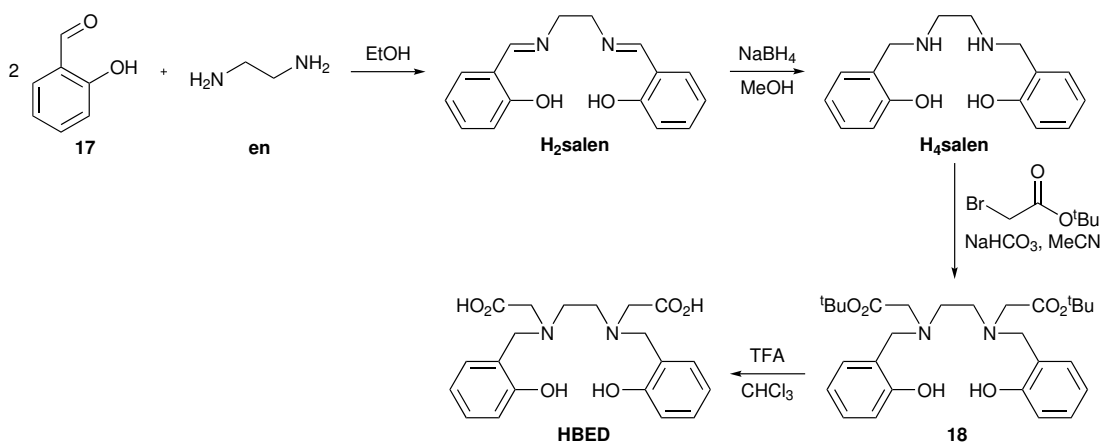


Figure 3.25: HPLC traces of the bridged cyclens **15b** and **16**. Eluent gradient: isocratic 100% A for 5 min; 0–100% B in A for 20 min; isocratic 100% for 5 min at a flow rate of 1 mL min^{-1} . UV absorption: $\lambda_{\text{abs}} = 250 \text{ nm}$.

3.4 HBED-Based Chelators: Attempted Syntheses



Scheme 3.10: Novel route to the synthesis of HBED developed during the preceding MRes Project, using a $H_4\text{salen}$ intermediate and the use of *tert*-butyl bromoacetate as a means of adding the carboxylate groups.

HBED was explored as a potential chelating backbone for the lipophilic and cationic gallium-68 chelators during my MRes project, due to the improved radiolabelling kinetics of acyclic chelators. During this project, a novel route to the synthesis of HBED (Scheme 3.10) was developed. The synthesis of the intermediate diimine $H_2\text{salen}$ and its subsequent reduction with sodium borohydride to form $H_4\text{salen}$ were performed according to Schmitt *et al.*, whilst the functionalisation with *tert*-butyl bromoacetate and subsequent deprotection with TFA were novel processes, albeit inspired by the DO3A triester synthesis described by Dadabhoy *et al.*^{20,21} Upon

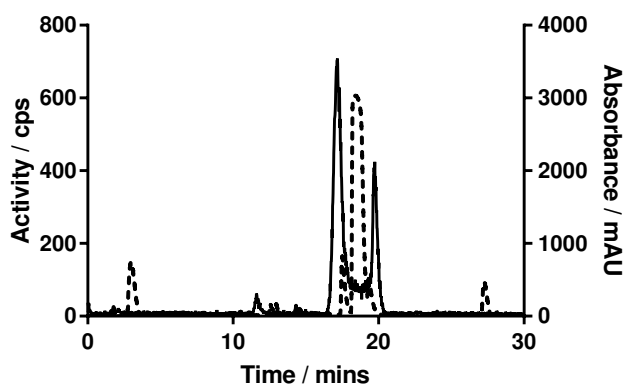
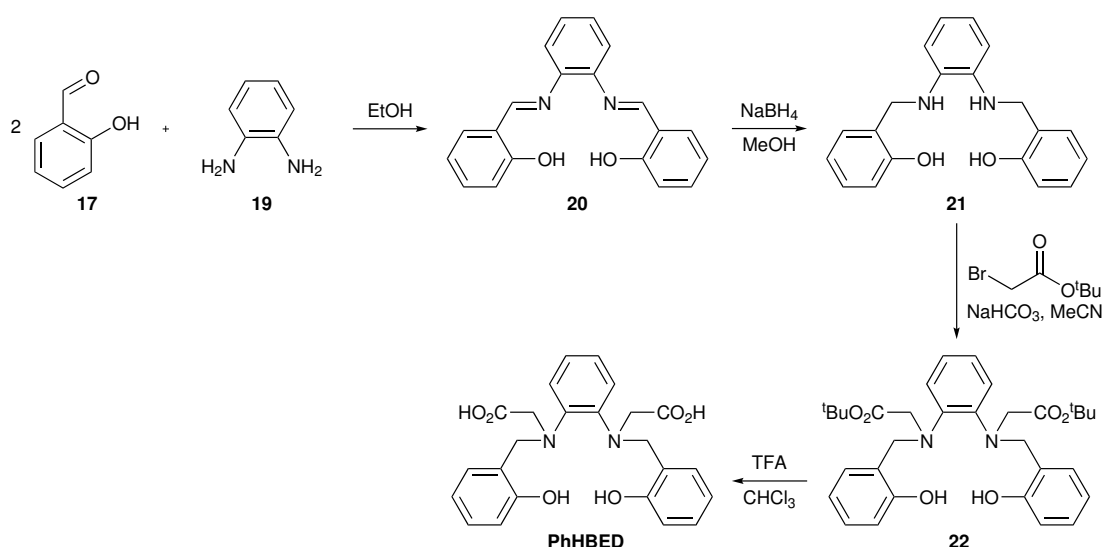


Figure 3.26: RadioHPLC trace of $[^{68}\text{Ga}]\text{Ga-HBED}$ obtained during the MRes project. Eluent gradient: isocratic 100% A for 5 min; 0–40% B in A for 20 min; isocratic 100% A for 5 min at a flow rate of 1 ml min^{-1} . Radioactivity shown as a solid line, UV absorption ($\lambda_{\text{ex}} = 250\text{ nm}$) shown as a dashed line.

radiolabelling with gallium-68 however, it can be seen by radioHPLC analysis (Figure 3.26) that despite the presence of a single main product by UV, there are multiple peaks referring to radiolabelled products. This result was explored further by Tsionou *et al.*, who confirmed the presence of multiple isomers in Ga-HBED complexes.²² Radiopharmaceutical regulators are reluctant to approve radiopharmaceuticals that cannot be isolated as a single species, and as such routes to the synthesis of more rigid HBED derivatives were explored.

3.4.1 Attempted Synthesis of Aromatic-Backboned HBED



Scheme 3.11: Proposed reaction scheme for the synthesis of PhHBED. The general synthesis is analogous to the synthesis of HBED shown in Scheme 3.10, albeit using *o*-phenylenediamine (**19**) instead of ethylenediamine.

PhHBED, HBED with an aromatic backbone, was devised as a way to rigidify the HBED framework whilst increasing the lipophilicity of the complex. The initial synthesis of this compound was designed as shown in Scheme 3.11, and was intended to be as similar as possible to that of HBED shown in Scheme 3.10.

3.4.1.1 Synthesis of Aromatic-Backboned Salen

As with HBED, the formation of the diimine salen **20** occurs through the reaction of a diamine, in this case *o*-phenylenediamine (**19**) with two equivalents of *o*-salicylaldehyde (**17**) in order to form the desired diimine. This step was found to be achievable in a manner entirely analogous to that used for the synthesis of H_2salen . In both cases, the colour of the reaction mixture

turns from colourless to yellow due to the alteration of electronic structure of the molecule. In addition, the product precipitated out of solution upon cooling of the reaction mixture, where it could be isolated as the pure compound by filtration.

The next step was reduction of the the two imine groups with sodium borohydride to form **21**. This process which unfortunately did not occur in an analogous manner to that of H₂salen, since cooling the reaction mixture to -18 °C did not form a precipitate, and simply removing the solvent under reduced pressure did not yield the pure product. Instead, an alternative procedure designed by Groysman *et al.* was used in which the methanolic reaction mixture was poured into water; the change in solvent polarity caused the product to precipitate out of solution such that it could be isolated by filtration.²³ Standard characterisation techniques demonstrated that the product was pure and ready for the next step.

3.4.1.2 Attempted Addition of the Carboxylate Arms

The next step as outlined in Scheme 3.11 was the addition of the carboxylate arms through the use of *tert*-butyl bromoacetate in the presence of inorganic base. For the synthesis of HBED, the equivalent intermediate **18** can be purified by taking the crude oil up in chloroform and removing the insoluble impurities by filtration. Unfortunately, the increased lipophilicity of the compound due to the aromatic groups increase the solubilities of the compounds in chloroform and as such they cannot be purified in this manner.

In order to determine the identities of the species formed in the reaction mixture, mass spectrometry was utilised. The mass spectrum shown in Figure 3.27 shows peaks which refer to the starting material, monosubstituted impurity and disubstituted product respectively. This observation was corroborated with ¹H NMR spectroscopy, which showed two peaks in the region for *tert*-butyl protons. Several purification methods were attempted, but unfortunately none were found to be successful. TLC using mixtures of DCM and acetone as mobile phases showed two spots, implying that the species could be separated by column chromatography, however use of a DCM/acetone gradient led to an increase in the number of species present. Even fractions with a single spot by TLC showed ¹H NMR spectra with multiple *tert*-butyl proton environments, which implied that column chromatography was causing the compound to decompose.

Secondly, trituration was attempted in order to purify the compound. The product oil

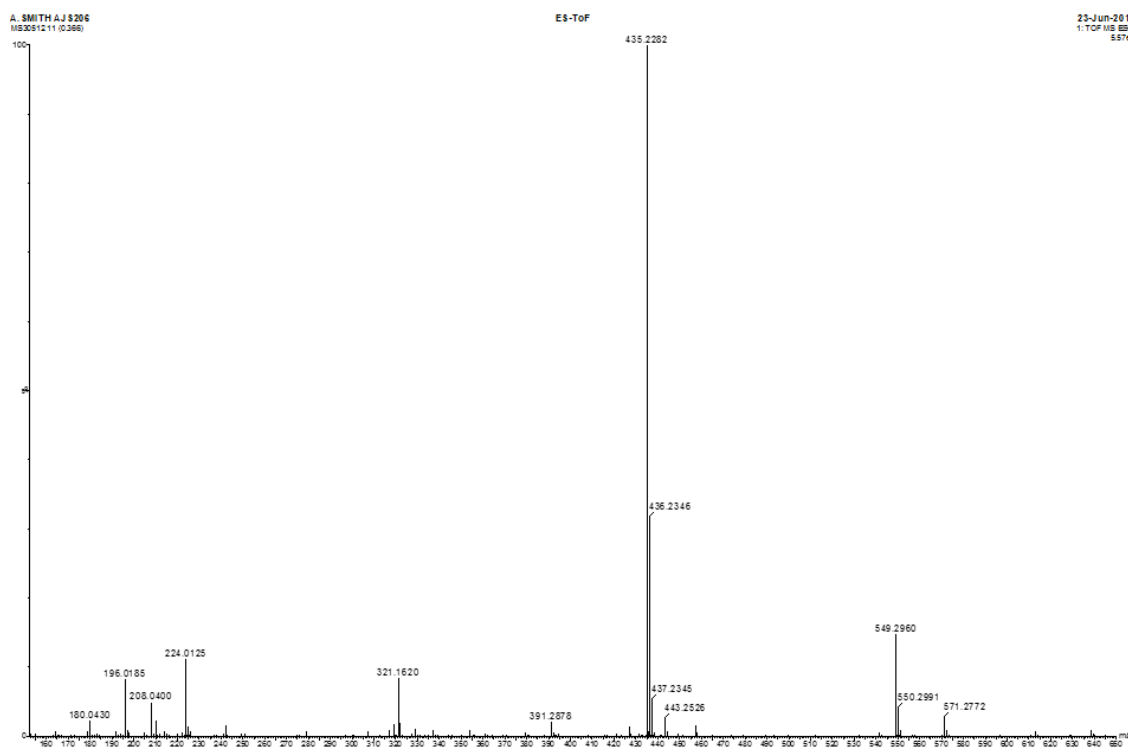
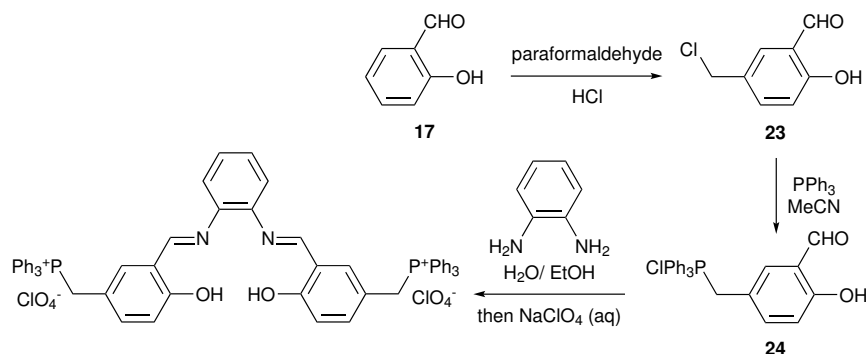


Figure 3.27: Mass spectrum of the result of the reaction between **20** and *tert*-butyl bromoacetate. A mixture of starting material, monosubstituted impurity and disubstituted product can be observed.

was taken up in minimal acetonitrile before excess diethyl ether was added. Addition of ether caused a white precipitate to form, which was isolated by vacuum filtration and dried *in vacuo*. This solid was found to turn pink overnight, and ^1H NMR spectroscopy showed that no purification had in fact taken place. Finally, recrystallisation in hot toluene was attempted. The impure pink solid dissolved to form a yellow solution, which promisingly turned cloudy as it cooled to room temperature. Left to cool further overnight however, the solution has turned red and no solid could be isolated. The results from the purification experiments indicate that **22** is an inherently unstable molecule, with the aromatic backbone proving incompatible with the alkylation of the nitrogen atoms. As such, PhHBED was not able to be synthesised by this method.

3.4.2 Attempted Synthesis of Bis-TPP Functionalised HBED

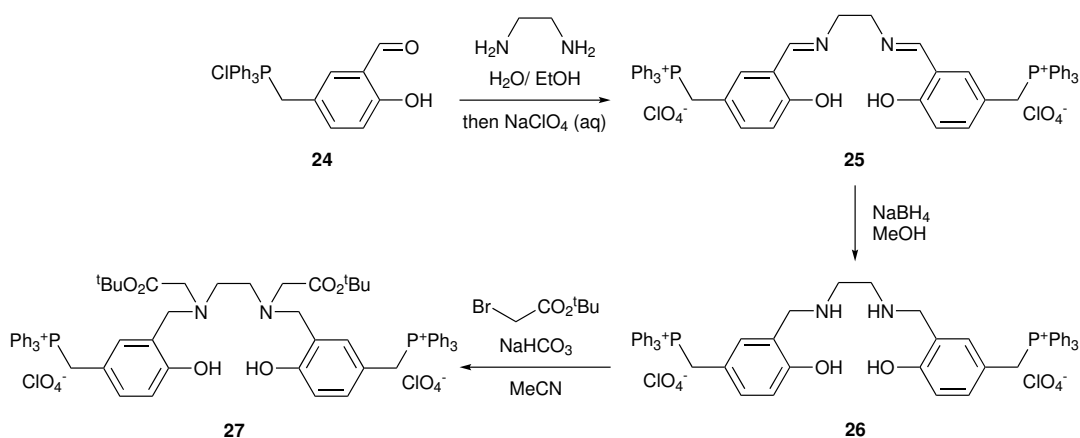
The second attempt at utilising a HBED-based chelator for the formation of gallium-68 labelled lipophilic cations involved bis-TPP functionalisation. The inspiration for this came from the bis-TPP functionalised Schiff bases designed by Asadi *et al.* for zinc and cobalt coordination



Scheme 3.12: Synthesis of a TPP-substituted Schiff base salen, designed by Asadi *et al.*²⁴

and DNA intercalation, the synthesis of which is outlined in Scheme 3.12.²⁴ The rationale behind this choice was that the TPP groups would provide lipophilicity and a cationic charge to the overall complex, whilst the additional steric bulk afforded by the TPP groups could lock the complex into a single conformation.

3.4.2.1 Synthesis Using TPP-Substituted Salicylaldehydes



Scheme 3.13: Proposed reaction scheme for the synthesis of bis-TPP functionalised HBED **27**.

The initial proposed reaction scheme for the synthesis of bis-TPP functionalised HBED (**27**) is shown in Scheme 3.13. The first step in its synthesis requires the use of a TPP-functionalised salicylaldehyde **24**, the synthesis of which is shown in Scheme 3.12. Asadi *et al.* perform this reaction in two steps, first by adding a benzyl chloride functional group using paraformaldehyde and conc. HCl, before the TPP functionalisation was added through S_N2 reaction with the benzyl chloride.²⁴ The benzyl chloride intermediate was formed as a pink viscous gum rather than the dark red powder as specified by the literature. This made it difficult to per-

form the aqueous washing steps, and recrystallisation from acetonitrile/petroleum ether did not form any precipitate. ^1H NMR spectroscopy showed that the desired product has indeed been formed, although there is a high degree of impurity.

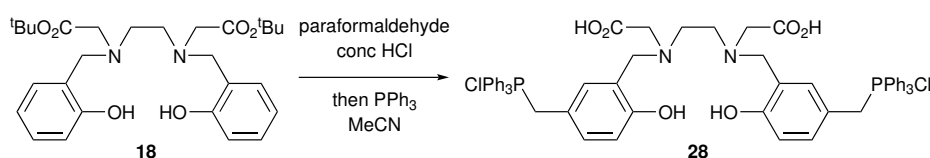
The next step was the formation of the TPP cation **24** through $\text{S}_{\text{N}}2$ reaction with triphenylphosphine. As was shown with analogous reactions with α,α' -dibromo-*p*-xylene, TPP cations are likely to precipitate out of low polarity solvents. Indeed, despite the reaction being performed in refluxing acetonitrile, the cationic product precipitated out of solution as a white solid in under two minutes. This product was found to be pure by ^1H NMR spectroscopy. The fact that this product is automatically purified by the reaction procedure, and the recrystallisation of **23** is not as efficacious as suggested by the literature, the chlorinated intermediate is best used without additional purification or characterisation.

Whilst Asadi *et al.* use an aromatic backbone in their Schiff base complexes, difficulties in functionalising these compounds with carboxylate arms led to the target compound **27** in Scheme 3.13 which uses an aliphatic backbone analogous to the salens shown in Scheme 3.10. The first step of the reaction is the formation of the two diimine bonds between the aldehydes and the amines performed in a water/ethanol solvent mixture, before the formed product is forced to precipitate out of solution using an aqueous solution of sodium perchlorate which induce anion exchange. The new perchlorate anions drastically lower the solubility of the product, to the point where DMSO-d_6 is required to obtain a ^1H NMR spectrum of the diimine product. This lowered solubility also hinders the next step, reduction of the imine groups with sodium borohydride to form **26**. Addition of sodium borohydride turned the solution from yellow to colourless, although 15 minutes later the solution was yellow again, either a result of further starting material dissolving or decomposition of the product. This process was repeated twice more before the product was isolated through addition of water, however ^1H NMR spectroscopy was inconclusive as to whether the desired product formed.

To facilitate the reduction process, it was decided to isolate the diimine **25** with its original chloride anions with a view to improving its solubility. Initial attempts at purifying the compound involved precipitating the product out of solution at low temperatures and column chromatography, however even temperatures of -78°C did not cause the product to precipitate, and all TLC mobile phases explored merely caused the product to streak from the baseline. Fortunately, recrystallisation in minimum chloroform and minimum diethyl ether was

found to be effective by ^1H and $^{31}\text{P}\{^1\text{H}\}$ NMR spectroscopy. Since these chlorides were soluble in methanol, the sodium borohydride reduction steps could now be attempted. As seen previously, reduction of the diimines caused the reaction mixture to turn from yellow to colourless as the degree of conjugation was decreased. Unfortunately, NMR was inconclusive as to whether the desired reaction had occurred, and no effective purification techniques could be discovered.

3.4.2.2 Synthesis Using TPP-Functionalisation of HBED



Scheme 3.14: Proposed synthesis of bis-TPP functionalised HBED (**28**) using premade *tert*-butyl protected HBED (**18**).

As an alternative to the synthetic methodology designed by Asadi *et al.* in which the salicylaldehyde is functionalised with TPP before the Schiff base is formed, the synthesis outlined in Scheme 3.14 was devised, in which pre-made *tert*-butyl protected HBED (**18**) is functionalised, firstly to form benzyl chlorides and secondly to form the TPP cations. **18** was synthesised according to Scheme 3.10, before the benzyl chloride groups were appended using paraformaldehyde and conc. HCl. Conc. HCl also has the potential to deprotect the carboxylate arms through removal of the *tert*-butyl groups; it was unclear at this point whether this process would hinder the subsequent formation of the TPP cations. This reaction underwent a series of colour changes as it progressed, ranging from yellow after 30 minutes to red after 72 h, indicating that the functionalisation was changing the electronic properties of the molecule. These benzyl chloride intermediates can be very difficult to definitively characterise by NMR, so instead mass spectrometry was used. A peak at $m/z = 597$ was expected in Figure 3.28 for the *tert*-butyl protected product, but instead a peak at 485 is observed, implying that the deprotection is a dominant reaction pathway.

This intermediate was not fully characterised or purified, since the formation of TPP cations tends to facilitate isolation of the product through changes in solubility. The synthesis of **28** involved taking the benzyl chloride intermediate and heating it at reflux in acetonitrile with

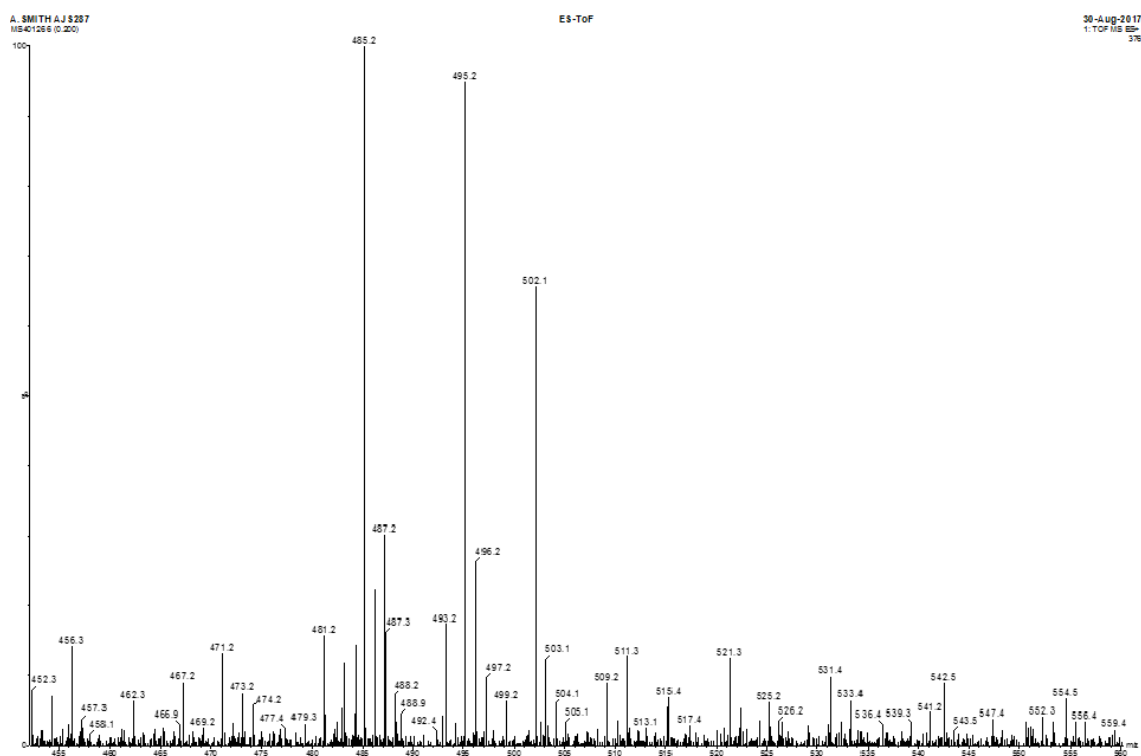


Figure 3.28: Mass spectrum of the product of the reaction between **18**, paraformaldehyde and conc. HCl. A peak at $m/z = 485.2$ can be observed for the desired product without *tert*-butyl deprotection.

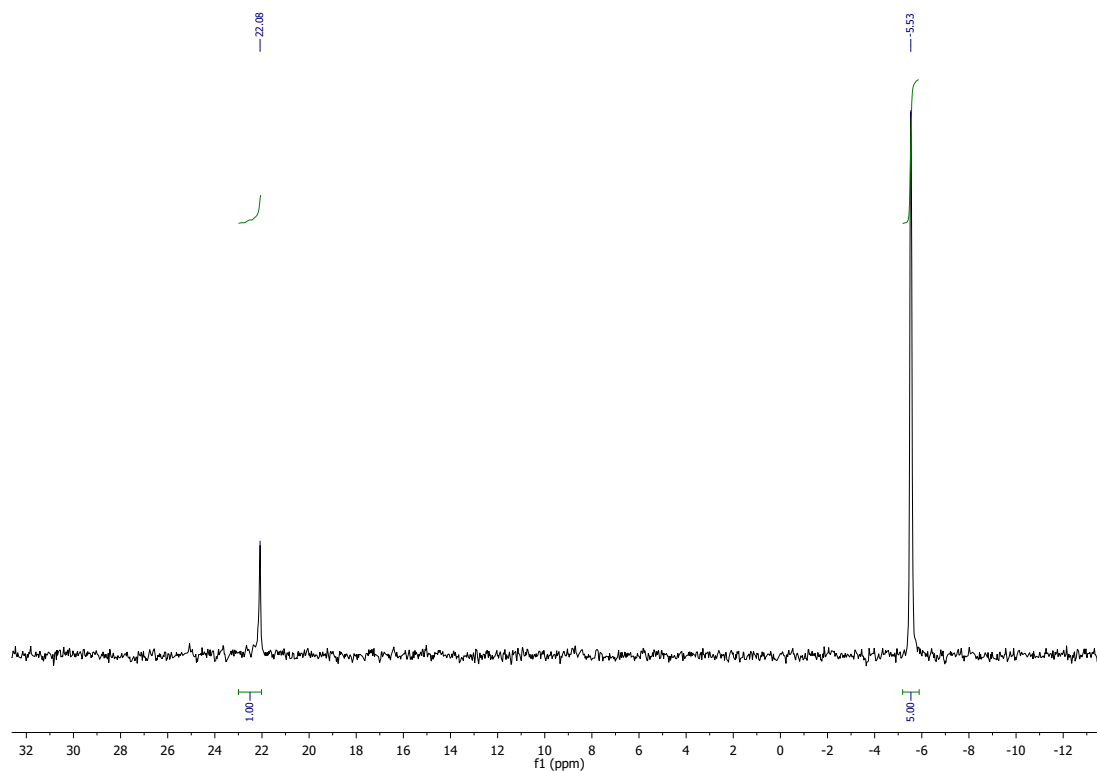


Figure 3.29: $^{31}\text{P}\{^1\text{H}\}$ NMR (162 MHz, 298 K, CDCl_3) of the product of the attempted formation of **28** according to Scheme 3.14.

triphenylphosphine. As the reaction mixture cooled, a white precipitate formed as expected, which could be isolated by vacuum filtration. Upon evaporation, the reaction mixture was found at this point to consist purely of unreacted triphenylphosphine. $^{31}\text{P}\{^1\text{H}\}$ NMR spectroscopy of the white solid (Figure 3.29) revealed that whilst some substitution had taken place as shown by the peak at $\delta_{\text{P}} = 22.1$ ppm, there is a much larger peak at $\delta_{\text{P}} = -5.5$ ppm referring to unreacted triphenylphosphine. This avenue of research was not pursued further, however it is expected that once a method to expedite the substitution process this chelator system shows promise for the formation of lipophilic and cationic gallium-68 complexes.

3.5 Concluding Remarks

This chapter outlines the synthesis of a series of chelators for the formation of lipophilic and cationic gallium-68 chelators. Initially, triarylphosphonium-functionalised DO3A chelators were considered, combining the chelator design by Wang *et al.* and Kardashinsky *et al.* with the lipophilicity tuning of Haslop *et al.*^{1,2,4} A novel route to the high-yield formation of pure TAP cations was developed on the way to the synthesis of a novel analogous series of chelators. The formation of non-radioactive reference complexes was also explored, with gallium nitrate hydrate being preferred as a Ga(III) source to gallium chloride due to its stability and ease of handling. HPLC experiments on the non-radioactive reference compounds, along with radiochemical and biological experiments discussed in the proceeding chapters, indicated that alteration of the TAP functionalisation had the desired effect on lipophilicity, as shown by the increase in retention time as the degree of functionalisation increased.

Following on from the synthesis of DO3A-based lipophilic cations, DO2A-based lipophilic cations were synthesised to take advantage of the small ionic radius of Ga(III) and its preferred hexadentate coordination geometry. The exploration of two separate routes to the synthesis of DO2A diester found that the route suggested by Hirayama *et al.* was superior due to its reliability, despite the use of chromatographic purification and hydrogen gas deprotection.¹⁴ Functionalisation with TAP groups was found to be entirely analogous to their DO3A counterparts, making the synthesis of a second analogous series simple. The DO2A diester also provided an excellent basis for the synthesis of a third analogous series of aryl-functionalised DO2A chelators, with a view to forming monocationic Ga(III) complexes.

Attempted syntheses of DO2Adiester and DO2A-Bn₂ derivatives using glyoxal-protected cyclen provided the inspiration for the synthesis of bridged cyclen chelators, with a view to forming Ga(III) complexes with high thermodynamic stability. Dicarboxylate and benzyl-functionalised variants were synthesised with the bridged cyclen thought to provide enough complex stability such that the carboxylate arms would not be needed, hence the benzyl-functionalised variant. The synthesis of a pyridyl-functionalised bridged cyclen was also explored in line with these principles, but to no avail. The radiochemistry and biological analysis of these ligands will be discussed in proceeding chapters.

Somewhat less successful was the synthesis of HBED derivatives, explored due to the favourable radiolabelling kinetics of acyclic chelators. The synthesis of PhHBED, HBED with an aromatic backbone, was initially explored, however the presence of the aromatic backbone hindered functionalisation of the nitrogen atoms with carboxylate groups. It would be interesting to see whether a rigid but non-aromatic backbone such as (R,R)-(-)-1,2-diaminocyclohexane would aid synthesis and complex rigidity. The functionalisation of HBED with TPP groups as inspired by Asadi *et al.* was also unsuccessful, with factors such as solubility and difficulties in characterisation hindering the synthesis. A logical next step for the synthesis of lipophilic and cationic gallium-68 chelators based on HBED could involve the use of HBED-CC (Figure 1.15), with the two pendant carboxylate groups providing a potential site for TAP-functionalisation.

3.6 References for Chapter 3

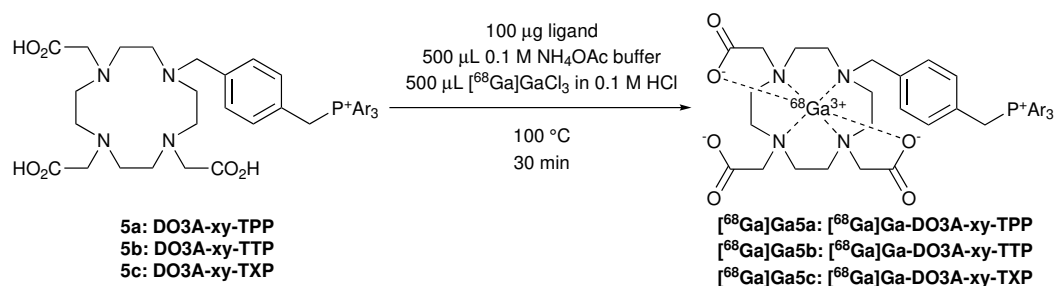
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Chapter 4

Radiolabelling of Lipophilic Cations with Gallium-68

Having described the synthesis of potential lipophilic and cationic chelators for gallium-68 in the previous chapter, the next step was to assess the radiolabelling properties of the chelators. Once effective radiolabelling strategies for the chelators were determined, their log *D* values were calculated as an initial assessment of their potential for mitochondrial uptake, both in myocardial tissue and in tumour cells.

4.1 [⁶⁸Ga]Ga-DO3A-xy-TAP



Scheme 4.1: Protocol for the radiolabelling of DO3A-xy-TAP compounds with generator-produced [⁶⁸Ga]GaCl₃. The use of 0.1 M NH₄OAc solution as a radiolabelling buffer was inspired by the use of a similar buffer for copper-64 chelation of DO3A-xy-TPP as explored by Wang *et al.*¹

Since analogues to DO3A-xy-TAP and appropriate radiolabelling strategies exist in the literature, they seemed like ideal starting points to explore gallium-68 radiolabelling.^{1,2} The radiolabelling protocol, adapted from the copper-64 labelled analogues demonstrated by Wang *et al.*, is outlined in Scheme 4.1.¹ Firstly, 100 µg of ligand was dissolved in 500 µL of 0.1 M NH₄OAc buffer, leading to an initial ligand concentration of approximately 20 µM. Ammonium acetate was chosen as a buffer due to its use in the copper complexation reactions, whilst a concentration of 0.1 M was chosen to be equimolar with the 0.1 M hydrochloric acid used to elute the generator. In accordance with Wang *et al.* and in order to ensure maximum radiochemical yield (RCY) was obtained for future biological experiments, the reaction mixtures were heated to 100 °C for 30 minutes, with the view that if the compounds showed appreciable mitochondrial uptake, optimisation of their radiolabelling would be explored afterwards.

The Eckert & Ziegler gallium-68 generator used in these experiments, similar to that shown in Figure 4.1 and functioning in a similar manner to the technetium-99m generator shown in Figure 1.7, is subject to a high degree of variability in the characteristics of the [⁶⁸Ga]GaCl₃ solution eluted. Firstly, research gallium-68 generators have an approximate functional life-



Figure 4.1: Eckert & Ziegler Model IGG100 Gallium-68 Generator, similar to the one used in this project. Picture taken from www.radiustech.it.³

time of one year, as the germanium-68 parent isotope depletes over time. It should be noted that a generator has a period of time dedicated to clinical use whilst it is at its most radioactive, before being transferred to research use. Once designated for research, a generator is capable of producing over 400 MBq of [^{68}Ga] GaCl_3 in 5 mL of eluate, whilst after a year it only produces 200 MBq in the same volume.

The amount of time between elutions also affects the eluate; the generator can be considered to be completely regenerated after three hours, as at this point the rates of formation and decay of gallium-68 are approximately equal. After about 24 hours however, the amount of non-radioactive zinc-68 starts to build up to the point where it competes for chelation with the gallium-68, potentially lowering the RCY values obtained. To this end, if a generator has not been eluted for a day, one pre-elution is performed to remove excess zinc. One pre-elution is considered enough for research purposes, however in a clinical setting multiple pre-elutions may be needed to further prevent the formation of non-radioactive zinc complexes in the administered formulation. Under GMP (Good Manufacturing Practice), the number of elutions a generator can undergo is limited, which may prove a problem if the generator is infrequently used. The generator is eluted with 5 mL of 0.1 M hydrochloric acid into five 1.5 mL Eppendorf tubes. Through careful administration and collection of the eluate, over 90% of the activity can be collected in two 1 mL fractions, and as such between 50–100 MBq is used in each

500 μL [^{68}Ga] GaCl_3 aliquot.

4.1.1 RadioHPLC Assessment

Initial analysis of the effectiveness of the radiolabelling of DO3A-xy-TAP compounds was performed by radioHPLC. In radioHPLC, the standard UV detector used by most HPLC set-ups is followed by a gamma detector to detect radioactivity. The gamma detector is surrounded by lead to reduce detection of the background radiation as much as possible, whilst a lead collimator is used to focus the detection over as thin a cross-section of the HPLC outlet tubing as possible. The section of tubing between the UV and gamma detectors means that it takes approximately 30 seconds for the eluate to pass between the two detectors; in practice the low concentration of radiolabelled compound means that it is not detected by UV.

4.1.1.1 RadioHPLC Method Development and Ligand Assessment

RadioHPLC traces of the three [^{68}Ga] Ga -DO3A-xy-TAP compounds are shown in Figure 4.2. Analytical C-18 reverse-phase HPLC was used, using a mobile phase of water with 0.1% TFA (A) and acetonitrile with 0.1% TFA (B). The traces in Figure 4.2a used a gradient of 0–50% B in A, following 5 minutes of isocratic 100% A and followed by another 5 minutes of 100% A. Returning to 100% A for 5 minutes at the end of each experiment allows for a higher throughput of compounds as less time is needed between experiments for the HPLC column to

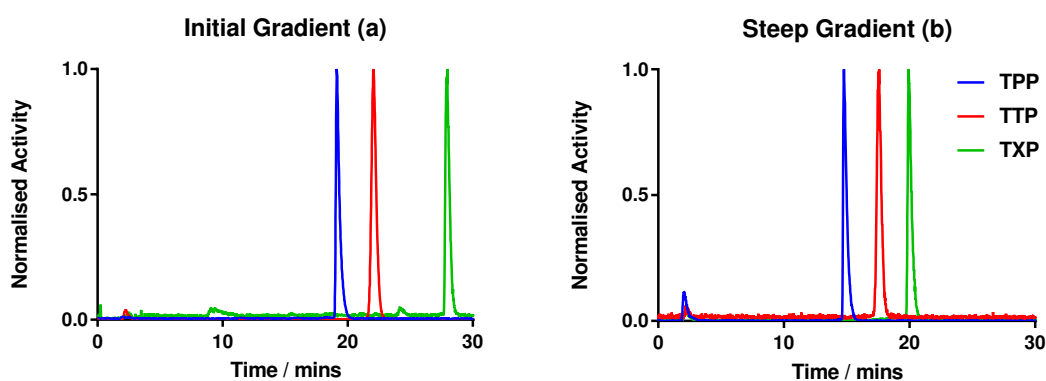


Figure 4.2: RadioHPLC traces of the three labelled [^{68}Ga] Ga -DO3A-xy-TAP compounds. Eluent gradient **a**: 100% A for 5 min; 0–50% B in A for 20 min; 100% A for 5 min at a flow rate of 1 mL min^{-1} . Eluent gradient **b**: 100% A for 5 min; 0–80% B in A for 20 min; 100% A for 5 min at a flow rate of 1 mL min^{-1} . All traces have been normalised such that the maximum activity measured for each compound is equal to 1.

equilibrate. Analysis of the HPLC traces shown in Figure 4.2a firstly allow for the estimation of RCY, since unchelated [^{68}Ga]Ga(III) is eluted with the solvent front with a retention time of two minutes. Secondly, the radioHPLC allows for the confirmation of radiochemical purity, as a single peak implies that only a single radiolabelled compound is formed. In Figure 4.2a, the TPP and TTP functionalised compounds seem to be pure, whilst the TXP functionalised compound has an impurity peak at approximately 24 minutes; the peak between 9–11 minutes in that trace was due to nearby high-activity fluorine-18 work causing a reading on the detector despite the lead shielding. Finally, a qualitative assessment of the lipophilicity of the three tracers can be determined from the retention times of the three tracers. Since a reverse-phase column consisting of silica functionalised with aliphatic C-18 chains is used as a stationary phase, tracers with a higher degree of lipophilicity adhere to the column more effectively and as such have a longer retention time. As can be seen from Figure 4.2a, the retention time increases as the degree of alkyl functionalisation increases, which is an initial indication that the alkyl functionalisation is having the desired effect.

Later HPLC analyses of [^{68}Ga]Ga-DO3A-xy-TXP gave highly variable results, with some experiments not resulting in the elution of the product after 30 minutes. This is likely a result of the fact that at this point in the gradient, the mobile phase consists of 100% water, which is of too low a solvent strength to move the product effectively through the column. In order to elute [^{68}Ga]Ga-DO3A-xy-TXP whilst the gradient still includes acetonitrile, the HPLC gradient had to be optimised. Whilst it is ideal if the product elutes from the column quickly, if this leads to peaks coeluting then it may be better to use a slower gradient to increase peak-to-peak resolution. Balancing these factors is the key to HPLC optimisation.

Following optimisation, a second set of analyses in which the mobile phase reached a peak of 80% acetonitrile rather than 50% were performed. As shown by Figure 4.2b, [^{68}Ga]Ga-DO3A-xy-TXP elutes with a retention time of 20 minutes whilst the acetonitrile concentration was still rising. The increase in retention time between the three compounds was also preserved, with the addition of three methyl groups leading to an increase in retention time of two minutes. The lack of obvious radiolabelled impurities in Figure 4.2b implies that each radiolabelling mixture only consists of one species, although the RCYs seem to be generally lower than in Figure 4.2a due to the larger [^{68}Ga]Ga(III) peak.

4.1.1.2 Comparison with Non-Radioactive Reference Compounds

In order to ensure that the radiolabelled species shown in Figure 4.2 refer to the [^{68}Ga]Ga-DO3A-xy-TAP species, their retention times were compared to the non-radioactive reference compounds synthesised and characterised in Section 3.1.4. The retention times for the non-radioactive and gallium-68 labelled compounds are summarised in Table 4.1; these values can be compared directly since the same HPLC gradient was used to analyse both sets of compounds. For the TTP-functionalised compounds, there is only 6 seconds difference between the retention times of the reference and radiolabelled compounds, meaning that it is highly likely that the radioactive species is in fact [^{68}Ga]Ga-DO3A-xy-TTP. There is a larger difference between the reference and radiolabelled compounds for the TXP-functionalised variant; this inconsistencies could arise through a variety of factors such as differences in eluent composition, internal differences in the HPLC column and even the laboratory temperature. Nevertheless, the fact that both peaks elute later than the TTP variant and have similar retention times is evidence enough that the radioHPLC peak does refer to [^{68}Ga]Ga-DO3A-xy-TXP.

Table 4.1: Comparison of the retention times for the non-radioactive reference Ga-DO3A-xy-TAP compounds (Figure 3.11a) and their radiolabelled analogues (Figure 4.2b).

	Retention Time / mins	
	DO3A-xy-TTP	DO3A-xy-TXP
Non-Radioactive	17.7	19.2
Radioactive	17.6	19.9

4.1.2 Development of an iTLC-Based QC Technique

Whilst radioHPLC is a powerful technique for the analysis of radiosyntheses due to its high sensitivity and resolution, it does have some drawbacks which preclude its use in all radiosyntheses. One such drawback is that it can be difficult to use for the calculation of RCYs, since only compounds which emerge from the column are detected, meaning that any radioactive compounds remaining on the column are not quantified in RCY calculations. On more practical notes, the popularity of radioHPLC means that opportunities to use it are sporadic, whilst the 30 minute run time coupled with the 30 minute synthesis time means that by the time the compound has been synthesised and analysed, roughly one half-life of gallium-68 has passed. This lowers the amount of activity available for biological experiments, meaning fewer experi-

ments can be performed from one radiolabelling sample. For quality control (QC) purposes in radiochemistry, Instant Thin Layer Chromatography (iTLC) is generally used instead. Whilst the resolution of iTLC is generally lowered when compared to traditional TLC, iTLC can be performed in about two minutes, which when coupled with a radioTLC plate reader allows for a fast confirmation of radiolabelling efficiency can be determined. TLC-based techniques are regarded as providing a more accurate readout of RCY than HPLC since when scanned by the detector, all the radiolabelled species initially applied on the baseline are detected by the detector.

4.1.2.1 Choosing an Appropriate Mobile Phase

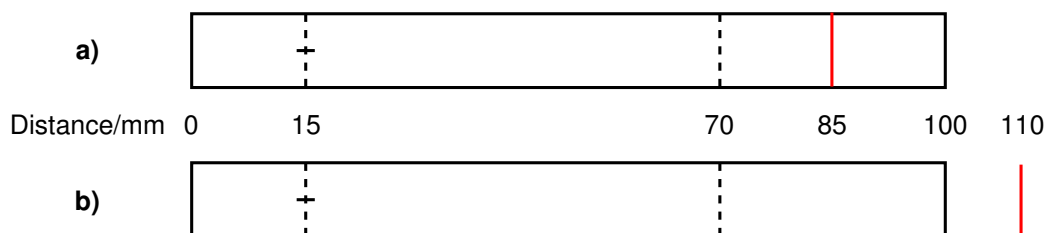


Figure 4.3: Diagrammatic representation of the iTLC-based QC methods used in this project. In both methods, a 100 mm length strip of iTLC paper is used. The radiotracer is dotted 15 mm from the bottom of the paper and the mobile phase is run to a distance of 70 mm, although residual mobile phase can push the mobile phase past this point. The limit of scanning is represented by the red lines, this lies at 85 mm for Method a and 110 mm for Method b.

The main part of using radioiTLC as a QC method for radiolabelling is the choosing of an appropriate mobile phase to provide separation between the radiolabelled compound and unchelated gallium. As shown by the work by Tsionou *et al.*, iTLC tends to use aqueous mobile phases such as sodium citrate or sodium phosphate when applied to gallium-68.⁴ Despite the intended lipophilicity of the tracers synthesised in this project, they still exhibit an overall cationic charge, and as such adhere very strongly to the polar silica gel on the iTLC plate. Instead of trying to move the compound from the baseline, chelating mobile phases were used to try to move the unchelated gallium-68. The iTLC methods used to visualise the compounds are shown in Figure 4.3. An aliquot of the radiolabelling mixture was spotted 15 mm from the bottom of the iTLC plate, before the mobile phase was run up to 70 mm. It was found that residual mobile phase caused the solvent front to keep moving up the plate even after it was removed from the TLC chamber, and as such the solvent front does show some variability.

One potential issue with this technique is the potential presence of colloidal $[^{68}\text{Ga}](\text{OH})_3$ in the reaction mixture, however as will be seen later control experiments can be performed to quantify the presence of this species.

Development of a QC method began with the iTLC traces shown in Figure 4.4, which were performed on $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TPP}$ alongside a control experiment of unchelated $[^{68}\text{Ga}]\text{Ga(III)}$. Firstly, 0.1 M trisodium citrate and 0.4 M monobasic sodium phosphate were used due to the fact that they were used by Tsionou *et al.* in their gallium-68 radiolabelling experiments.⁴ As expected, neither of these mobile phases moved the radiolabelled product from the baseline (Figures 4.4a and c), however in the control experiments (Figures 4.4b and d) neither mobile phase was found to efficiently move the unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ from the baseline. Since there is no difference between the traces in the product and control experiments, these mobile phases were deemed unsuitable for this procedure.

Since aqueous mobile phases were not found to move the cationic product from the baseline, it was decided instead to use a mobile phase with a higher degree of chelating ability in order to move the unchelated $[^{68}\text{Ga}]\text{Ga(III)}$. Initially, the 0.1 M sodium citrate solution was mixed in a 1:1 ratio with a 0.1 M solution of disodium ethylenediaminetetraacetic acid (EDTA),

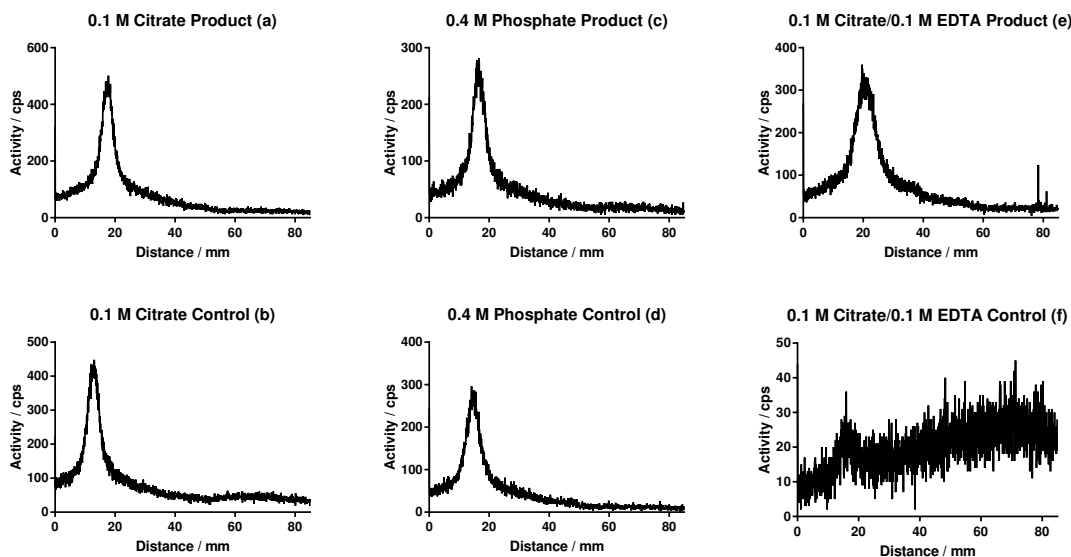


Figure 4.4: Initial development of an iTLC-based QC method for assessing the radiolabelling efficiency of $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TPP}$ (Product, **a,c,e**). This was compared to control experiments involving unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ (**b,d,f**). Mobile phases tested include: 0.1 M trisodium citrate (**a,b**), 0.4 M monobasic sodium phosphate (**c,d**), and a 1:1 mixture of 0.1 M trisodium citrate and 0.1 M disodium EDTA (**e,f**). The iTLC plates were scanned using Method a from Figure 4.3 and a gamma detector.

which as expected left the product on the baseline (Figure 4.4e). The trace obtained for the control experiment is shown in Figure 4.4f, where it can be seen that whilst some activity remains on the baseline, there is an increase in activity towards the solvent front, indicating that the presence of EDTA in the mobile phase does cause separation. Performing this iTLC gave two factors which were taken into consideration for all other iTLCs performed during this project. Firstly, the low amount of activity means that the trace is noisy, and therefore it is difficult to discern the retention of the tracer over the background radiation. As such, from this point onwards at least 400 Bq of radiation were applied to the iTLC plate before the chromatogram was run, and a beta detector was used rather than a gamma detector due to the lower prevalence of beta particles in the background radiation caused by beta decay. Secondly, despite running the solvent front to a height of 70 mm and scanning to a height of 85 mm, it seems that the residual mobile phase on the plate causes the solvent front to keep rising, meaning that some activity is not detected. This was changed by using the scanning method shown in Figure 4.3b, which scans to a height of 110 mm and therefore is certain to detect all the activity on the plate.

4.1.2.2 Calculation of RCY by iTLC

Successful separation of product and unchelated [^{68}Ga]Ga(III) was achieved using a mobile phase of 0.1 M disodium EDTA, as shown by the iTLC traces in Figure 4.5. Figure 4.5d shows the trace obtained from the control experiment, here most of the activity has moved with the solvent front, demonstrating the required separation. This trace shows some residual activity at the baseline, which refers to insoluble colloidal [^{68}Ga]Ga(OH)₃ that forms when gallium is in aqueous solution. This peak coincides with the product peak which resides on the baseline, however since most of the unchelated gallium moves this technique still provides an acceptable method for calculating RCY. RCY values were calculated from Figure 4.5a-c, with the maximum values reported in Table 4.2. The three compounds show maximum RCYs of over 97%, which is acceptable for imaging. Since all the tracers exhibit an overall positive charge and will

Table 4.2: Maximum RCYs obtained for the three [^{68}Ga]Ga-DO3A-xy-TAP compounds, as shown by the iTLC traces in Figure 4.5a-c.

[^{68}Ga]Ga-DO3A-xy-TAP	TPP	TTP	TXP
RCY / %	98	100	97

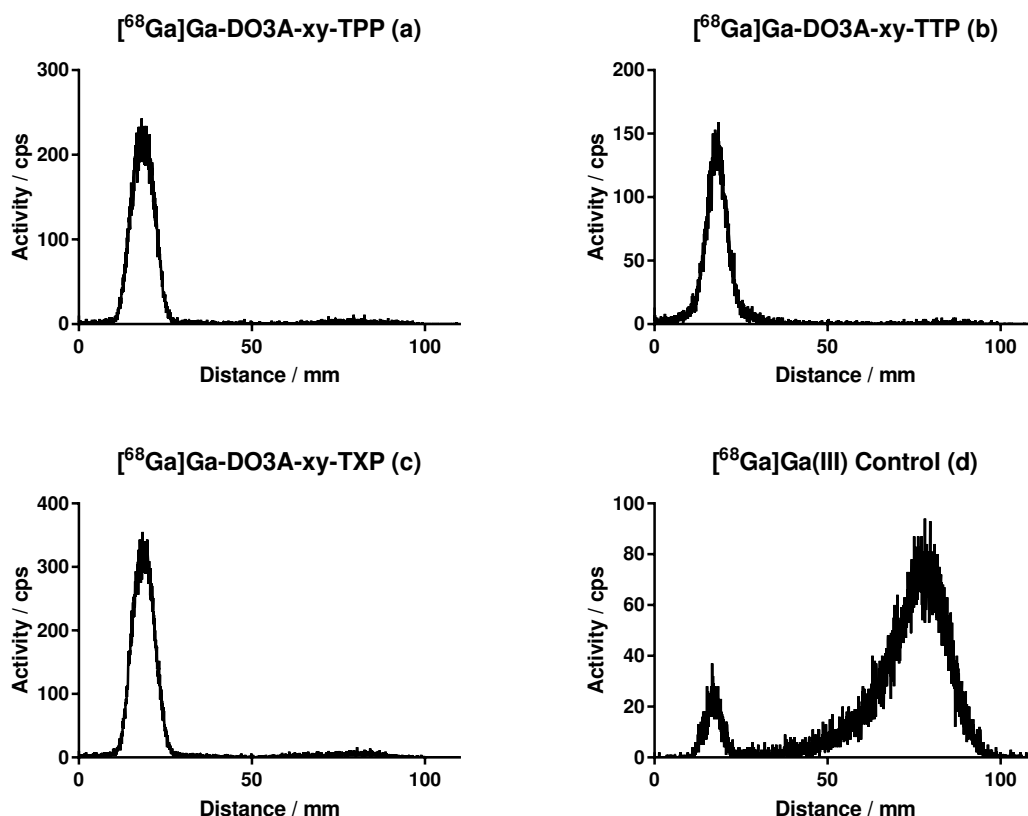


Figure 4.5: iTLC traces of the $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TAP}$ compounds (a-c) compared to a control experiment of unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ (d). A mobile phase of 0.1 M disodium EDTA was used, and the plates were scanned using the method outlined in 4.3b using a beta detector.

likely adhere to the baseline, the work done devising this mobile phase will be applicable for QC throughout this project.

4.1.3 Log D Determination

As an index for the lipophilicity of the radiotracers, octanol/phosphate-buffered saline (PBS) distribution coefficients or log D values were determined. This characteristic is calculated by taking a sample containing a 1:1 mixture of *n*-octanol and PBS, which is chosen to mimic the physiological pH of 7.4. Aliquots of the radiotracer are added to these mixtures, before they are mixed thoroughly to allow the radiotracer to become distributed according to its solubility, with more lipophilic radiotracers favouring the octanol layer. After centrifugation to separate the two immiscible layers, samples of identical volume were taken from each layer, and the activity of each sample was measured. Log D values were calculated according to the following equation:

$$\text{Log } D_{7.4} = \log_{10} \left(\frac{A_{\text{octanol}}}{A_{\text{PBS}}} \right) \quad (4.1)$$

Whilst $\log D$ is defined as a concentration ratio, equation 4.1 can be derived since samples of equal volume are taken, thus the volume terms cancel each other out. In order to obtain an accurate value for the $\log D$ value of a compound, six replicate values were obtained, and the $\log D$ is reported as the mean $\pm$ standard deviation (SD). Since lipophilic compounds are preferentially dissolved in the non-polar octanol layer, compounds with higher lipophilicity report higher $\log D$ values.

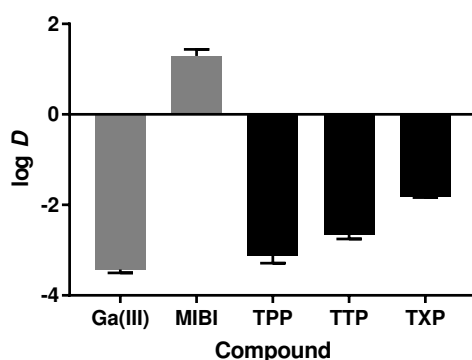


Figure 4.6: Graph showing the $\log D$ values of the $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TAP}$ compounds (black), compared to the control experiments of unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ and the literature value for MIBI as obtained by Kim *et al.*⁵ (grey). Data are mean $\pm$ SD ($n = 6$ for all compounds except MIBI, $n = 3$ for MIBI).

The $\log D$ values for the $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TAP}$ compounds are shown in Figure 4.6, alongside control values for unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ and a literature $\log D$ value for the clinically used lipophilic cation MIBI as obtained by Kim *et al.*⁵ Unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ shows a $\log D$ value of -3.43 ± 0.07 , which indicates that it shows a slight propensity, no matter how small, to dissolve in octanol. MIBI has a $\log D$ value of 1.29 ± 0.15 , which acts as a positive control and a target for the gallium-68 labelled compounds to reach. The $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TAP}$ compounds show $\log D$ values of -3.12 ± 0.07 , -2.66 ± 0.09 and -1.81 ± 0.02 for the TPP, TTP and TXP-functionalised compounds respectively. These compounds do show a trend in which the $\log D$ value increases with an increase in the degree of alkyl functionalisation, which is a promising result for further compounds which utilise this motif; however the $\log D$ values for these compounds are low, especially when compared to the gold standard MIBI. This is a likely result of the unbound carboxylate arm in these compounds (Figure 3.12) which

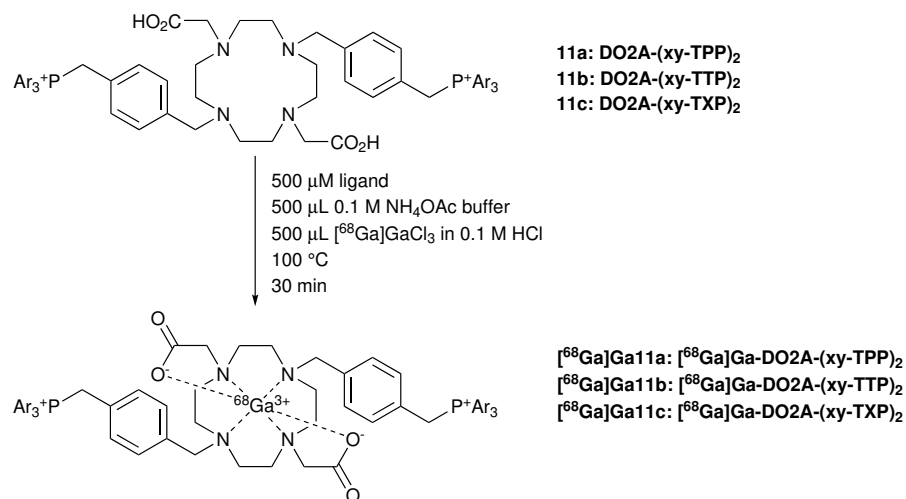
serves to increase the hydrophilicity of the tracers.

4.2 [^{68}Ga]Ga-DO2A-(xy-TAP) $_2$

The next set of compounds to be assessed was the DO2A-(xy-TAP) $_2$ series, as they do not suffer from the unbound carboxylate arm present in the DO3A-based analogues. In addition, it was interesting to see whether increasing the overall charge to +3 increases the hydrophilic interactions to the point where the lipophilicity gains due to loss of the unbound carboxylate arm are mitigated. DO2A-(xy-TPP) $_2$ was radiolabelled with copper-64 by Kim *et al.*, which provided a basis for the radiolabelling of these compounds with gallium-68.⁶ As of the time of writing, there is only one paper in the literature regarding the radiolabelling of DO2A-based chelators with gallium-68,⁷ so a novel radiolabelling method was devised within this project.

4.2.1 Proof of Concept Experiments

4.2.1.1 Initial Radiolabelling Experiments



Scheme 4.2: Protocol for the radiolabelling of DO2A-(xy-TAP) $_2$ compounds with generator-produced [^{68}Ga]GaCl $_3$, adapted from the protocol shown in Scheme 4.1.

As a starting point for the radiolabelling of the DO2A-based compounds, it was decided to use the 0.1 M NH_4OAc radiolabelling buffer employed successfully for the DO3A-based compounds and in line with the copper-64 chelation experiments performed by Kim *et al.*⁶ As shown in Scheme 4.2, the heating temperature and time were initially kept constant at 100 $^\circ\text{C}$

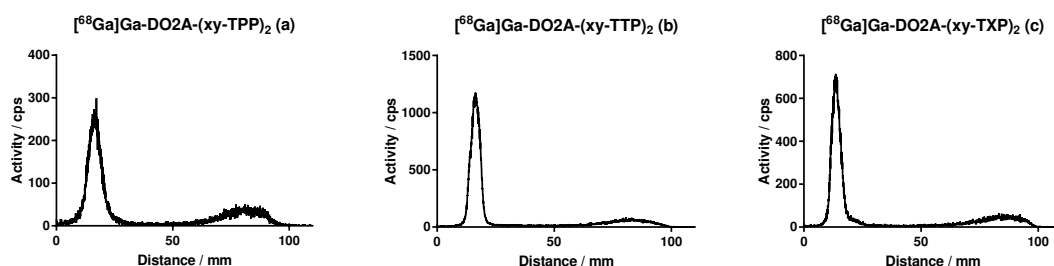


Figure 4.7: iTLC traces of the initial radiolabelling experiments of $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ in 0.1 M NH_4OAc .

Table 4.3: RCY values obtained from the iTLC traces shown in Figure 4.7.

$[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$	TPP	TTP	TXP
RCY / %	72	85	80

for 30 minutes to give the best possible chance of radiolabelling.

The initial test reaction was performed on $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$, which as can be seen from Table 4.3 and the iTLC trace shown in Figure 4.7a, gave a RCY of 72%. This reaction procedure gave similar results for the TTP- and TXP-functionalised variants as shown by Figure 4.7b and c. This shows that DO2A is a viable chelator for gallium-68, and that neither the addition of a second bulky TAP group nor the addition of extra methyl groups hinders radiolabelling; the latter observation in line with the RCY values observed for DO3A-xy-TAP discussed previously.

4.2.1.2 Initial Log D Estimation

For biological imaging studies, the use of radiotracers consisting of a single radiolabelled product with no unchelated radiometal is expected, as this limits the amount of activity non-specific to the radiotracer and thus to parts of the body not relevant to diagnosis. Despite the fact that quantitative RCY values were not initially obtained for the DO2A-(xy-TAP)_2 compounds, it was decided to calculate an estimate of their log D values as an estimate of their potential as mitochondrial imaging agents, and in order to determine whether it was worth pursuing the radiochemical analysis of these compounds further. The log D values were calculated using the conventional shake-flask method and Equation 4.1, although the activity in the octanol and PBS layers contributed by the residual unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ could theoretically be accounted for by using the log D value for $[^{68}\text{Ga}]\text{Ga(III)}$ shown in Figure 4.6.

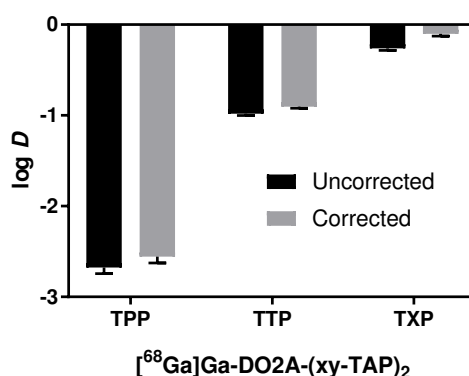


Figure 4.8: Estimates of the log D values for the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ compounds, using the impure compounds outlined in Figure 4.7 and Table 4.3. Log D values calculated from the raw data are in black, values corrected by the log D value of the unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ from Figure 4.6 are in grey. Data are mean $\pm$ SD ($n = 6$).

Both the uncorrected and corrected log D values for the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ compounds are shown in Figure 4.8. All three compounds show more favourable log D values than their DO3A-based analogues, showing that the removal of the unbound carboxylate arm and the addition of another lipophilic triarylphosphonium group were enough to overcome the increase in Born energy. Indeed, for the DO2A compounds, increases in the degree of alkyl functionalisation lead to more pronounced increases in log D values since double the amount of alkyl groups are being added, and the hydrophilicity provided by the unbound carboxylate arm does not have to be overcome. These proof of concept experiments show that DO2A-(xy-TAP) $_2$ ligands can be radiolabelled with gallium-68, and that they show more favourable log D values than their DO3A-based counterparts.

4.2.2 Optimisation of RCY Through Buffer Alteration

4.2.2.1 pH Assessment of Potential Radiolabelling Buffers

As discussed previously, for biological and clinical studies with radiometal-based radiotracers, the radiotracer should consist of a single species, with no free radioisotope in order to limit both the amount of non-specific imaging and the overall patient dose. To this end, it is prudent to increase the RCY obtained in the radiolabelling reaction. For radioisotopes with longer half-lives such as fluorine-18 and technetium-99m, semi-prep HPLC can be used to increase the radiochemical purity, however this comes at the cost of lowering the specific activity of

radiotracer obtained *i.e.* the amount of activity per unit mass of chelator, through decay of the radioisotope. Increased specific activity also lowers the amount of chelator that must be administered to the patient, lowering the likelihood of potential toxic side effects. The relatively short half-life of gallium-68 meant that in the first instance, RCY was attempted to be increased through the reaction instead of through chromatography.

The gallium-68 radiolabelling properties of DO3A-xy-TAP were not explored in great detail due to the prevalence of the ligand in the literature, however it is understood that its radiolabelling characteristics are highly sensitive to the pH of the reaction mixture. This is because in order to achieve favourable interactions between the radiometal and the ligand, the amine and carboxylate groups must be deprotonated. A variety of radiolabelling buffers have been used in radiometal chelation experiments, from the 0.1 M NaOAc buffer used by Kim *et al.* to the 0.2 M solutions of NH₄OAc and NaOAc used by Tsionou *et al.*^{4,6} As a starting point for the optimisation of the radiolabelling procedures for the DO2A-based ligands, varying concentrations of both NH₄OAc and NaOAc were mixed with equal volumes of 0.1 M hydrochloric acid in order to simulate the reaction mixture used in the radiolabelling experiments, and the pHs measured. These data are summarised in Figure 4.9 and Table 4.4.

At low and high acetate concentrations, there is little variation between the pH values of the two buffers, however when both the acid and the acetate are at 0.1 M concentration, there

Table 4.4: Summary of the data shown in Figure 4.9.

Concentration / M	0.05	0.1	0.2
NH ₄ OAc	1.67	3.34	4.93
NaOAc	1.59	2.17	4.53

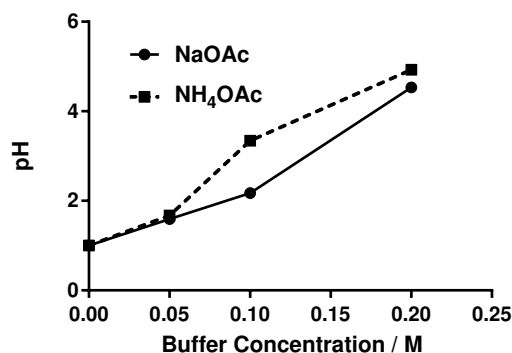


Figure 4.9: pH values of the mixtures obtained by mixing equal volumes of 0.1 M HCl and the stated acetate solution. The pH values at 0 M buffer concentration refers to that of 0.1 M HCl.

is high variability between the pH values of the two resulting solutions. Performing radiolabelling experiments using all six of the buffers shown in Table 4.4 allows for the elucidation of the effect of both the pH and the acetate counterion on the RCY, as well as the optimum radiolabelling conditions for later experiments. To ensure that these experiments are accurate, the ligand concentration used in all experiments was 500 μM in line with the maximum concentration used by Tsionou *et al.* in their radiolabelling experiments. This corresponded to approximately 300 μg of ligand, with the actual value depending on the molecular weight of the ligand used.⁴ It is assumed that the presence of the ligand in the radiolabelling mixtures will not affect the overall pH too dramatically, since a 500 μM ligand concentration is four orders of magnitude lower than that of the acetate. All reactions were heated at 100 $^{\circ}\text{C}$ for 30 minutes, before the RCY was calculated using iTLC.

4.2.2.2 Effect of Radiolabelling Buffer on RCY

Data from these experiments are summarised in Figure 4.10. Both Figures 4.10a and 4.10b show the same data, but visualised using two different characteristics. Figure 4.10a, which plots the RCYs against the concentration of acetate buffers, shows that 0.2 M acetate concentration leads to quantitative RCY values for both ammonium and sodium acetate. As the acetate concentration is lowered to 0.1 M, there is a significant difference between the RCY values obtained in ammonium and sodium acetate buffer, with the sodium counterion lead-

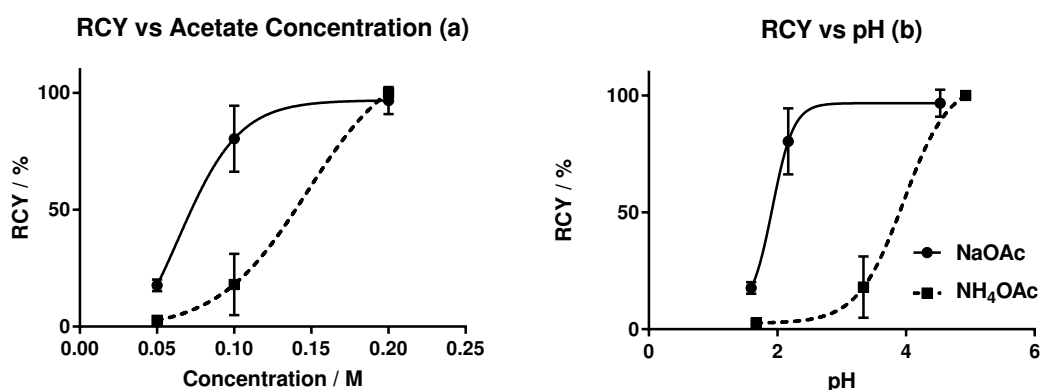


Figure 4.10: RCY values for the acetate buffer alteration experiments for the optimisation of DO2A-(xy-TPP)₂ radiolabelling. RCY is compared to the acetate buffer concentration (a) and to the pH of the resulting solution as shown in Table 4.4 (b). RCYs calculated by iTLC. Data are mean $\pm$ SD ($n = 3$) and fitted using a sigmoidal curve.

ing to a higher RCY. Figure 4.10b plots the RCY values against the pH of the radiolabelling mixture as shown in Table 4.4. This graph shows that the RCY does not solely depend on the pH of the radiolabelling mixture, since 0.1 M NaOAc gives a higher RCY than 0.1 M NH₄OAc despite its lower pH, which shows that the radiolabelling process is counterion dependant. It is postulated that the protons in the ammonium cation serve to protonate the ligand lone pairs and inhibit radiolabelling. Whilst there is some variability in the RCYs obtained for 0.2 M NaOAc compared to 0.2 M NH₄OAc, it was still chosen as the preferential radiolabelling buffer because it is less sensitive to changes in buffer concentration and pH.

4.2.3 Application of 0.2 M NaOAc Buffer to DO2A-(xy-TAP)₂ Compounds

4.2.3.1 Effect of Buffer on RCY Across the DO2A-(xy-TAP)₂ Series

Experiments performed on the DO3A-xy-TAP series showed that the addition of alkyl functionalisation does not negatively impact the RCY values obtained, and this was expected to continue for the DO2A-(xy-TAP)₂ series. The three DO2A-based compounds were radiolabelled in 0.2 M NaOAc at 100 °C for 30 minutes, before QC was performed using the iTLC traces shown in Figure 4.11a-c. The TPP and TXP variants were found to radiolabel in quantitative yield, whilst the TTP variant gave a 98% RCY. In order to determine that the strong baseline activity peak was not due to the more basic pH of the radiolabelling mixture forming large amounts of colloidal [⁶⁸Ga]Ga(OH)₃, a control reaction was performed in an identical reaction mixture to that used in the other experiments, although without the presence of a chelator. The iTLC trace obtained from this process is shown in Figure 4.11d; this shows that a large amount of unchelated [⁶⁸Ga]Ga(III) remains in the solution, and that the baseline peaks in Figure 4.11a-c do in fact refer to radiolabelled product.

4.2.3.2 Log *D* Determination from the 0.2 M NaOAc-Buffered Reactions

Having established that the use of 0.2 M NaOAc gives high RCY values for the [⁶⁸Ga]Ga-DO2A-(xy-TAP)₂ compounds, the log *D* values were recalculated and are shown in Figure 4.12. The [⁶⁸Ga]Ga-DO2A-(xy-TAP)₂ compounds show log *D* values of -2.59 ± 0.03 , -1.23 ± 0.02 and -0.28 ± 0.01 for the TPP, TTP and TXP variants respectively. These values are in line with the values estimated in Figure 4.8, and indicate that [⁶⁸Ga]Ga-DO2A-(xy-TXP)₂ shows more

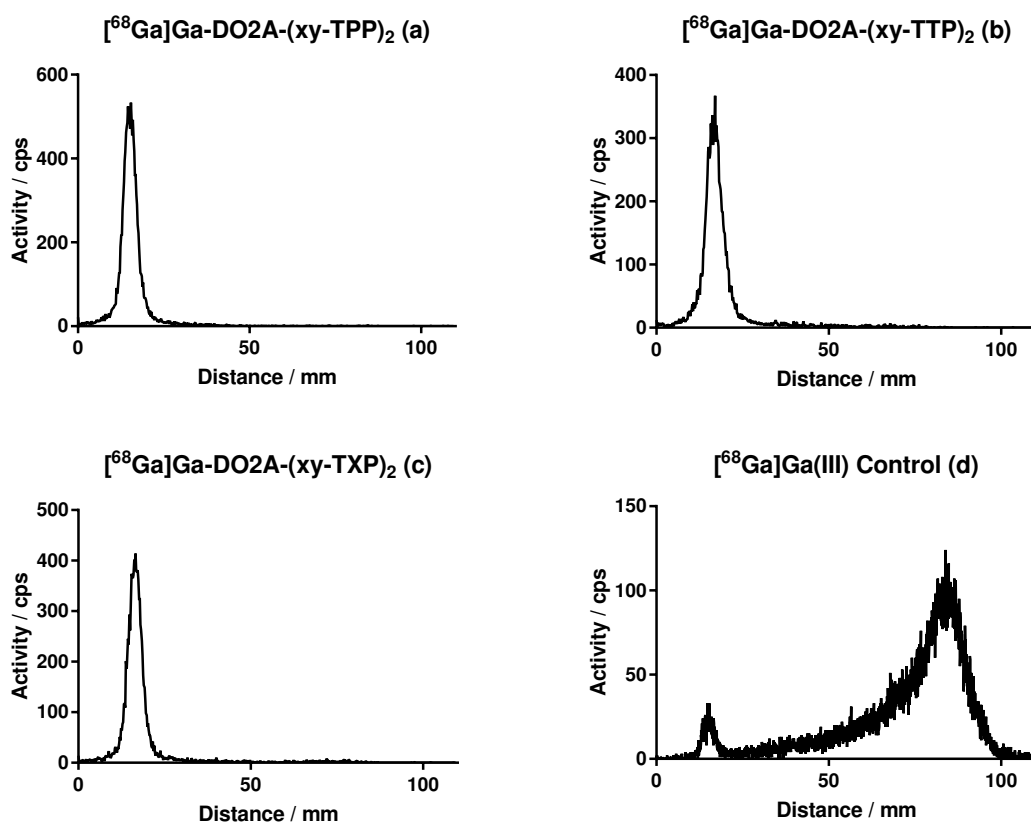


Figure 4.11: iTLC traces of the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ series of compounds having been labelled with a 0.2 M NaOAc buffer (a-c), and a control experiment of unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ having been heated in a 0.2 M NaOAc solution in an analogous manner to the radiolabelling procedure (d).

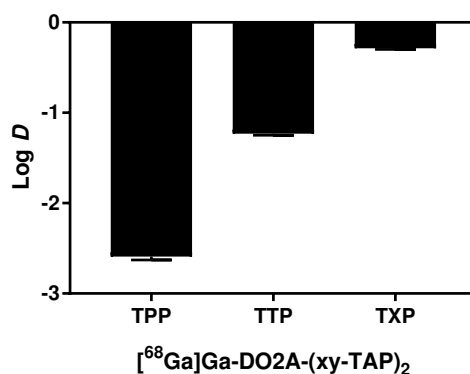


Figure 4.12: Log D values for the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ compounds as shown in Figure 4.11a-c.

potential than its DO3A-based analogue ($\log D = -1.81$) as a mitochondrial imaging agent, however there is still improvement to be made before the tracers are as lipophilic as MIBI ($\log D = 1.29$).⁵

4.2.3.3 RadioHPLC Analysis of the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ Series

The next step was to perform radioHPLC analysis of the three $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ compounds, in order to assert that there is only one radiolabelled species formed in each reaction mixture, as was seen for the $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TAP}$ compounds in Figure 4.2. A more polar HPLC gradient was used in order to ensure that these more lipophilic radiotracers would elute from the column in good time, which increased to 100% MeCN after 25 minutes rather than 80% and remained at 100% MeCN for the final five minutes of the run. The requirement to ensure that all radiolabelled species eluted from the column also meant, however, that changing the radiolabelling gradient prevented direct radioHPLC comparison between the two sets

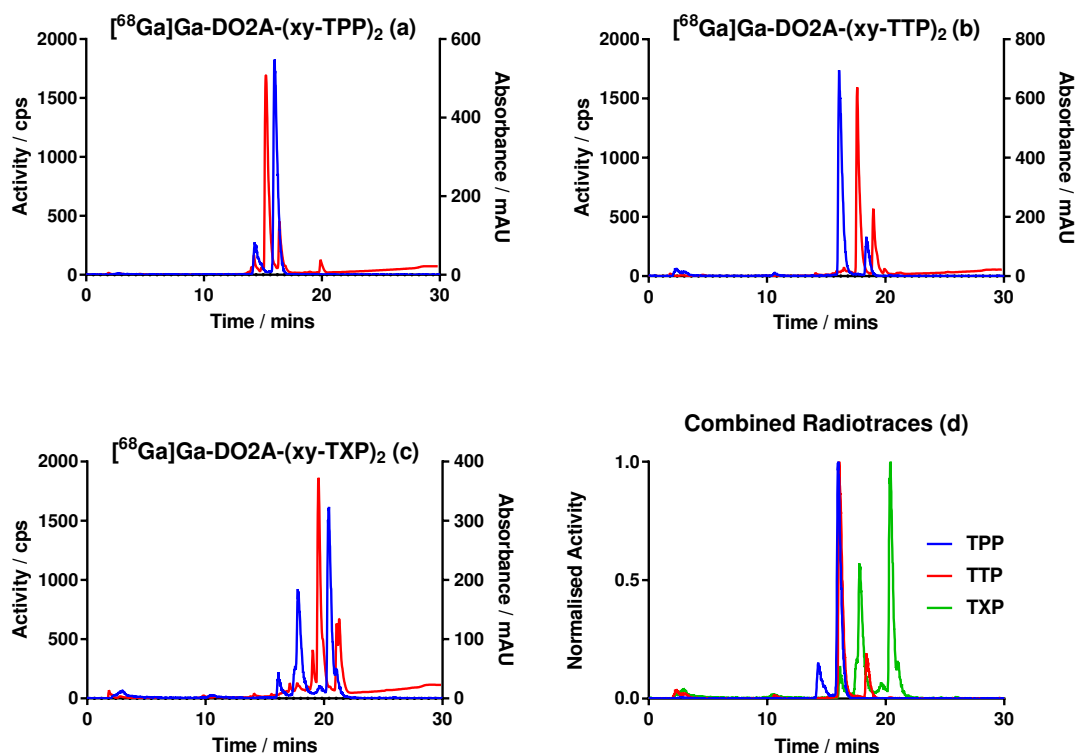


Figure 4.13: RadioHPLC traces of the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ compounds. Eluent gradient: 100% A for 5 min; 0–100% B in A for 20 min; isocratic 100% B for 5 min at a flow rate of 1 mL min^{-1} . **a-c:** Radioactivity shown in blue, UV absorption ($\lambda_{\text{abs}} = 250 \text{ nm}$) shown in red. **d:** Overlaid radiotracers of the three compounds. Activity normalised with respect to the highest value in each trace.

of compounds, and causes a seven minute downtime between runs whilst the column reequilibrates.

Unfortunately, as shown by the radioHPLC traces in Figure 4.13a-c, there are multiple radiolabelled species in each reaction mixture. For the TPP and TTP variants, there is one main peak and a second smaller peak nearby. The normalised and combined radiotracers in Figure 4.13d show another surprising result; despite having differing $\log D$ values as shown by Figure 4.12, the two main peaks co-elute. This result is reproducible even after extensively washing the HPLC syringe between runs. The trace for the TXP variant (Figure 4.13c) is particularly egregious, with at least three radiolabelled species visible.

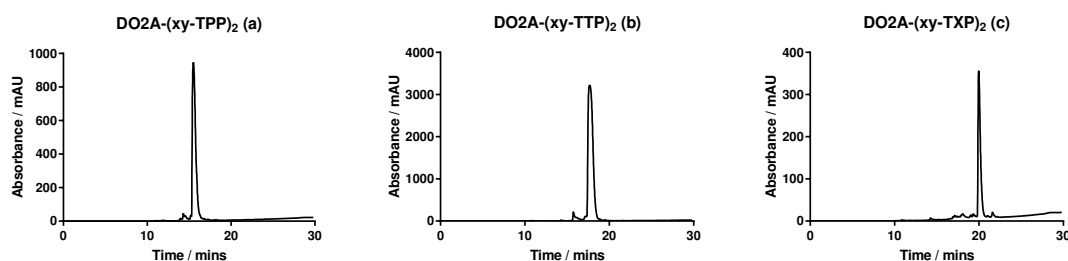


Figure 4.14: HPLC traces of the ligands post-filtration. Gradient and UV absorption as in Figure 4.13.

Initially, it was thought that the ligand solution was impure, which was implied by the formation of insoluble precipitate over time despite the fact that the solution was stored at 5 °C. As such, the precipitated impurities were removed by filtration, and the product was lyophilised in order to prevent further decomposition in solution; stock solutions from this point on were made up on the day of radiolabelling. In order to ensure that these products were indeed pure, the ligands were reassessed by HPLC; the traces are shown in Figure 4.14. Each product exists as one major product, a result which is corroborated by the ^1H and $^{31}\text{P}\{^1\text{H}\}$ NMR spectra of DO2A-(xy-TPP)_2 shown in Figure 4.15. This was thought to rule out impurity as a reason for the multiple radiolabelled species shown in Figure 4.13.

4.2.4 Analysis of Radiolabelling and Speciation in $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$

Having decided on the use of 0.2 M NaOAc as a radiolabelling buffer, the other three factors which could be altered in the reaction were ligand concentration, temperature and time. The next step was to alter these parameters in order to determine how they affect the RCY of the re-

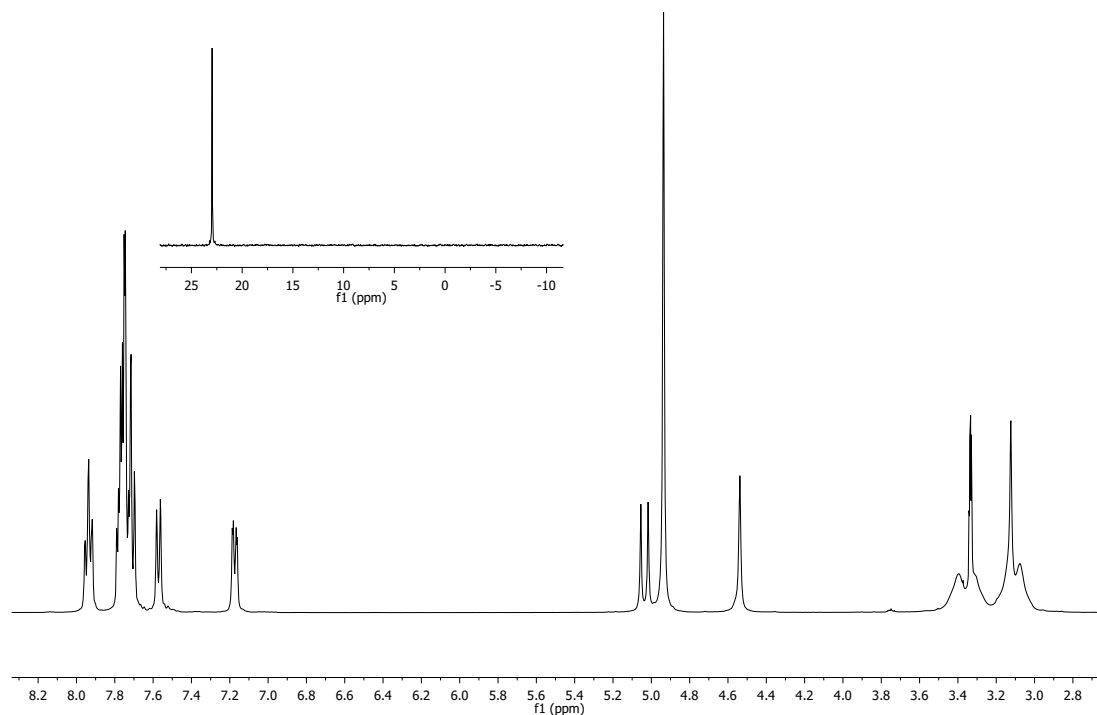
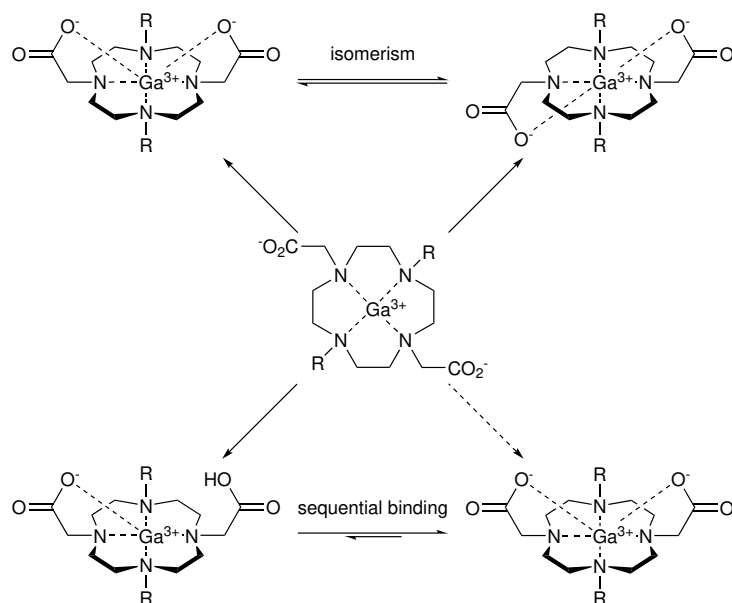


Figure 4.15: ^1H NMR spectrum of the DO2A-(xy-TPP)_2 post-filtration and lyophilisation (400 MHz, 298 K, MeOD). Inset: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of the same compound (162 MHz, 298 K, MeOD).

action, as well as the speciation in the reaction mixture, two possible explanations for which are outlined in Scheme 4.3. Firstly, there exists the possibility of *cis/trans* isomerism in the complex depending on which face of the macrocycle the two arms bind on. It is thought that these two isomers would be interconvertible as well as existing as a pair of kinetic/thermodynamic products. Secondly, it is thought that the binding of the two carboxylate arms could occur sequentially. Whilst it is plausible that this process would be reversible, it is likely that the second binding event acts as a thermodynamic sink for the reaction. In order to probe these hypotheses further, iTLC and HPLC experiments were performed using DO2A-(xy-TPP)_2 as a test case, since it has the simplest chemical structure and was the simplest to synthesise. As these experiments were preliminary, no repeats were performed, however in the future in order to fully quantify the Ga(III) chelation of DO2A repeat measurements for these values and others in this chapter should be obtained.



Scheme 4.3: Schematic proposing two explanations for the speciation seen in Ga-DO2A complexes such as those in Figure 4.13. Either the complex formation involves the binding of the two carboxylate arms in *cis/trans* conformations, or the sequential binding of the carboxylate arms to the metal centre. R = non-chelating functionalisation.

4.2.4.1 Ligand Concentration Alteration Experiments

The first parameter to be studied was the ligand concentrations. In their radiolabelling experiments, Tsionou *et al.* used ligand concentrations of 500, 50, 5, 0.5 and 0.05 μM , which seemed like a sensible range to study initially.⁴ It should be noted that the paper is unclear on whether this ligand concentration refers to the concentration of the overall reaction mixture or the concentration before the generator eluate is added, which in effect halves the stated ligand concentration. In this study, the latter set-up was used, as this would provide a more exacting assessment of the chelating capabilities of the ligand. Parity was maintained with previous DO2A radiolabelling experiments by heating the reaction mixture for 30 minutes at 100 °C. The data is summarised in Figure 4.16.

As shown in Figure 4.16a and summarised in Figure 4.16c, only a ligand concentration of 500 μM gave any radiolabelling, albeit in quantitative RCY. The other concentrations tested, 50, 5 and 0.5 μM , all gave very low RCY values. In order to more accurately assess this process, four concentrations between 500 and 50 μM , namely 400, 300, 200 and 100 μM , were used. In this case, only 100 μM did not display a quantitative RCY, instead showing 63% RCY. It seems from these experiments, a 200 μM ligand concentration can be used rather than the

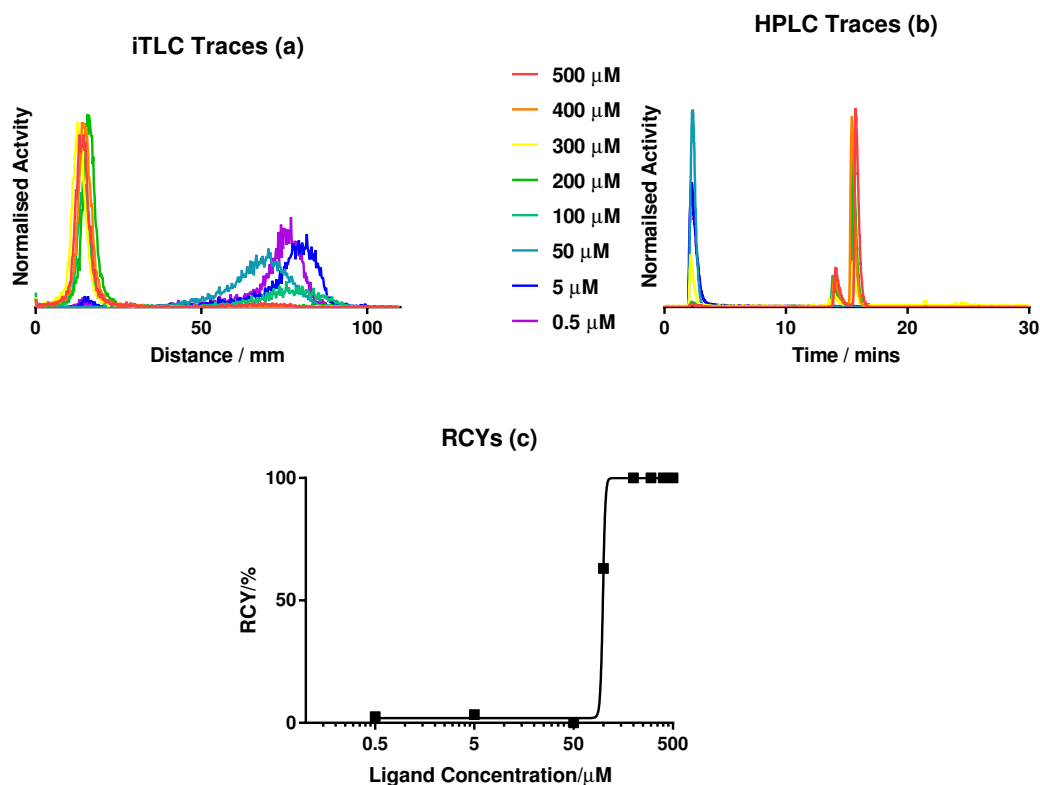


Figure 4.16: **a:** iTLC traces and **b:** radioHPLC traces of the concentration alteration experiments. Gradient as described in Figure 4.13. **c:** Summary of the RCY values from the iTLC traces shown in Figure 4.16a. Data are fitted using a sigmoidal curve.

500 μM concentration normally used, which potentially increases the number of radiolabelling experiments which can be performed with a batch of ligand by 250%.

The second part of these experiments was to determine whether the alteration of the radiolabelling parameters would lead to a change in the speciation of the radiotracers. As shown by the HPLC traces in Figure 4.16b, once the ligand concentration is high enough to allow for radiolabelling, a similar radiotrace is observed in all cases, with two species formed at all concentrations. As such, whilst these experiments have allowed for the reduction in the amounts of ligand needed for radiolabelling, they do not allow for the isolation of a single species.

4.2.4.2 Varying Reaction Times at 100 °C

As discussed previously, the speciation is potentially a result of kinetic and thermodynamic products, arising from either formation of interconvertible isomers or an activation energy barrier to the binding of the second carboxylate arm to the metal centre. In both cases, one

species forms at a faster rate due to a lower activation energy, whilst the other product is more stable. In order to probe whether this happens to the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$, the effect of reaction time on the RCY and speciation was explored. Once again, parity was maintained by previous radiolabelling experiments by using a $500\ \mu\text{M}$ ligand concentration and a $100\ ^\circ\text{C}$ reaction mixture temperature. Data from these experiments are shown in Figure 4.17.

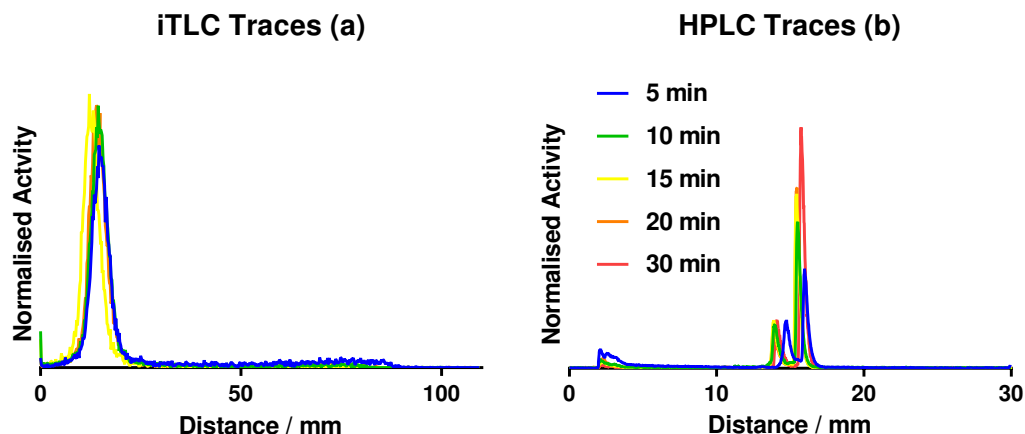


Figure 4.17: a: iTLC traces and b: radioHPLC traces of the time-varied radiolabelling experiments at $100\ ^\circ\text{C}$. Gradient as described in Figure 4.13.

The iTLC traces in Figure 4.17a show that even after 5 minutes, an 86% RCY is observed, and after 10 minutes the RCY is quantitative. The HPLC traces appear to show a change in the isomer profile as the length of heating time is increased. This data is quantified in Figure 4.18, which shows how the ratio of the integrals of the two peaks change with time and the differences in the integral of the unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ peak. Figure 4.18 shows that as the

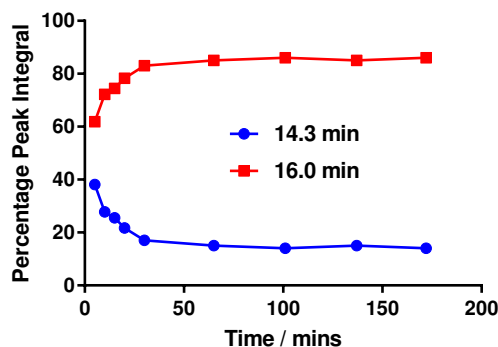


Figure 4.18: Summary of the HPLC data for the $100\ ^\circ\text{C}$ heating experiments, some of which is shown in Figure 4.17b, showing the ratio of the integrals of the main two isomer peaks.

heating time increases from 5 to 30 minutes, the 16.0 min peak grows larger, whilst the 14.3 min peak gets smaller. This appears to indicate that the 16.0 min peak refers to the thermodynamic product as it takes longer to form, with the 14.3 min peak being the kinetic product. After 60 minutes however, the speciation remains unchanged no matter how long heating is performed for. This leads to an approximately 85% abundance of the thermodynamic product, and seems to indicate that longer heating times can cause one of the two isomers to become more prevalent.

4.2.4.3 Performing Radiolabelling at Room Temperature

Studying the radiolabelling procedures at room temperature had two main purposes; firstly in order to determine whether the reaction would proceed at all at room temperature. Room temperature radiolabelling has myriad advantages, since it does not require heating equipment in order to perform the radiolabelling and the reaction mixture does not need to cool down before it can be administered to the patient in a clinical setting. Secondly, by using a lower temperature it was thought that the interconversion of any isomerism would be slowed down, which would allow for the confirmation of the theories proposed in the 100 °C experiments. In order to maintain parity with the previous radiolabelling experiments, a 500 μM ligand concentration was used in each experiment, and since heating was not a factor in these experiments care had to be taken to perform the iTLC and HPLC analyses as close to the stated times as possible. Data from these experiments are shown in Figure 4.19.

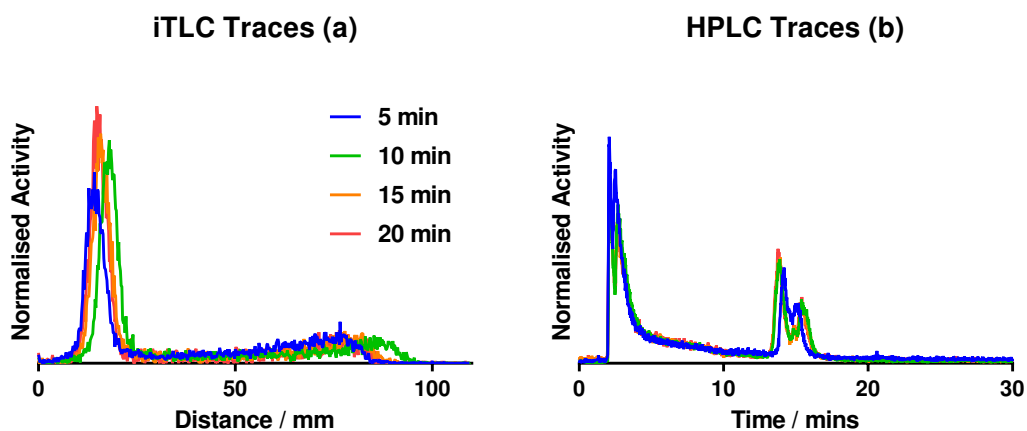


Figure 4.19: a: iTLC traces and b: radioHPLC traces of the room temperature radiolabelling experiments. Gradient as described in Figure 4.13.

The iTLC traces in Figure 4.19a all show similar profiles, with a 70% RCY achieved after 5 minutes, but only reaching approximately 85% RCY after 20 minutes. Unfortunately this result suggests that room temperature radiolabelling is out of the question for the DO2A-based compounds. The HPLC traces in Figure 4.19b confirm that radiolabelling here is less effective than at 100 °C, due to the large activity peak at 2 minutes referring to unchelated [^{68}Ga]Ga(III). For the two peaks, the 14.3 min peak is more intense, corroborating the conclusions drawn from Figure 4.17b. The 14.3 min peak, being the kinetic product, is formed more readily at room temperature due to the lower activation energy of its formation. Nevertheless, the purpose of these experiments was to determine whether slowing down the complexation kinetics would result in the formation of a single species, which is not the case.

4.2.4.4 Varying Reaction Temperatures

Having performed experiments at 100 °C and at room temperature, the next series of experiments to perform was to vary the reaction temperature between these two points. This would allow for a more granular exploration of the factors explored previously, both in order to perform the radiolabelling at as low a temperature as possible and to explore the kinetics of speciation. In order to maintain parity with previous radiolabelling experiments, 500 μM of ligand was used in all experiments, and the iTLC and HPLC analyses were performed as close to 30 minutes after the start of the reaction as practicable.

The iTLC data from these experiments is shown in Figure 4.20a, and is summarised in

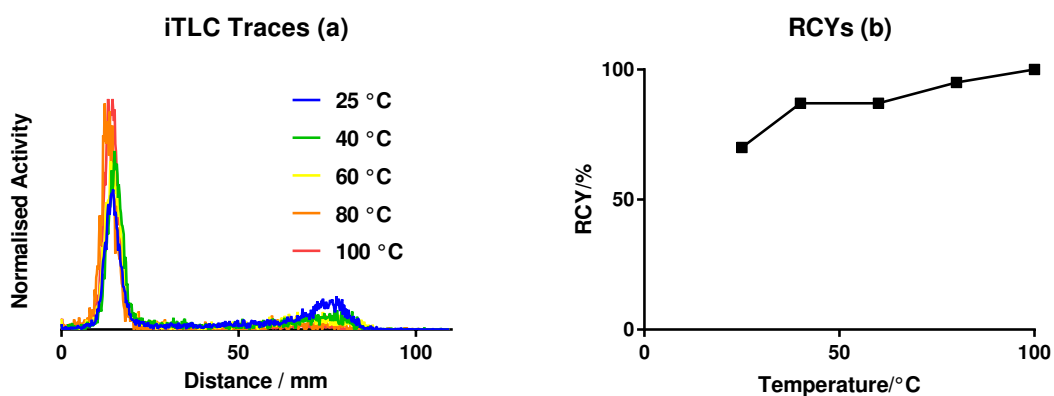


Figure 4.20: a: iTLC traces of the temperature alteration experiments. b: Summary of the RCY values calculated from Figure 4.20a.

Figure 4.20b. At room temperature, a 70% RCY is observed after 30 minutes, which serves to highlight the variability of RCY values when compared to the values shown in Figure 4.19a. There is then an increase in RCY as the radiolabelling temperature increases, ending with the quantitative RCY observed at 100 °C. This means that 100 °C is still the optimal temperature for radiolabelling due to the fact that it gives quantitative RCY values, despite requiring cooling to room temperature before imaging can be performed with the radiotracer. The HPLC traces from these experiments are shown in Figure 4.21, which show how the speciation changes with temperature. At room temperature, the 14.3 min peak is more intense as shown in Figure 4.19b, whilst as the temperature increases the 16.0 min peak begins to increase in intensity. This data is quantified in Figure 4.21b, which shows that the 16.0 min peak generally grows more intense at higher temperature. These observations fit with the kinetic/thermodynamic product formation theory proposed previously.

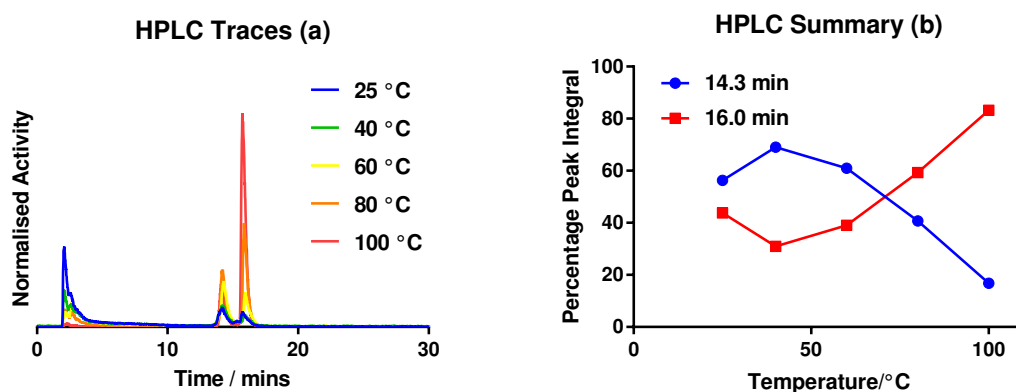


Figure 4.21: **a:** HPLC traces of the temperature alteration experiments. Gradient as described in Figure 4.13. **b:** Integrals of the two peaks in each trace in Figure 4.21a, expressed as a percentage of the total area underneath the two peaks. It can be seen that the use of a higher reaction temperature favours formation of the species represented by the 16.0 min peak.

4.2.4.5 Long-Term Stability of Speciation at Room Temperature

It has so far been established that $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ exists as two different species which form a kinetic/thermodynamic product pair. In order to probe the reversibility of the complex formation and thus the stability of the thermodynamic product, an experiment was designed in which a reaction mixture at equilibrium formed after 30 minutes at 100 °C was allowed to stand at room temperature, with HPLC measurements taken at several timepoints.

It was hypothesised that if the energetic barrier to interconversion was low, there would be an increase in the integral of the HPLC peak at 14.3 min with time.

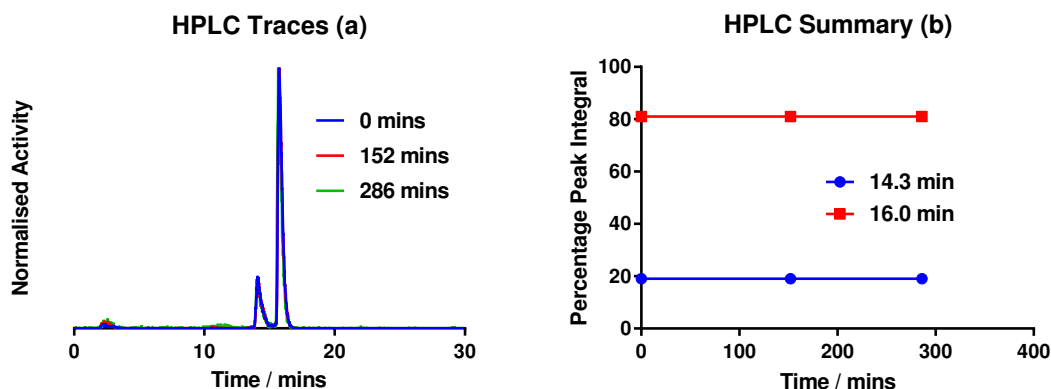


Figure 4.22: a: HPLC traces obtained from allowing a sample of $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ to stand at room temperature. The integrals of the two peaks at the three timepoints are summarised in b. Gradient as described in Figure 4.13.

HPLC data from these experiments are shown in Figure 4.22a and summarised in Figure 4.22b. It is fairly obvious that there is no change over time at room temperature, with an 19:81 peak integral ratio observed after both 152 minutes and 286 minutes. This seems to indicate that whilst the 16.0 min peak is the thermodynamic product of the reaction, the activation energy barrier for conversion between the kinetic and thermodynamic products of the reaction must be very high to forbid alteration of the speciation profile at room temperature. It is also interesting that even at 100 °C, full conversion to one species is not obtained, indicating that there is likely some equilibrium between the two species at higher temperatures.

4.2.4.6 Varying the Sodium Acetate Buffer Concentration

As carboxylate groups can only bind to metal centres when they are deprotonated, it follows that the pH of the reaction mixture will change the way the DO2A chelator will bind to Ga(III). This is especially worth studying as one of the speciation regimes being considered is that of successive binding events, which will likely not proceed to full hexadentate coordination if the second carboxylate group is protonated. To this end, it was decided to repeat the pH alteration experiments discussed previously in terms of RCY using different concentrations of sodium acetate buffer, but this time see how this effects the speciation in the reaction mixture by HPLC. The HPLC data is shown in Figure 4.23a and summarised in Figure 4.23b.

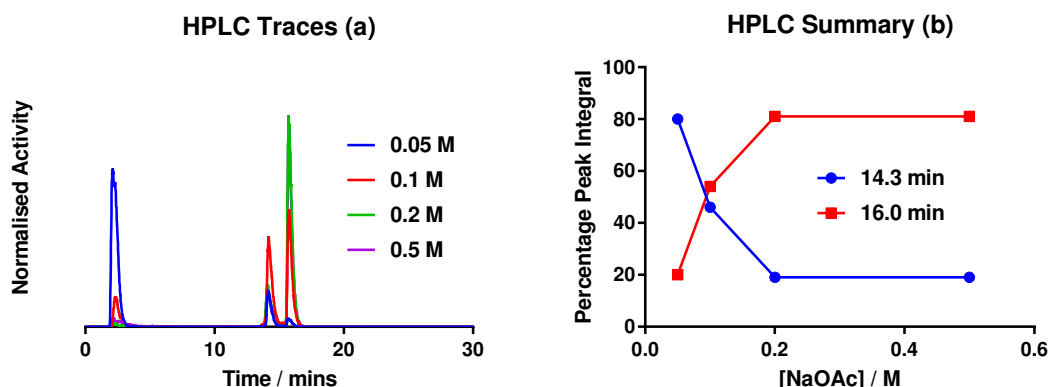


Figure 4.23: a: HPLC traces from the sodium acetate buffer concentration alteration experiments. b: Summary of the data from Figure 4.23a. Gradient as described in Figure 4.13.

The RCY results shown in Figure 4.10 are corroborated here, as can be seen by the decrease in the $[^{68}\text{Ga}]\text{Ga}(\text{III})$ peak at 2 minutes with increasing sodium acetate concentration. It can also be seen that at low pH values, the 14.3 min peak dominates, whilst as the pH value increases the 16.0 min peak begins to dominate. This result seems to suggest that the sequential binding event pathway is most likely to be the case, as higher pH values cause the deprotonation of the second carboxylate arm and allow it to bind to the Ga(III) centre. As has been seen previously, binding of pendant carboxylate arms increases the hydrophobicity of the complex and thus their retention times by reverse phase HPLC. There is no change in isomer profile upon increasing the sodium acetate concentration above 0.2 M though, and as such 0.2 M sodium acetate was deemed optimal for future DO2A-(xy-TAP)₂ radiolabelling experiments.

4.2.5 Optimising Isomer Profiles of $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TTP/TXP)}_2$

4.2.5.1 HPLC Analysis

Having extensively studied the radiolabelling characteristics of $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$, the next step was to analyse the TTP- and TXP-functionalised variants. As discussed in the previous section, 100 °C heating and 0.2 M sodium acetate were used as they led to the most favourable RCY values and speciation profiles. HPLC measurements were taken from 30 minutes of heating onwards, in order to most effectively determine at which time point a stable speciation profile is obtained. This data is summarised in Figure 4.24. For the TTP-functionalised variant, shown in Figures 4.24 a and b, equilibrium seems to be obtained after 30 minutes, with

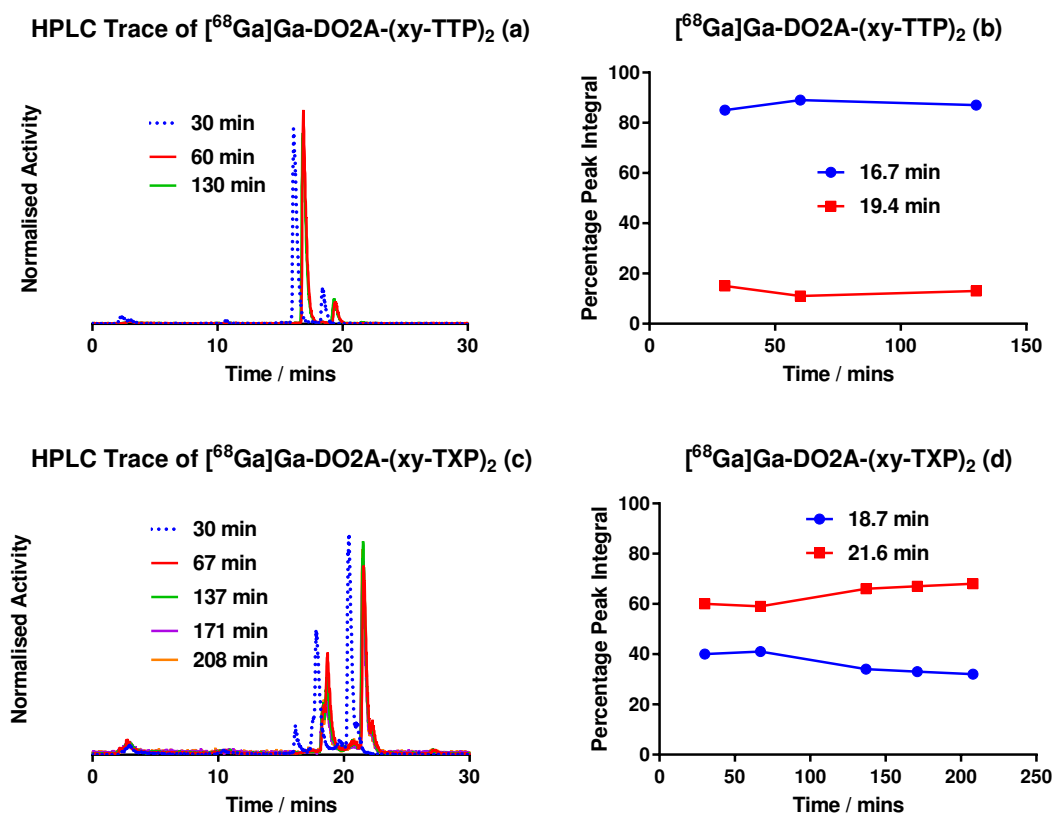


Figure 4.24: Summary of the HPLC isomer optimisation experiments on $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TTP)}_2$ and $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TXP)}_2$. **a and c:** HPLC traces of the TTP- and TXP- functionalised variants respectively. Gradient as described in Figure 4.13. **b and d:** Summary of the relative peak integrals from the HPLC traces for the TTP- and TXP-functionalised variants respectively.

the HPLC traces seeming to be functionally identical after this point. This result also seems to go against the conclusion found in the previous section, with the peak with the later retention time being less prevalent, indicating that speciation does not completely arise from successive binding events, as this would lead to the peak with the longer retention time increasing in intensity as the second binding event occurs with time. It should be noted, however, that once again there seems to be co-localisation issues between the traces for the 30 minute experiments, as these were taken from the historical experiments in Figure 4.13.

The TXP-functionalised tracers, shown in Figure 4.24c and d, show a different change in isomer profile over time. As the radiotracer is heated at 100 °C for 3.5 h, the 21.6 min HPLC peak appears to still be increasing in size, indicating that equilibrium between the two species has not been reached, as in Figures 4.18 and 4.24b. This is in line with the theory that the two peaks refer to kinetic and thermodynamic products, as the 21.6 min peak that refers to the

thermodynamic product keeps increasing in size with prolonged heating. Unfortunately, the 3.5 h of heating required to affect this slight favouring of one species is impractical, especially given the short 68 minute half-life of gallium-68.

4.2.5.2 Log D Analysis of [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$ with Prolonged Heating

Due to their different retention times on the reverse-phase HPLC column, the two peaks of [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$ can be considered to have different lipophilicities, and thus different log D values. For the TPP and TTP variants, this is not especially important as equilibrium in isomer profile and thus overall log D value is obtained after 30 minutes, however it can be inferred that the overall log D value of the TXP-functionalised variant will change over prolonged heating as the more lipophilic thermodynamic product is formed. It should be noted, however, that the actual compounds themselves are not changing to become more lipophilic; instead that a change in log D value merely reflects the prevalence of one more lipophilic isomer over the other. To probe this, a standard DO2A-(xy-TXP) $_2$ labelling reaction was set up, with aliquots of the reaction mixture taken at 5 minutes and 120 minutes and the log D values calculated. Data for these experiments are summarised in Figure 4.25.

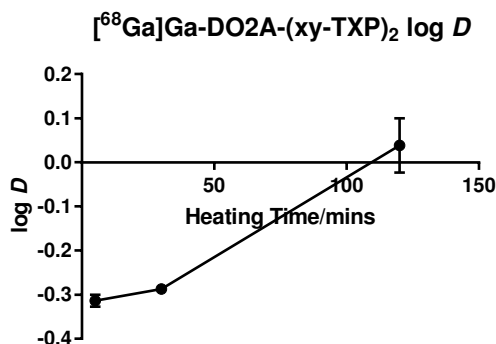
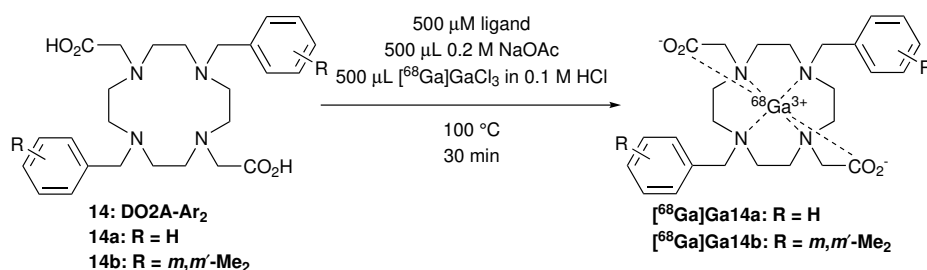


Figure 4.25: Variation in the log D value of the [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$ radiolabelling mixture with time heated at 100 °C. Data are mean $\pm$ SD ($n = 6$).

From 5 to 30 minutes, the log D values show a slight but significant increase from -0.31 ± 0.01 to -0.29 ± 0.01 . As heating is performed for 120 minutes however, there is a dramatic increase to 0.03 ± 0.06 , which is the first positive log D value observed for a gallium-68 labelled compound in this project. As throughout this project, the log D values were measured on samples of the radiolabelling reaction mixture, which will contain both of the isomers. This means that the increase in observed log D value refers mainly due to the prevalence of the more

lipophilic thermodynamic isomer in the reaction mixture, rather than any specific change in structure increasing the lipophilicity of the product. Another observation from this experiment is that of the two isomers, one must have $\log D$ value less than -0.29 whilst the other must have a $\log D$ value greater than 0.03. Whilst it was not possible in these experiments to truly isolate these isomers and calculate their individual $\log D$ values, there is still enough evidence to suggest that these compounds are more lipophilic than their DO3A-based counterparts.

4.3 [^{68}Ga]Ga-DO2A-Ar₂



Scheme 4.4: Protocol for the radiolabelling of DO2A-Ar₂ chelators DO2A-Bn₂ (**14a**) and DO2A-Xy₂ (**14b**).

The first question intended to be answered through the gallium-68 labelling of the DO2A-Ar₂ chelators DO2A-Bn₂ (**14a**) and DO2A-Xy₂ (**14b**) was whether the lower overall cationic charge of the complex would decrease the Born interactions and the hydrophilicity when compared to analogous TAP-functionalised chelators. Secondly, it was hypothesised that the smaller aryl groups would allow for more facile radiolabelling due to their lower steric bulk when compared to TAP functionalisation, and secondly whether this lowered steric bulk would facilitate the formation of a single radiolabelled species. It was thought that most of the work done so far on DO2A-based chelators would apply here, and as such radiolabelling was generally performed in 0.2 M sodium acetate buffer, as outlined in Scheme 4.4.

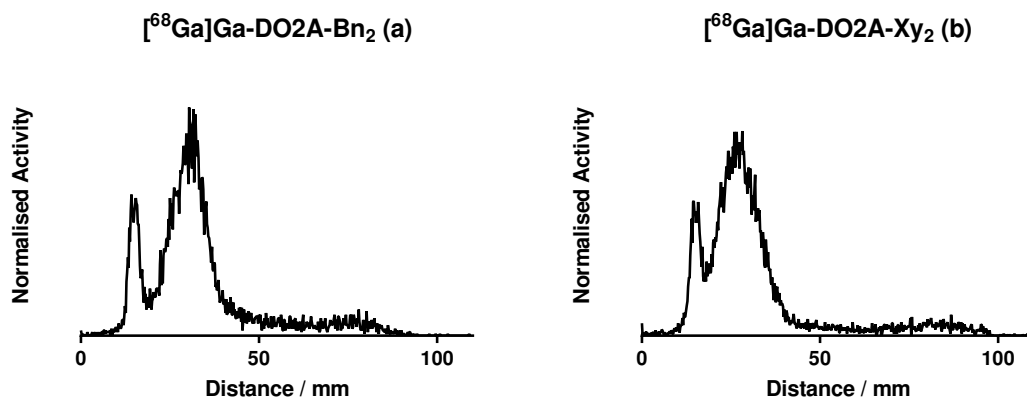
4.3.1 Initial Radiolabelling Experiments

4.3.1.1 Initial iTLC Measurements

The initial radiolabelling experiments were performed using a ligand concentration of 500 μM in 0.2 M NaOAc buffer, with heating for 30 minutes at 100 °C. iTLC traces using a mobile phase

Table 4.5: Summary of the integrals of the iTLC traces shown in Figure 4.26.

Species	RCY at R _f value / %		
	0	0.27	1
[⁶⁸ Ga]Ga-DO2A-Bn ₂	17	75	7
[⁶⁸ Ga]Ga-DO2A-Xy ₂	15	79	6

**Figure 4.26:** iTLC traces obtained for [⁶⁸Ga]Ga-DO2A-Ar₂ radiolabelling experiments at 500 μM ligand concentration.

of 0.1 M disodium EDTA of both [⁶⁸Ga]Ga-DO2A-Bn₂ and [⁶⁸Ga]Ga-DO2A-Xy₂ are shown in Figures 4.26a and 4.26b respectively. Unlike in previous iTLC measurements using an EDTA mobile phase in which the product remained on the baseline whilst unchelated [⁶⁸Ga]Ga(III) moved with the solvent front, in these iTLC traces there is a third peak with a retention factor (R_f) value of 0.27. The integrals of the three peaks is summarised in Table 4.5.

It is likely that the activity at the solvent front still refers to unchelated [⁶⁸Ga]Ga(III), and as such the overall RCY values can be calculated as 93% and 94% for [⁶⁸Ga]Ga14a and [⁶⁸Ga]Ga14b respectively. The proposed lipophilicity of the compounds might explain why the radiolabelled compounds move from the baseline, as they exhibit a lower degree of interaction with the polar stationary phase. The peak for the activity that remains on the baseline seems to be too intense to solely consist of colloidal [⁶⁸Ga]Ga(OH)₃ when compared to the control experiment in Figure 4.11d. As such, it seems that the two iTLC peaks likely refer to speciation in the reaction mixture in line in the TAP-functionalised variants discussed previously, with the more lipophilic species being not as adherent to the stationary phase. The fact that speciation in [⁶⁸Ga]Ga-DO2A-Ar₂ complexes is already exhibited, however, does confirm that the use of less sterically bulky functional groups has not prevented speciation in the reaction mixture.

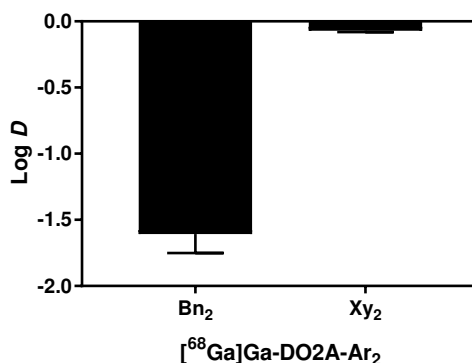
4.3.1.2 Log *D* Determination

Figure 4.27: Log *D* values of [⁶⁸Ga]Ga-DO2A-Bn₂ and [⁶⁸Ga]Ga-DO2A-Xy₂. Data are mean ± SD (n = 6).

Despite the unexpected iTLC traces obtained for [⁶⁸Ga]Ga-DO2A-Ar₂, it was decided to obtain a log *D* value for the reaction mixture in order to determine whether further study of these complexes is warranted, in line with the experiments shown in Figure 4.25. As seen in Figure 4.27, [⁶⁸Ga]Ga-DO2A-Bn₂ shows a log *D* value of -1.61 ± 0.14 (n = 6), whilst [⁶⁸Ga]Ga-DO2A-Xy₂ shows a log *D* value of -0.07 ± 0.01 (n = 6).

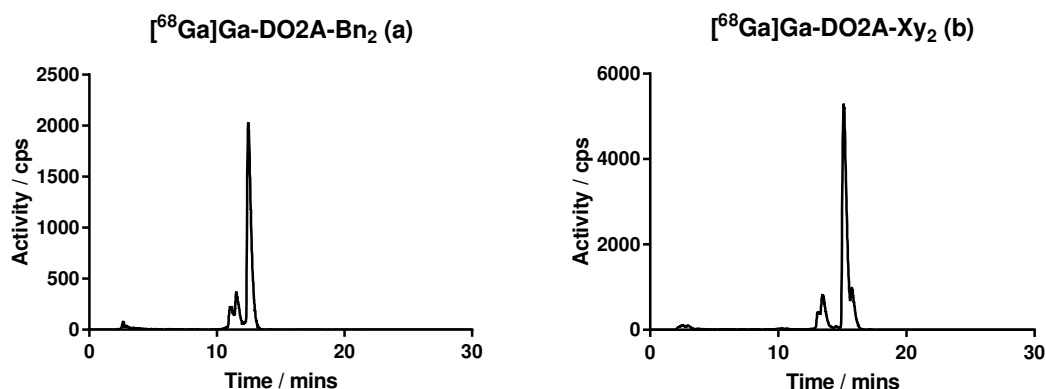
When considering complexes formed after 30 minutes of heating at 100 °C, these complexes show a marked improvement in log *D* values when compared to their [⁶⁸Ga]Ga-DO2A-(xy-TAP)₂ counterparts, with the phenyl variant showing an improvement of an order of magnitude. The improvement is less dramatic in the xylyl-functionalised compounds, which is likely a result of the fact that DO2A-(xy-TXP)₂ has 12 methyl groups contributing to improve the lipophilicity of the complex whilst DO2A-Xy₂ only has 4. Nevertheless, these results show the effects that lowering the overall cationic charge of the complexes has on their hydrophilic interactions and thus their lipophilicities. The improved log *D* of these complexes, coupled with their smaller sizes, could potentially lead to improved cell uptake, and thus these chelators are worthy of further study.

4.3.1.3 Initial HPLC Analyses

Whilst the iTLC traces in Figure 4.26 hint to the degree of speciation in the complexes, radioHPLC is considered to provide a more accurate assessment of speciation since it has increased

Table 4.6: Comparison of the relative integrals of the peaks in the HPLC and iTLC traces of $[^{68}\text{Ga}]\text{Ga-DO2A-Bn}_2$ and $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$, shown in Figures 4.26 and 4.28.

Species	Peak Ratio	
	HPLC	iTLC
$[^{68}\text{Ga}]\text{Ga-DO2A-Bn}_2$	16:84	18:82
$[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$	17:83	16:84

**Figure 4.28:** RadioHPLC traces of the $[^{68}\text{Ga}]\text{Ga-DO2A-Ar}_2$ compounds as prepared for Figure 4.26. Gradient as described as Figure 4.13.

peak resolution and integration. It will also be interesting to discover whether the integration of the peaks by iTLC will correlate to the integration of the HPLC peaks. The radioHPLC peaks for $[^{68}\text{Ga}]\text{Ga14a}$ and $[^{68}\text{Ga}]\text{Ga14b}$ are shown in Figures 4.28a and 4.28b respectively. As expected, $[^{68}\text{Ga}]\text{Ga14b}$ has a longer retention time than $[^{68}\text{Ga}]\text{Ga14a}$ by virtue of its higher degree of lipophilicity, whilst each chromatograph has a similar two peak structure.

In order to determine whether the multiple peaks observed in the iTLC spectra correlate to the speciation observed by HPLC, the ratios of the integrals of the peaks to each other were calculated and are reported in Table 4.6. These results show an acceptable correlation between the integrals of the iTLC and HPLC traces, which also implies that the peak on the baseline in fact refers to a $[^{68}\text{Ga}]\text{Ga-DO2A-Ar}_2$ species and not colloidal $[^{68}\text{Ga}]\text{Ga}(\text{OH})_3$. Whilst it is likely that iTLC measurements could be used as a shortcut to the analysis of speciation in this case, the increased resolution afforded by radioHPLC makes it a more accurate technique. It is interesting that the speciation ratio for both compounds is similar, implying that the increased degree of functionalisation does not provide a steric favourability for one species.

4.3.2 Analysis of Radiolabelled Product Speciation

In a similar vein to the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ compounds, it was decided to analyse the radiolabelling characteristics of $[^{68}\text{Ga}]\text{Ga-DO2A-Ar}_2$ in greater detail to characterise the radiolabelling kinetics and speciation. Unlike in the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ experiments, it was decided to focus on $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$, since it will be used in biological experiments due to its more favourable lipophilicity. It was also decided to focus three experiments which yielded the most striking results from the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ experiments: namely ligand concentration, reaction temperature and reaction time.

4.3.2.1 Concentration Alteration Experiments

In order to gain a view into the potential optimisation of the radiolabelling of $[^{68}\text{Ga}]\text{Ga-DO2A-Ar}_2$ compounds, experiments were performed using differing concentrations of ligand. Concentrations were chosen in line with the analogous experiments performed on $[^{68}\text{Ga}]\text{Ga-}$

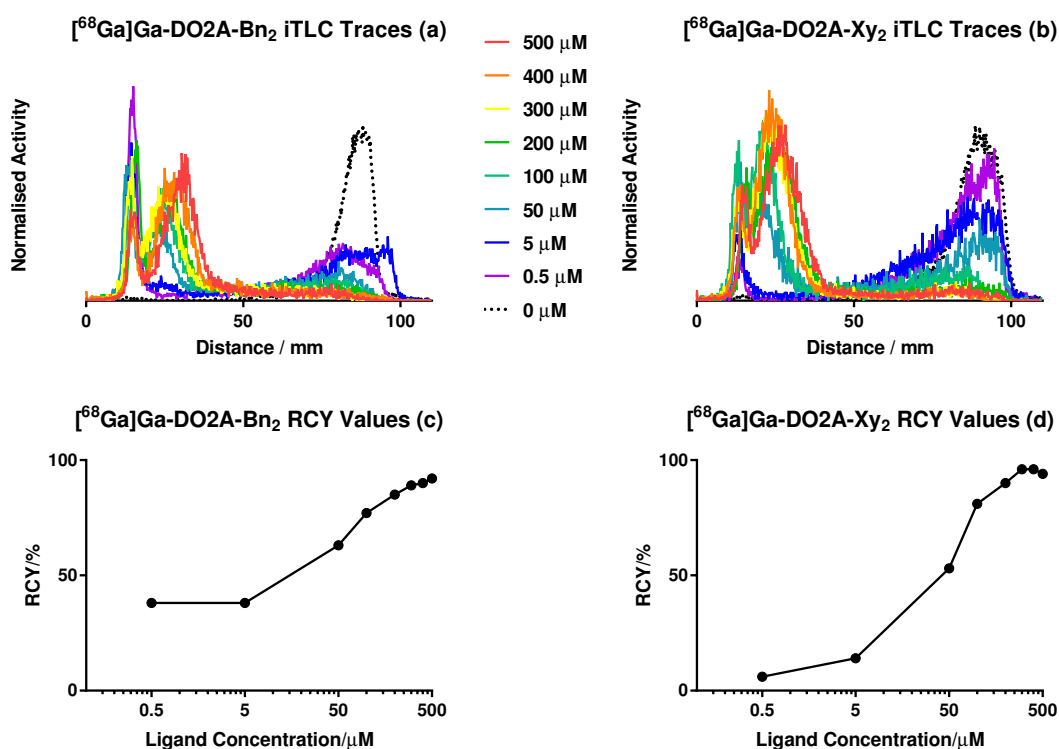


Figure 4.29: a and b: iTLC traces of the concentration alteration experiments performed on $[^{68}\text{Ga}]\text{Ga14a}$ and $[^{68}\text{Ga}]\text{Ga14b}$ respectively. c and d: Summary of the RCY values calculated from the iTLC traces.

DO2A-(xy-TPP)₂, with reaction conditions of 0.2 M NaOAc buffer and 30 minutes of heating at 100 °C. iTLC traces of these reactions, as well as their summaries are shown in Figure 4.29.

[⁶⁸Ga]Ga-DO2A-Xy₂ gives perhaps the more conventional result, with the RCY summary data (Figure 4.29d) fitting a sigmoidal curve. The decrease in RCY with concentration is far less abrupt than is observed for [⁶⁸Ga]Ga-DO2A-(xy-TPP)₂ in Figure 4.16c, with 81% RCY observed at 100 μM ligand, and 53% observed at 50 μM, compared to the 0% radiolabelling observed for [⁶⁸Ga]Ga-DO2A-(xy-TPP)₂. The result for [⁶⁸Ga]Ga-DO2A-Bn₂ is far more unexpected; the decrease in RCY at higher ligand concentrations is slower, with a 63% RCY observed at 50 μM ligand concentration, however the more surprising result is that 38% RCY is observed at concentrations as low as 0.5 μM. Whilst this result will require repeats to establish its true nature, it highlights that these chelators may show preferential radiolabelling kinetics compared to the TAP-functionalised variants.

A useful aspect of the fact that the three species are observable by iTLC is that the speciation profile can be calculated from iTLC measurements. Relative peak integrals for the iTLC traces in 4.29a and b are shown in Figure 4.30. In both cases, the amount of uncoordinated [⁶⁸Ga]Ga(III) in each case decreases as expected with ligand concentration. For **14b** (Figure 4.30b), the activity on the baseline seems to be stable at around 15%, with the activity in the middle peak increases with ligand concentration to a maximum of 80%. These results seem to imply that the 80:15 ratio between the two peaks is formed at equilibrium. This equilibrium is more difficult to see for **14a** (Figure 4.30b), as it seems that a stable equilibrium has not

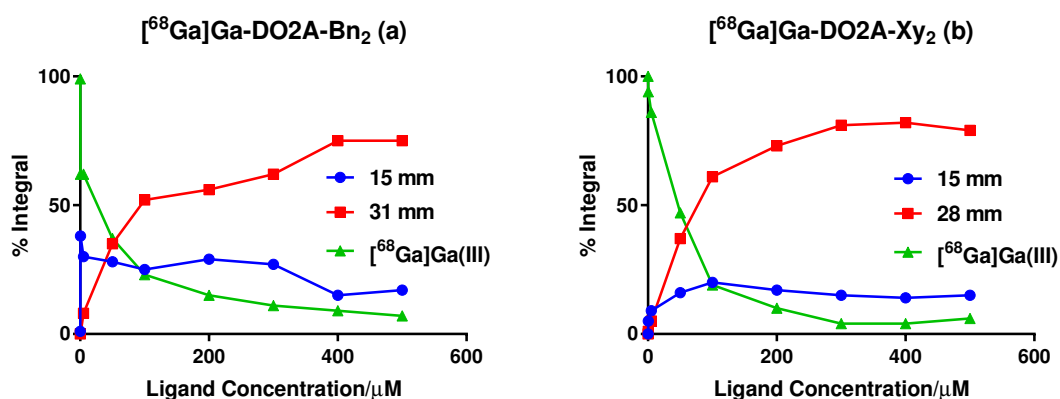


Figure 4.30: Integrals of the peaks in the iTLC traces in Figure 4.29a and b, expressed as a percentage of the total area under the trace.

yet been reached even at 500 μM ligand concentration. At 500 μM however, a 75:15 ratio between the two peaks is formed, a similar result to **14b**, implying that this is an equilibrium position for this reaction, although repeats are needed to smooth out the curve and allow for the corroboration of this trend.

4.3.2.2 Reaction Temperature Alteration Experiments

The next experiments to be performed were on the radiolabelling of $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ at different temperatures in order to determine how it effects the RCY and speciation of the reaction. Reactions were performed in 0.2 M NaOAc for 30 minutes. iTLC traces from these reactions are shown in Figure 4.31a, and the RCY values are shown in Figure 4.31b. There seems to be a linear relationship between reaction temperature and the RCY observed, which is in contrast to the results for $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ shown in Figure 4.20b in which over 80% RCY was observed at all temperatures used. In addition, this result contradicts the concentration alteration experiments, which indicated that DO2A-Xy₂ shows superior radiolabelling kinetics.

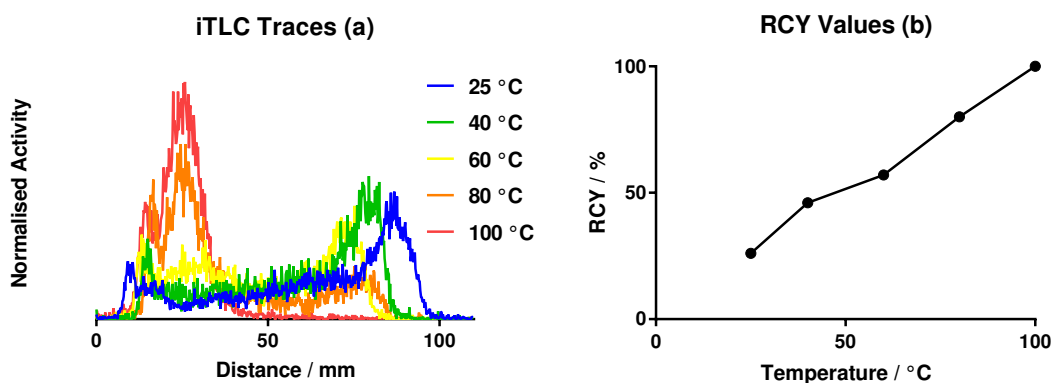


Figure 4.31: **a:** iTLC traces obtained from the radiolabelling of $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ at different temperatures. **b:** Summary of the RCY values of the iTLC traces in Figure 4.31a, expressed in terms of the integrals of the peaks at 15 and 28 mm.

HPLC traces were also obtained of these reaction mixtures; the traces are shown in Figure 4.32a and are summarised in 4.32b. One interesting aspect of these traces is that the peak at 2 minutes which normally refers to unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ has split into two separate peaks. It is unlikely that this refers to a product bound to a lipophilic chelator as these complexes show much longer retention times by HPLC. One possibility is that the two peaks refer to

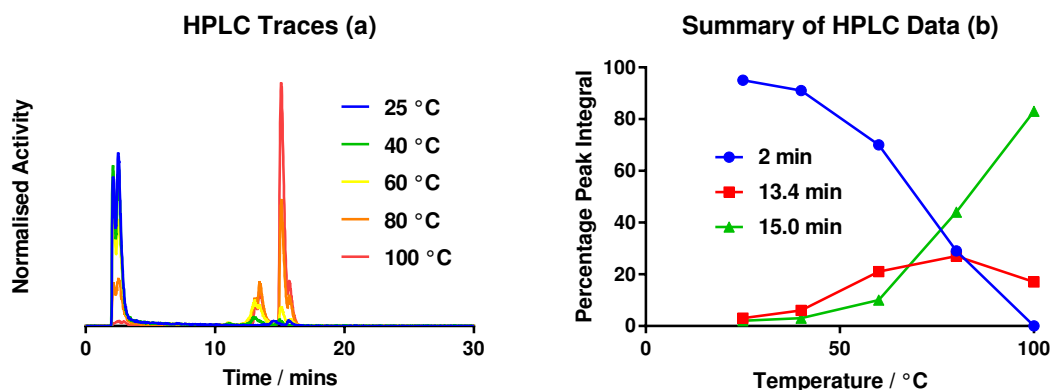


Figure 4.32: **a:** HPLC traces obtained from the radiolabelling of $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ at different temperatures. Gradient as described as Figure 4.13. **b:** Summary of the HPLC data shown in Figure 4.32a, expressed in terms of the relative integrals of the three peaks.

$[^{68}\text{Ga}]\text{Ga(III)}$ centres with different counteranions such as chloride or acetate, but then it would be strange that this is the first time this effect has been manifested.

As shown by the summary in Figure 4.32b, the integral of the peak at 2 minutes decreases in a linear fashion with time, reflective of the iTLC data shown previously (Figure 4.31). At lower temperatures, the species at 13.4 minutes is more dominant than the species at 15.0 minutes, before its prevalence in the HPLC remains constant at higher temperatures. This indicates that the species at 13.4 minutes is likely the kinetic product. On the other hand, at higher temperatures the product at 15.0 minutes starts to be formed in greater concentration, implying that it is the thermodynamic product of the reaction. Nevertheless, as with the TPP-functionalised chelators discussed previously, it was not possible to completely favour one species through alteration of the reaction temperature.

4.3.2.3 Reaction Time Alteration Experiments

The final radiolabelling experiment performed on $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ was altering the amount of time the reaction was performed for, both to get an insight into speciation kinetics and into whether this reaction could be optimised for a biological setting. Each reaction was performed in 0.2 M NaOAc at 100 °C. iTLC traces from these experiments are shown in Figure 4.33a, and the RCY values are shown in Figure 4.33b. Once again, these results indicate that $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ shows poorer radiolabelling kinetics than $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$, which showed near-quantitative RCY values after 5 minutes of reaction. Nevertheless,

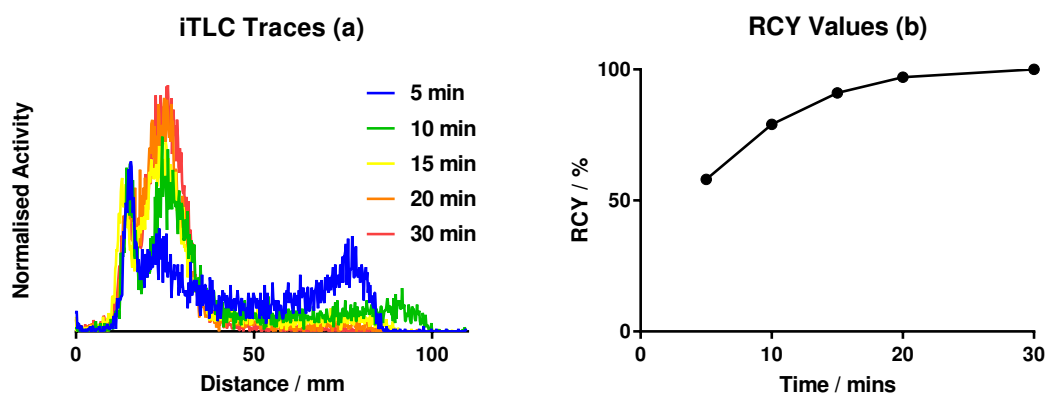


Figure 4.33: **a:** iTLC traces obtained from the radiolabelling of $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ for different lengths of time. **b:** Summary of the RCY values of the traces in Figure 4.33a, expressed in terms of the integrals of the peaks at 15 and 28 mm.

there is scope to optimise this reaction since 97% RCY is obtained after 20 minutes.

The HPLC traces of these reactions are shown in 4.34a and are summarised in Figure 4.34b. Once again, the peak at 2 minutes is split into two overlapping peaks. In line with the RCY data in 4.33b, the amount of unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ decreases with increasing reaction time. In addition, the size of the 13.4 min peak seems to remain constant at around 20% after 10 minutes, whilst the integral of the 15.0 min peak seems to increase in line with the decrease in $[^{68}\text{Ga}]\text{Ga(III)}$. These observations are in line with the formation of a kinetic/thermodynamic species pair, with the 13.4 minute peak forming at a faster initial rate and the 15.0 minute product forming with increased reaction time.

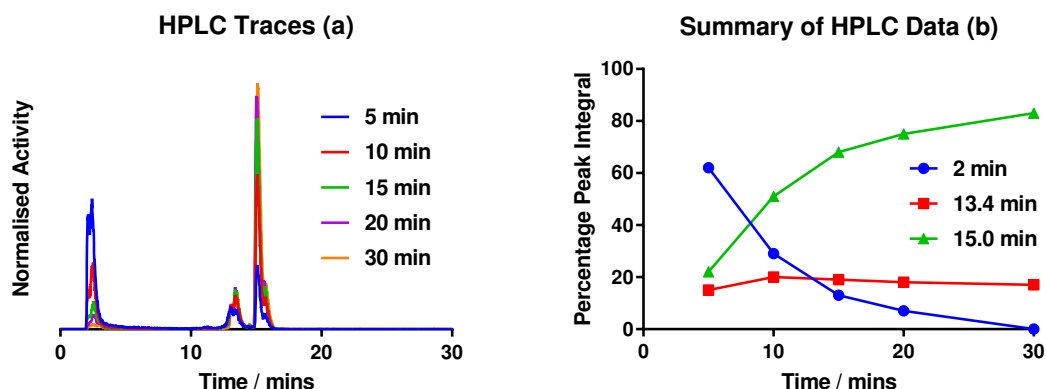
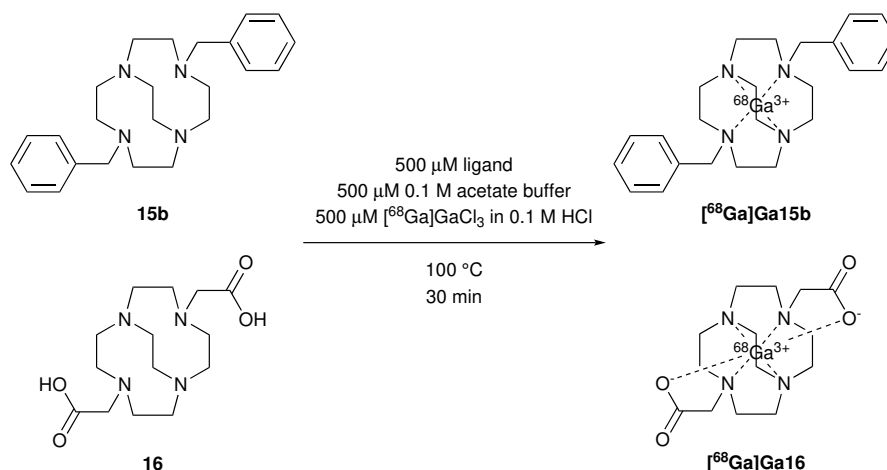


Figure 4.34: **a:** HPLC traces obtained from the radiolabelling of $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ for different lengths of time. Gradient as described as Figure 4.13. **b:** Summary of the HPLC data shown in Figure 4.34a, expressed in terms of the relative integrals of the three peaks.

As with the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TXP)}_2$ compounds discussed previously, it has not been found possible to only form one species of $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ through the alteration of reaction conditions. It was, however, found that the formation of a more lipophilic species could be favoured through the use of thermodynamically forcing conditions. Due to its comparable $\log D$ value and smaller molecular size, $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ was carried forward to biological evaluation.

4.4 Bridged Cyclens

There is no literature precedent on the gallium-68 radiolabelling of bridged cyclens, and as such differing radiolabelling buffers were explored in order to initially determine whether radiolabelling was possible, before they could be optimised later. In accordance with the initial work performed on $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ in section 4.2.1.1 and as shown in Scheme 4.5, 0.1 M NH_4 and 0.1 M NaOAc were used, as they highlight the effects of differing pHs and counter-cations and pHs on the RCY. Due to the preliminary nature of these experiments, relatively harsh radiolabelling conditions of heating to 100 °C for 30 minutes to give radiolabelling the best possible chance of occurring.



Scheme 4.5: Initial protocol for the radiolabelling of the Bn_2 bridged cyclen **15b** and the $(\text{CO}_2\text{H})_2$ bridged cyclen **16** with generator-produced $[^{68}\text{Ga}]\text{GaCl}_3$.

4.4.1 Dibenzyl-Functionalised Bridged Cyclen

Due to its dibenzyl functionalisation, the dibenzyl-functionalised bridged cyclen **15b** was thought likely to be more lipophilic than its diacid counterpart and therefore a good target for the formation of lipophilic cations. Whilst the four nitrogen atoms present in the chelator limit the potential coordination number, the preorganisation of the ligand through the bridge may provide an entropic boost to gallium coordination. As can be seen by the iTLC traces in Figure 4.35 however, the majority of the activity in the iTLC is located at the solvent front as seen in the control iTLC in Figure 4.4d, which implies that neither 0.1 M NH_4OAc or 0.1 M NaOAc facilitates radiolabelling.

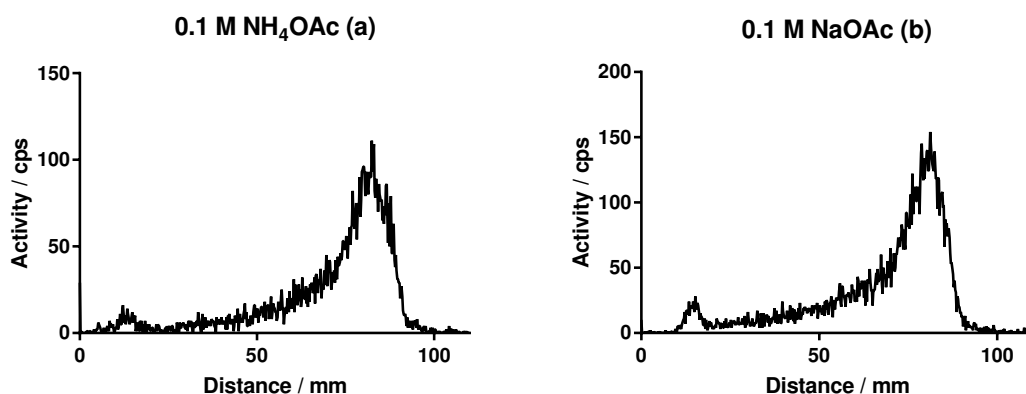


Figure 4.35: iTLC traces of the initial radiolabelling experiments of dibenzyl-functionalised bridged cyclen **15b** in 0.1 M NH_4OAc and 0.1 M NaOAc . Mobile phase: 0.1 M disodium EDTA.

The difficulty in deprotonating bridged cyclens is due to the fact that they act as proton sponges, since the cavity surrounded by nitrogen-centred lone pairs is an ideal place for a proton to reside. Removal of this proton would require such harsh base that it would hinder the biological suitability of the radiotracer formulation. As such, work on the radiolabelling of the dibenzyl-functionalised bridged cyclen was not pursued further.

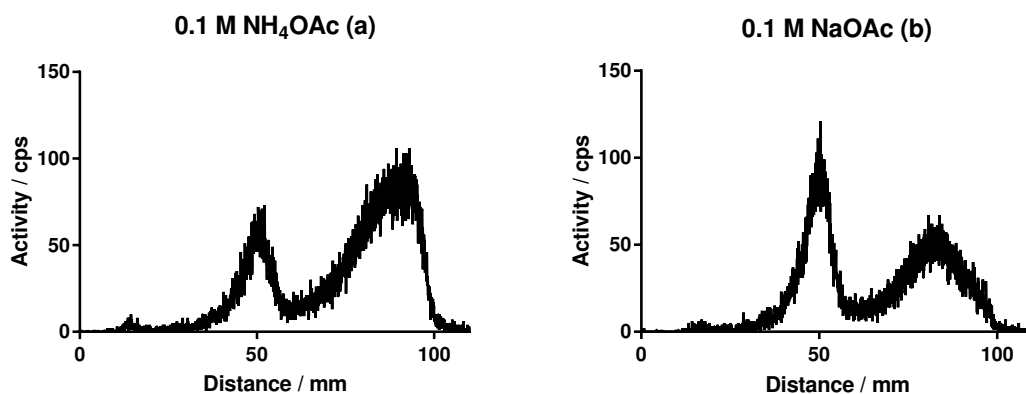
4.4.2 Diacid-Functionalised Bridged Cyclen

4.4.2.1 Initial Radiolabelling Experiments

Since the four nitrogen atoms present in the bridged cyclen are not enough to efficiently chelate Ga(III) centres, the diacid variant **16** was designed to increase the possible coordi-

Table 4.7: Radiochemical yields obtained from the iTLC traces of the diacid-functionalised bridged cyclen **16** radiolabelling experiments in Figure 4.36.

Species	R_F Range	RCY / %	
		0.1 M NH_4OAc	0.1 M NaOAc
$[^{68}\text{Ga}]\text{Ga}(\text{OH})_3$	<0.06	1	1
$[^{68}\text{Ga}]\text{Ga16}$	0.06–0.81	25	46
$[^{68}\text{Ga}]\text{Ga(III)}$	>0.81	74	53

**Figure 4.36:** iTLC traces of the initial radiolabelling experiments of diacid-functionalised bridged cyclen **16** in 0.1 M NH_4OAc and 0.1 M NaOAc . Mobile phase: 0.1 M disodium EDTA.

nation number to six. Once again, the initial radiolabelling experiments were performed in 0.1 M NH_4OAc and 0.1 M NaOAc , with heating at 100 °C for 30 minutes. The iTLC traces for these experiments (Figure 4.36) give traces where the product is located in the middle of the chromatogram, a result not exhibited by any other compound radiolabelled in this project so far. These iTLC traces allow for the analysis of not only the desired radiolabelled compound and the unchelated $[^{68}\text{Ga}]\text{Ga(III)}$, but for the first time allow for the quantification of colloidal $[^{68}\text{Ga}]\text{Ga}(\text{OH})_3$, which is normally obscured by the product peak. The RCY values from these iTLC experiments is summarised in Table 4.7. For both 0.1 M NH_4OAc and 0.1 M NaOAc , only 1% colloidal $[^{68}\text{Ga}]\text{Ga}(\text{OH})_3$ is observed. As with the $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ discussed previously, 0.1 M NaOAc provides a higher RCY of 46%, compared to the 25% RCY for 0.1 M NH_4OAc . The fact that the diacid-functionalised bridged cyclen move on silica iTLC either implies that the compound is quite lipophilic since it shows weak interactions with the polar stationary phase, or quite hydrophilic since it interacts strongly with the polar aqueous mobile phase. The only way to determine which is true is to firstly obtain the product in quantitative RCY, then determine their $\log D$ values.

4.4.2.2 Optimisation of RCY

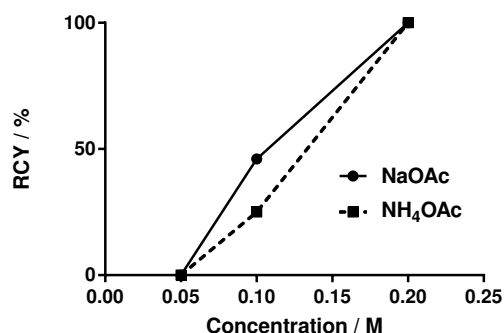


Figure 4.37: RCY values for the buffer alteration experiments for the optimisation of diacid-functionalised bridged cyclen **16**.

As performed for [^{68}Ga]Ga-DO2A-(xy-TAP₂) in Figure 4.10, the next step was to determine whether the concentration of the acetate buffer will alter the obtained RCY. The data from these experiments is summarised in Figure 4.37. These results are far less dramatic than in the [^{68}Ga]Ga-DO2A-(xy-TAP₂) case, with both ammonium and sodium acetate facilitating quantitative RCY at 0.2 M, and neither achieving any chelation at 0.05 M. The main difference between the two solutions is thus at the 0.1 M acetate concentrations, where sodium acetate is favourable over ammonium acetate. It is easier to deprotonate the diacid functionalised bridged cyclen than the dibenzyl functionalised analogue since the carboxylate groups make it easier to remove the proton in the cavity by shuttling the proton out, lowering the energetic barrier for deprotonation and thus gallium complexation. It was decided to use 0.2 M NaOAc for future radiolabelling experiments of bridged cyclens, as it shows a lower amount of dependence on the radiolabelling pH.

4.4.2.3 Log *D* Calculation

In order to determine whether the optimisation of the radiolabelling of diacid-functionalised bridged cyclen **16** was worth pursuing, the log *D* values were calculated. Since radiolabelled [^{68}Ga]Ga**16** was deemed pure by iTLC, its log *D* value could be determined directly. As seen in Figure 4.38 however, [^{68}Ga]Ga**16** has a log *D* value of -3.88 ± 0.22 ($n = 6$), which is in fact lower than the log *D* value for unchelated [^{68}Ga]Ga(III) of -3.43 ± 0.07 ($n = 6$) determined previously. As such, it seems that the additional functionalisation present in the DO3A- and

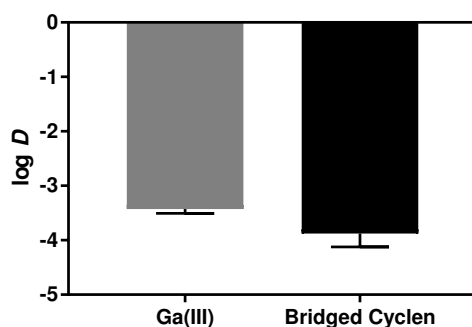


Figure 4.38: Log D value of the gallium-68 labelled diacid-functionalised bridged cyclen [^{68}Ga]Ga15 (black) compared to the control experiments for unchelated [^{68}Ga]Ga(III) (grey) taken from Figure 4.6. Data are mean $\pm$ SD ($n = 6$).

DO2A-based ligands discussed previously is in fact vital to shielding of the cationic Ga(III) centre and the polar carboxylate arms from hydrophilic interactions and the lowering of the overall tracer lipophilicity. This disappointing result for the radiolabelling characteristics of bridged cyclen chelators meant that this avenue of research was not pursued further.

4.5 Concluding Remarks

This chapter outlines the radiolabelling of four analogous series of chelators with generator-produced gallium-68. The first series of chelators studied were the DO3A-xy-TAP chelators, originally considered for copper-64 and gallium-67 chelation by Wang *et al.* and Kardashinsky *et al.* respectively.^{1,2} These compounds showed acceptable radiolabelling kinetics as well as one radiolabelled product in each reaction mixture which correlated to the non-radioactive reference compound by HPLC. This initial work allowed for the development for a fast iTLC QC method for RCY calculations of radiolabelled lipophilic cations. Unfortunately, these complexes showed low log D values as a result of the unbound carboxylate arm present in the complex, which may prohibit mitochondrial uptake.

To attempt to circumvent the unbound carboxylate arm, DO2A-based chelators were explored next. Inspired by the TAP functionalisation already utilised, the radiochemistry of DO2A-(xy-TAP)₂ was first studied under the hypothesis that two lipophilic cationic groups and removal of a potential unbound carboxylate group would improve the log D values of the complexes. Due to the dependence of radiolabelling efficiency on the pH of the reaction mix-

ture, the radiolabelling of DO2A-(xy-TAP)₂ in a series of concentrations of acetate buffers were studied, with 0.2 M NaOAc being found the most effective. The log *D* values of the complexes were found to be more favourable than their DO3A-based counterparts.

HPLC studies of these complexes found that multiple species are observed upon radiolabelling with gallium-68, despite the apparent purity of the chelators. Two explanations were proposed for this observation: that the chelator was not binding completely to the Ga(III) centre, or that a *cis/trans* isomer pair was forming. Through systematic alteration of the reaction conditions, it was established that the two species formed a kinetic/thermodynamic product pair, and as such one species could be somewhat favoured by using thermodynamically forcing conditions. Similar results were observed for an analogous series of DO2A chelators with aryl functionalisation, although whilst these complexes showed comparable log *D* values to their TAP-functionalised counterparts, they displayed less favourable radiolabelling kinetics despite the lower degree of steric hindrance and repulsive cationic charge afforded by their functionalisation. Looking forward to biological evaluation, [⁶⁸Ga]Ga-DO3A-xy-TXP will be studied due to its prevalence in the literature despite its low lipophilicity, [⁶⁸Ga]Ga-DO2A-(xy-TXP)₂ due to its more favourable lipophilicity and tricationic charge, and [⁶⁸Ga]Ga-DO2A-Xy₂ due to its lower cationic charge, both in spite of their speciation upon radiolabelling.

The final series of compounds studied, based on bridged cyclens, were far less successful. Use of lipophilic benzyl pendant arms under the assumption that the bridged cyclen chelator alone could bind gallium did not provide any radiolabelling. An analogous chelator with pendant carboxylate arms allowed for high RCY values, however the log *D* values observed were worse than those observed for unchelated [⁶⁸Ga]Ga(III). Due to their inability at the current time to form lipophilic cations, bridged cyclens were not pursued further in this project.

4.6 References for Chapter 4

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Chapter 5

Biological Evaluation of Radiolabelled Lipophilic Cations

5.1 Introduction

Having synthesised a series of chelators for the formation of lipophilic and cationic complexes with Ga(III), and analysed their radiochemistry with gallium-68, three potential candidates were proposed for biological evaluation. These compounds are shown in Figure 5.1.

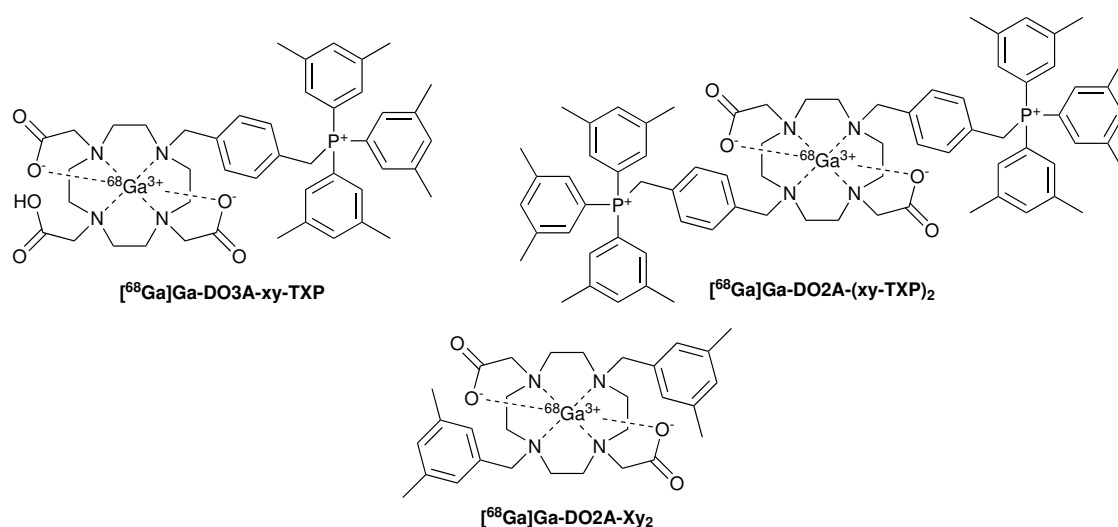


Figure 5.1: The three gallium-68 radiolabelled complexes brought forward for biological evaluation.

In order to assess whether these complexes showed mitochondrial uptake in a manner dependant on $\Delta\Psi_{\text{m}}$, two preclinical biological models were used. The propensity of lipophilic cations to accumulate in tumour mitochondria as a result of their elevated $\Delta\Psi_{\text{m}}$ was discussed in the introduction to this thesis, measuring this uptake is easily performed using *in vitro* cell culture preparations. Due to the high energy demand of the heart, the myocardium exhibits a correspondingly high concentration of mitochondria, making it a prime target for lipophilic cationic radiotracers.¹ The Langendorff isolated perfused heart model is ideal for studying the kinetics of radiotracer uptake, retention and washout in myocardial tissue, as it allows for the accurate induction of pathological conditions on the heart which would be lethal to an animal *in vivo*, such as ischemia and hypoxia, whilst also maintaining a high degree of *in vivo* relevance.^{2,3} Previous work in the Southworth group has shown that the model is fast, simple and highly reproducible.⁴⁻⁷

5.1.1 Coronary Circulation *In Vivo*

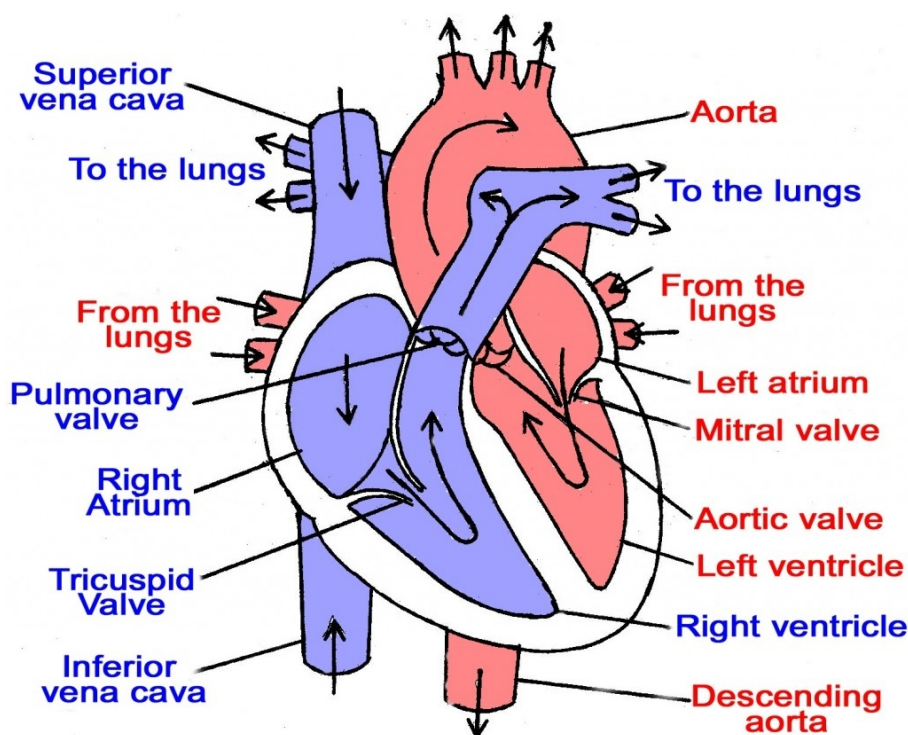


Figure 5.2: Schematic of the heart, showing the direction of blood flow through the four chambers. Picture taken from www.humananatomychart.com.⁸

Before the Langendorff perfusion apparatus itself can be introduced, the basic biology of the heart must be revisited. The heart itself consists of four muscle-encased chambers as outlined in Figure 5.2 which pump blood around the body. Each chamber has an inlet valve and outlet valve to increase circulation pressure and prevent backflow of blood. Systole occurs when the chambers of the heart contract and the pressure increases, causing the inlet valves to shut and the outlet valves to open, pumping blood around the circulatory system. The opposite process, diastole, occurs when the chambers of the heart relax, decreasing the pressure inside the heart and opening the inlet valves, subsequently refilling the heart with blood.

During the cardiac cycle *in vivo*, deoxygenated blood from the systemic circulation returns to the right atrium through the venae cavae. The blood then passes through the tricuspid valve into the right ventricle, then pumped through the pulmonary valve into the pulmonary artery. The blood is then oxygenated in the pulmonary capillary bed before returning to the left atrium of the heart through the pulmonary veins, before being pumped through the mitral valve into the left ventricle. Left ventricular contraction pumps the blood through the aortic

valve and into the aorta. During subsequent diastole, the high aortic pressure coupled with the low left ventricular pressure forces the aortic valve shut, meaning that the blood is forced into the two coronary ostia arising from the descending aorta. These lead to the coronary arteries, which perfuse the myocardium with oxygenated blood before it is drained back into the right atrium, primarily through the coronary sinus.⁹

5.1.2 Langendorff Isolated Heart Perfusion

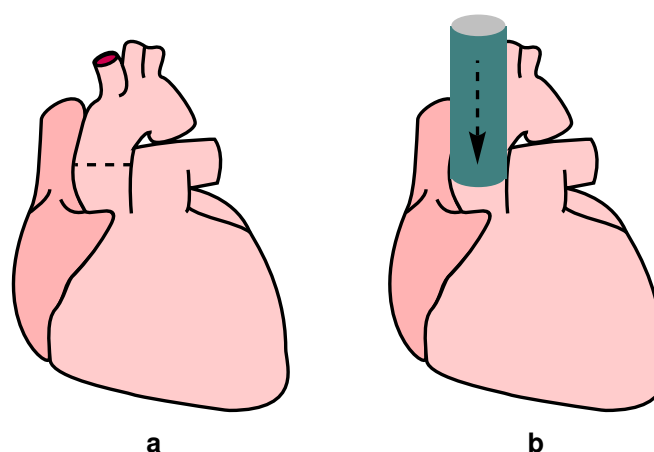


Figure 5.3: Cartoon schematic showing **a**: the incision to be made in the aorta to allow Langendorff mode perfusion shown by the dashed line, and **b**: insertion of the cannula through which Krebs buffer is perfused.

In Langendorff mode heart perfusion, coronary circulation is exploited through aortic cannulation to provide a viable, beating controllable and physiologically relevant model for a variety of applications in cardiovascular research, as shown in Figure 5.3. An incision is made in the aorta (Figure 5.3a), through which a cannula is inserted to a point just above the coronary ostia (Figure 5.3b) through which the heart is perfused in a retrograde manner.

Due to the pressure in the aorta, the aortic valve is forced shut, thereby preventing backflow of perfusate into the left ventricle. In order to allow the perfusate to flow freely through the coronary vasculature, an incision is made at the base of the pulmonary artery so that the perfusate can be pumped out of the heart through the right ventricle. This is also performed since it is possible to tie the pulmonary artery closed during cannulation. In contrast to *in vivo* perfusion, in which the left ventricle is full of blood and is performing active work, the left ventricle is empty in Langendorff mode perfusion and as such the physiological workload

on the ventricle is reduced. This workload can be simulated through the removal of the left atrium and insertion of a water-filled balloon into the left ventricle through the mitral valve; the balloon is then connected to pressure transducer which allows for readout of left ventricular pressure.

Optionally, the final step of Langendorff perfused heart experiments is external electrical pacing. Whilst the sinoatrial node continues to trigger the heart to beat *ex vivo*, external electrical pacing allows for the maintenance of a consistent heart rate throughout the experimental protocol, ensuring a consistent energetic demand and thus improving the reproducibility achievable using the model. Experimentally, pacing is achieved by hooking a silver wire through the right atrium near to the sinoatrial node, with the other end of the circuit connected to the metal cannula. These are connected to a pacing unit, which allows the alteration of the pacing frequency and voltage.

Langendorff isolated heart perfusion was chosen over *in vitro* cardiac cell preparations since there is more clinical relevance in using actual cardiac tissue when considering future translatability. For example, *in vitro* preparations do not take into account variations in membrane potentials throughout the cardiac cycle. In addition, since uptake is a function of the time a tracer spends near a tissue of interest, the Langendorff model takes into account the small amount of time the tracer spends in proximity to the heart as it flows through the coronary vasculature, as opposed to an *in vitro* preparation where the tracer is incubated with the cells. There are some downsides to an *ex vivo* approach, however, as it does not take into account biodistribution effects like *in vivo* imaging whilst also requiring the sacrifice an animal per experiment, unlike *in vitro* studies.

5.1.3 Carbonyl Cyanide *m*-Chlorophenyl Hydrazone (CCCP)

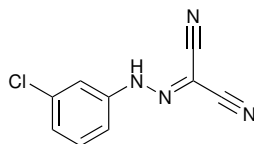


Figure 5.4: CCCP, a chemical inhibitor of oxidative phosphorylation which serves to uncouple the proton gradient in mitochondria and thus reduce $\Delta\Psi_m$ to zero.

One pharmacological intervention commonly used in isolated perfused heart studies is carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), shown in Figure 5.4. CCCP is a chemical

inhibitor of oxidative phosphorylation, which acts as a protonophore with a high degree of lipophilicity. As such, it is capable of shuttling protons across the inner mitochondrial membrane at a faster rate than they are used by the ATP synthase, which has the effects of halting ATP production and uncoupling the proton gradient, thereby reducing the magnitude of $\Delta\Psi_m$ in a dose-dependant manner. Since the uptake of lipophilic cations in heart tissue is dependant on the magnitude of $\Delta\Psi_m$, this intervention can be exploited to determine the relative contribution of active retention of tracers within the mitochondria by virtue of $\Delta\Psi_m$, as opposed to their passive non-selective trapping in cell membranes as a result of their lipophilicity.¹⁰

Previous work by Saffee *et al.* has used CCCP as a method of assessing radiotracer uptake in hearts in which $\Delta\Psi_m$ has been compromised.¹¹ Using the clinical SPECT radiopharmaceutical MIBI as validation, Saffee *et al.* found that a 600 nM solution of CCCP was optimal to effect complete $\Delta\Psi_m$ depolarisation, as well as to cause significant cessation of cardiac contractility. A range of CCCP concentrations between 0–600 nM can be used to establish a more granular assessment of $\Delta\Psi_m$ depolarisation on mitochondrial uptake of lipophilic cations.

5.2 Materials and Methods

5.2.1 Isolated Perfused Heart Apparatus

A schematic representation of the isolated perfused heart apparatus is shown in Figure 5.5, with the triple γ -detector system highlighted in red. For a comprehensive discussion of the experimental procedures used in this chapter, see Chapter 7.3.1.

5.2.2 Establishment of Exclusion Criteria

To confirm proficiency in isolated heart perfusion, a series of stability studies were performed to determine how long the preparations were stable for, and to establish exclusion criteria for all future experiments. If a preparation did not meet the exclusion criteria within 10 minutes post-cannulation, the experiment was halted and another preparation was made. Isolated rat hearts were excised, cannulated, paced and perfused as described as above. The haemodynamic parameters: LVDP, PP, heart rate and LVDP, were monitored and recorded for 90 minutes. The average haemodynamic parameters 10 minutes post-cannulation from 6 hearts which were

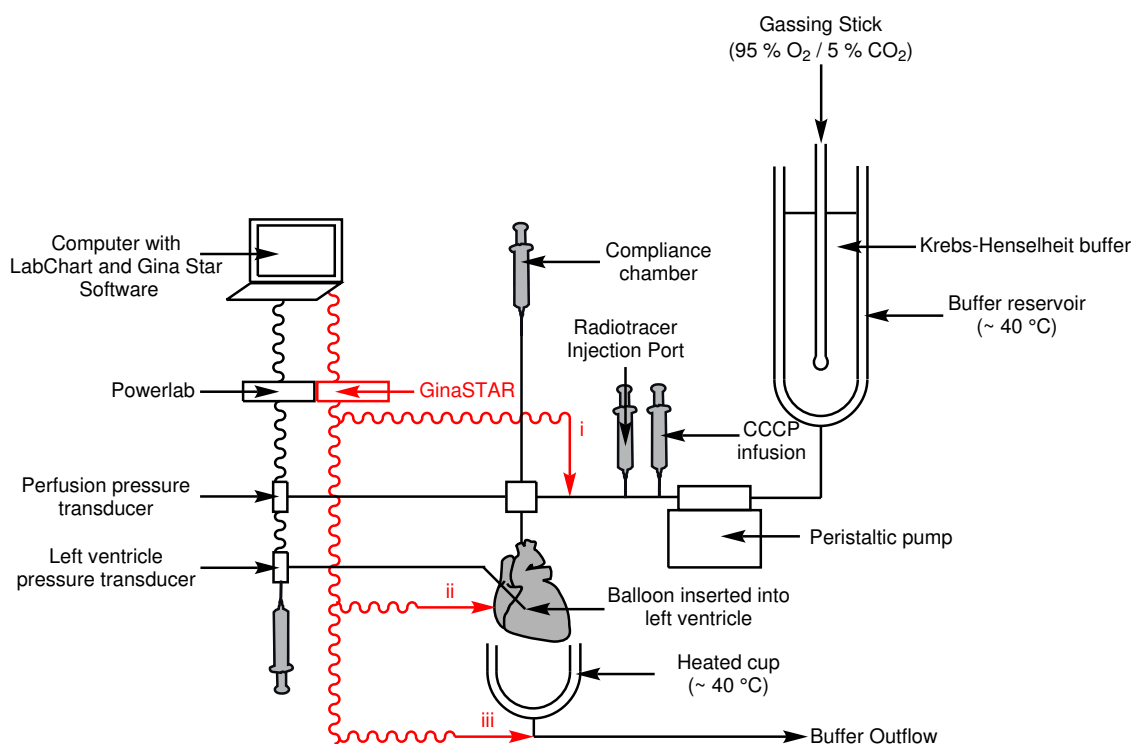


Figure 5.5: Schematic representation of the Langendorff isolated perfused heart apparatus, showing the triple γ -detector system in red.

recorded as still capable of developing >120 mmHg LVDP 90 minutes post-cannulation were used as the exclusion criteria for all future experiments. The data from the stability study are summarised in Figure 5.6, and the exclusion criteria derived from these data are shown in Table 5.1. The values stated are $\pm 10\%$ of the mean values from the stability study. In future experiments, hearts which do not show these levels of function at $t = 10$ minutes will not be used for radiotracer uptake experiments, as it cannot be certain that the hearts will still be functioning effectively at the end of the experiment.

Table 5.1: Exclusion criteria for the measured haemodynamic parameters at $t = 10$ minutes, derived from the stability study data shown in Figure 5.6.

Parameter	Acceptable Values at $t = 10$ min	
	Min	Max
Perfusion Pressure / mmHg	64	78
LVDP / mmHg	153	187
LVEDP / mmHg	6.6	8.1
Heart Rate / bpm	303	371

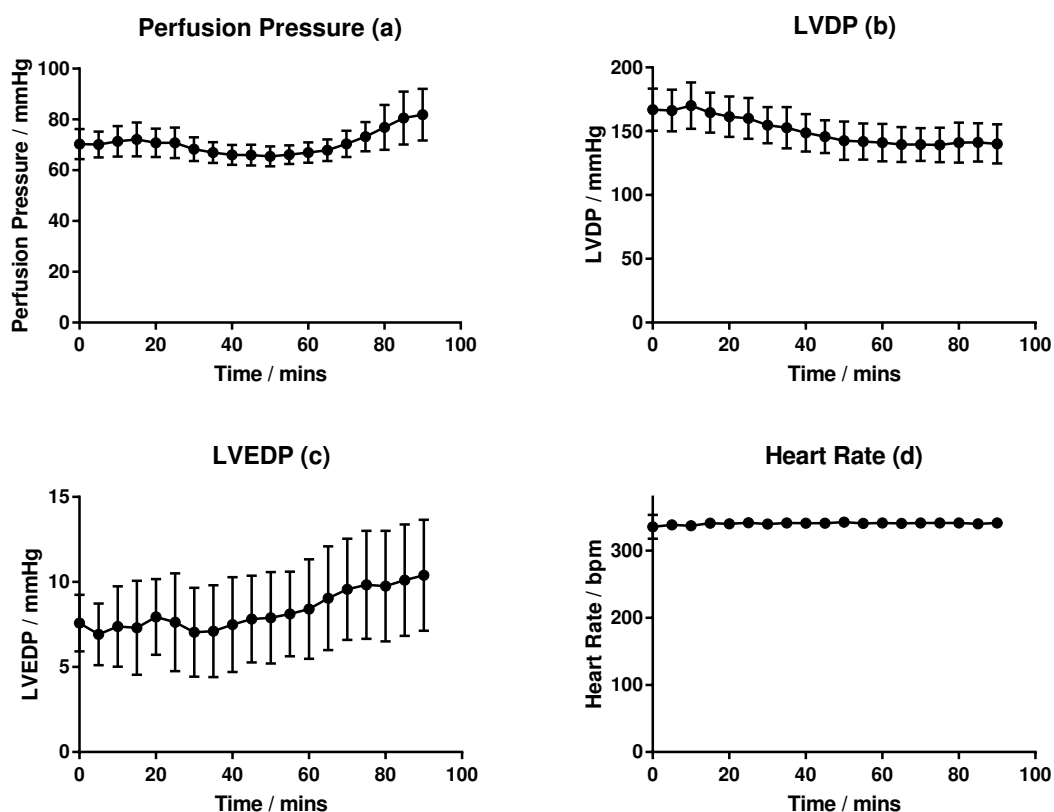


Figure 5.6: Stability study for the cardiac function of isolated perfused hearts. **a:** perfusion pressure, **b:** LVDP, **c:** LVEDP and **d:** heart rate. Data are mean $\pm$ SD ($n = 6$).

5.2.3 Measurement of Radiotracer Kinetics in Isolated Perfused Hearts

For these experiments, the basic Langendorff perfusion apparatus was coupled with a triple γ -detector system previously developed in the Southworth group.⁴ This system is highlighted in red in Figure 5.5, and is shown further in Figure 5.7. Further discussion is shown in Chapter 7.3.2.

5.2.4 Experimental Protocols for Biological Studies

Comprehensive discussion of the Langendorff-based and *in vitro* cancer cell-based radiotracer uptake experiments are provided in Chapters 7.3.4.1 and 7.3.4.2 respectively. A timetable for the Langendorff-based uptake experiments is included in Figure 5.8 for reference.

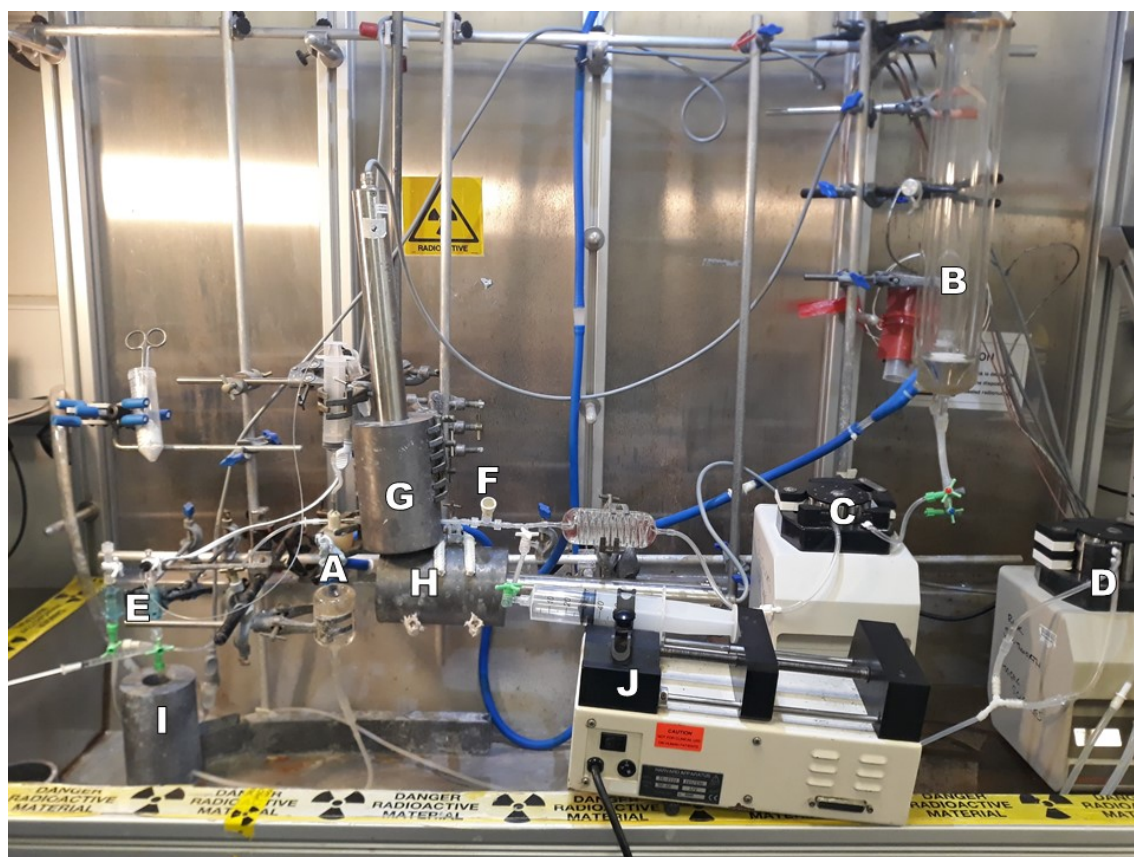


Figure 5.7: The triple detector system for monitoring radiotracer retention in isolated hearts. Hearts were cannulated (A), and then perfused with mKHB held in reservoirs maintained at 37 °C (B). Coronary flow was maintained constant by peristaltic pump (C) and venous outflow was removed via the heated cup connected to the second peristaltic pump (D). Cardiac haemodynamic parameters were monitored by two pressure transducers (E). Radiotracer boluses were injected into the injection port (F), and their passage through the perfusion system and the heart were monitored by three Na/I detectors located on the arterial line (G), the heart (H) and the venous outflow line (I). CCCP was infused into the arterial line using a syringe pump (J).

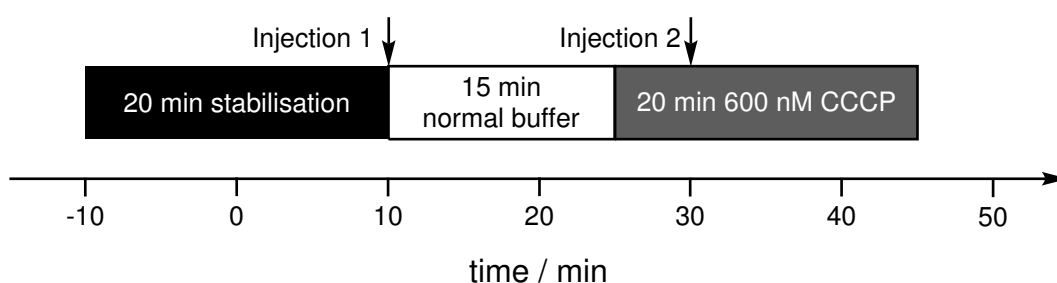


Figure 5.8: Schematic representation of the experimental protocol for the two-injection protocol for the assessment of radiotracer uptake in isolated perfused rat hearts. Black arrows indicate radiotracer injections.

5.3 Results

5.3.1 MIBI

5.3.1.1 Time-Activity Curve Retention and Clearance Analysis

Typical kinetic profiles of radiotracers moving through the heart and perfusion apparatus are shown in Figure 5.9. The radiochromatogram in Figure 5.9a shows the time-activity curves observed in the arterial line representing the injection of the tracer into the system *via* the injection port, Figure 5.9b shows radiotracer retention and washout by the heart, whilst Figure 5.9c shows the washout of tracer in the coronary effluent line. Attempts have been made to use the chromatograms arising from Figure 5.9a as an input function for the pharmacokinetic modelling of radiotracer retention in Figure 5.9b, however this proved challenging and as such only the results shown in Figure 5.9b are shown and analysed in the rest of this thesis.¹² All

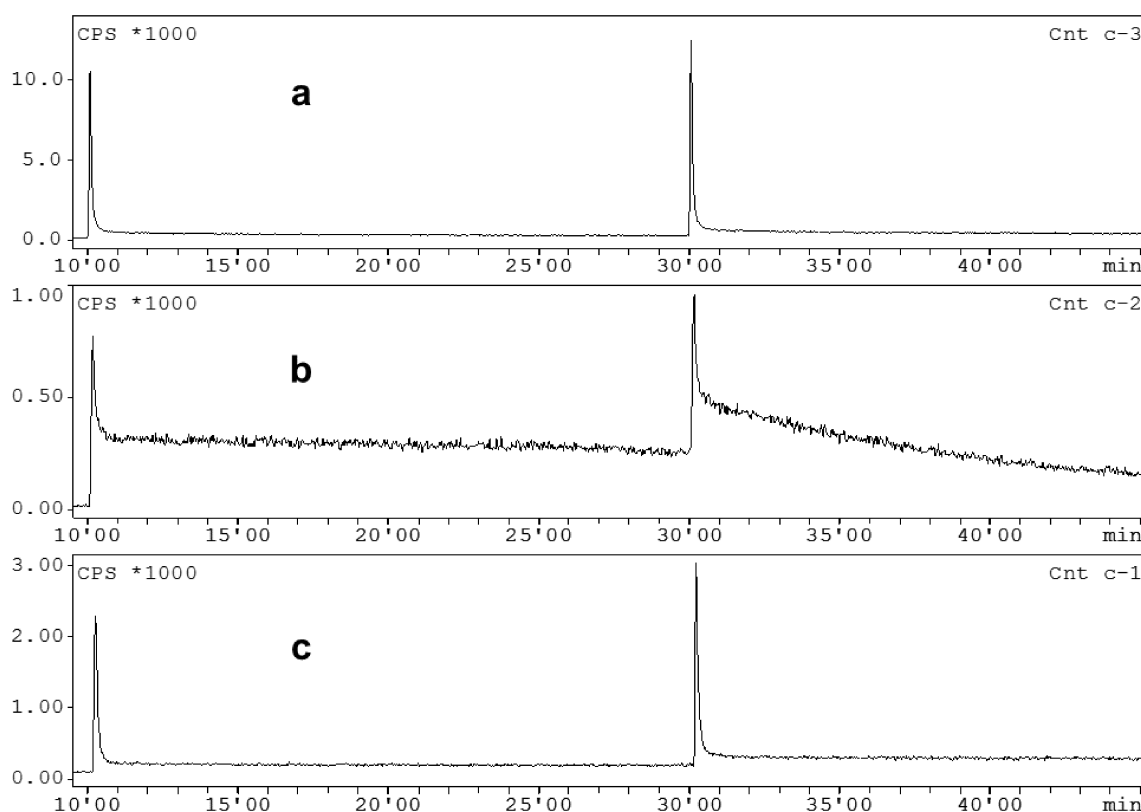


Figure 5.9: Representative time-activity curves demonstrating MIBI injection, uptake and washout in **a:** arterial inflow, **b:** heart and **c:** venous outflow. The first injection is into a healthy heart, the second into a heart infused with 600 nM CCCP (discussed later).

datasets were corrected for radioactive decay.

5.3.1.2 Cardiac Retention of MIBI in Control and CCCP-Infused Hearts

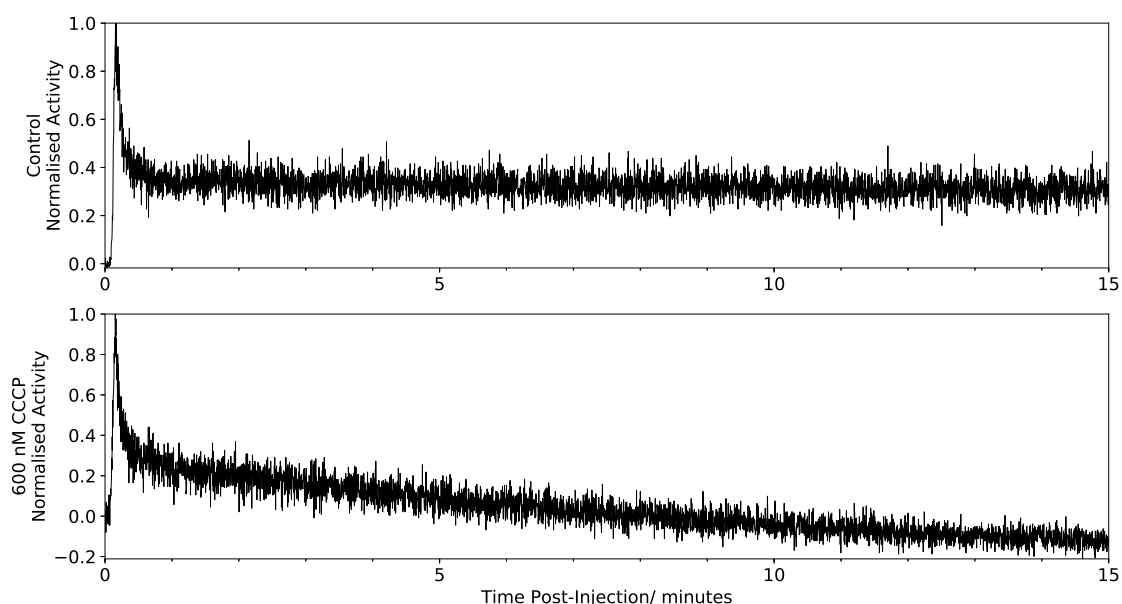


Figure 5.10: Example of the graphical output of the Python script in Appendix A.1 having analysed the data in Figure 5.9b.

The protocol described in Section 7.3.4.1 was followed using the clinical gold-standard lipophilic cationic radiotracer MIBI in order to ensure validity of this model for this set of experiments. As shown by the time-activity curves in Figure 5.10 and summarised in Figure 5.11, MIBI demonstrates $30 \pm 3\%$ ($n = 4$) radiotracer retention after 15 minutes in hearts under normal conditions, but 0 ± 4 ($n = 4$, $P < 0.05$) retention after 15 minutes in hearts under depolarising conditions achieved by the infusion of 600 nM CCCP. This negative retention refers to the fact that retained radiotracer in the heart from the first injection starts to leach out of the mitochondria as it is depolarised.

5.3.1.3 Cardiac Functional Parameters in Hearts Infused with 600 nM CCCP

Figure 5.12 displays the cardiac haemodynamic parameters observed during the same protocol compared to those obtained from the stability study (Figure 5.6). Before the commencement of CCCP infusion at 25 minutes, there is no difference in haemodynamic parameters between the two experiments. After 5 minutes of 600 nM CCCP infusion, perfusion pressure

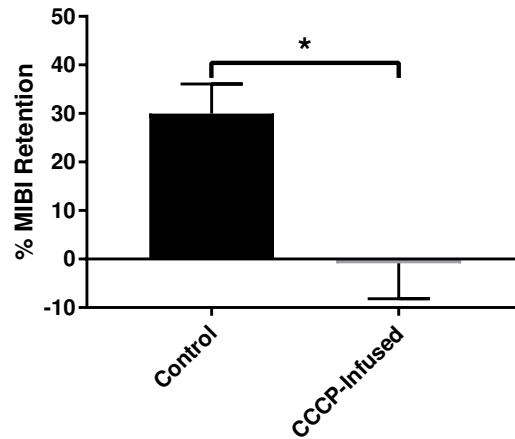


Figure 5.11: Retention values obtained using the two-injection protocol 15 minutes post-injection for MIBI. Control data were obtained from the injection before CCCP infusion, whilst CCCP data were obtained during 600 nM CCCP infusion. Data are mean $\pm$ SD (n = 4). * = $P < 0.05$.

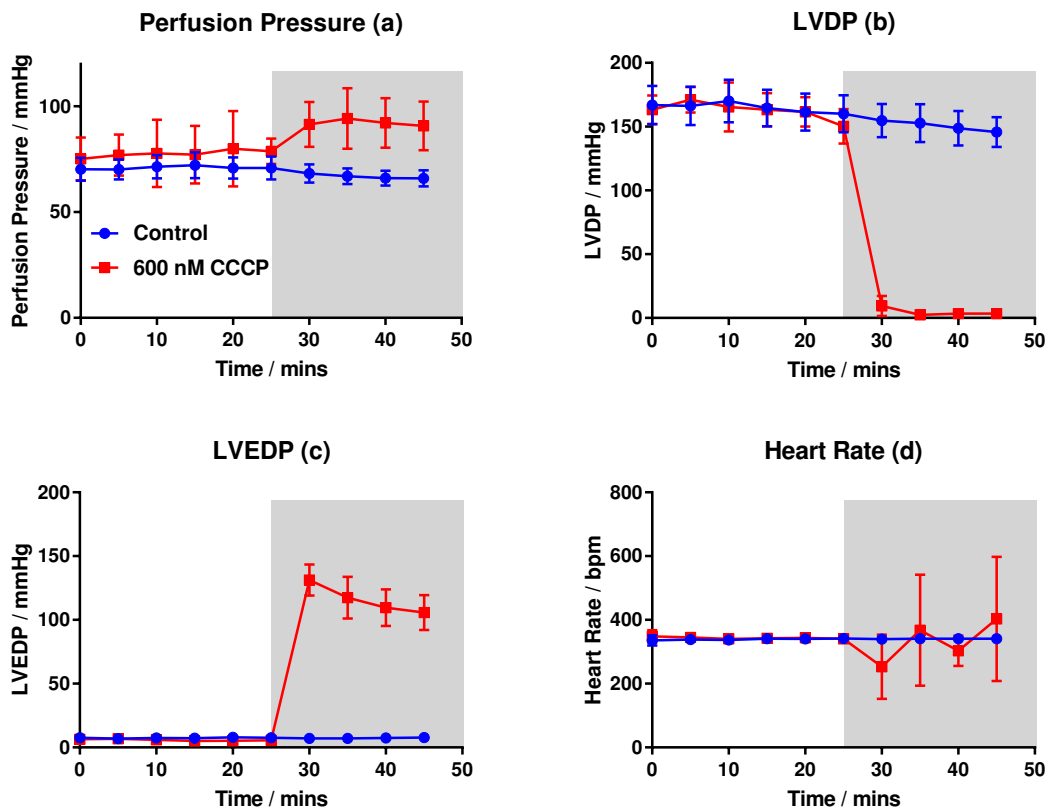


Figure 5.12: Haemodynamic parameters for control hearts undergoing normal function for 45 minutes (n = 6, blue) and hearts undergoing normal function for 25 minutes followed by infusion 600 nM CCCP for 20 minutes (n = 4, red). **a:** perfusion pressure, **b:** left ventricular developed pressure, **c:** left ventricular end diastolic pressure and **d:** heart rate. Data are mean $\pm$ SD at each time point.

increases to 91.5 ± 10.6 mmHg, compared to 68.3 ± 4.3 mmHg observed at the same time-point in the stability study. After 20 minutes of CCCP infusion, perfusion pressure measures 90.7 ± 11.5 mmHg. LVDP decreases dramatically from 150.3 ± 13.5 mmHg at the start of CCCP infusion to 9.5 ± 8.0 mmHg within 5 minutes, and does not increase above 5 mmHg for the rest of the experiment. LVEDP shows the opposite trend, increasing from 5.6 ± 3.0 mmHg at the start of CCCP infusion to 131.2 ± 12.2 mmHg within 5 minutes, before decreasing to 105.8 ± 13.6 mmHg over 20 minutes of CCCP infusion, reflecting significant vasoconstriction. Heart rate grows erratic as a result of CCCP infusion, maintaining mean values of 252.9–402.7 bpm, but with the standard deviation increasing from an average of 2.0 bpm before CCCP infusion to 58.8 bpm after CCCP infusion.

5.3.2 [^{68}Ga]Ga-DO3A-xy-TXP

5.3.2.1 Cardiac Retention of [^{68}Ga]Ga-DO3A-xy-TXP in Control and CCCP-Infused Hearts

The protocol described in Section 7.3.4.1 was followed using the most lipophilic example of the DO3A-based radiotracers, [^{68}Ga]Ga-DO3A-xy-TXP, with a $\log D$ value of -1.81. As shown by the representative time-activity curves in Figure 5.13 and summarised in Figure 5.14a, [^{68}Ga]Ga-DO3A-xy-TXP demonstrates $1.3 \pm 0.3\%$ ($n = 3$) retention after 15 minutes in hearts under normal conditions, and $1.5 \pm 0.3\%$ ($n = 3$) retention after 15 minutes in hearts under depolarising conditions. No statistically significant difference is observed between the two retention values. In addition, [^{68}Ga]Ga-DO3A-xy-TXP shows a 30-fold decrease in uptake in control hearts than MIBI under the same conditions.

5.3.2.2 *In Vitro* Quantification of [^{68}Ga]Ga-DO3A-xy-TXP Uptake in Tumour Cells

Due to the lack of observable heart uptake of [^{68}Ga]Ga-DO3A-xy-TXP, the protocol described in Section 7.3.4.2 was followed to determine whether the tracer showed appreciable uptake in tumours. Intracellular/extracellular concentration ratios for [^{68}Ga]Ga-DO3A-xy-TXP and unchelated [^{68}Ga]Ga(III) in cultured breast cancer cells (MDA-MB 231) *in vitro* were calculated in triplicate, and are summarised in Figure 5.14b. [^{68}Ga]Ga-DO3A-xy-TXP shows a ratio of 10.0 ± 0.5 , indicating that the tracers localise inside the tumour cells. In comparison, [^{68}Ga]Ga(III) shows a ratio of 3.4 ± 0.4 , indicating that the chelator facilitates a three-fold

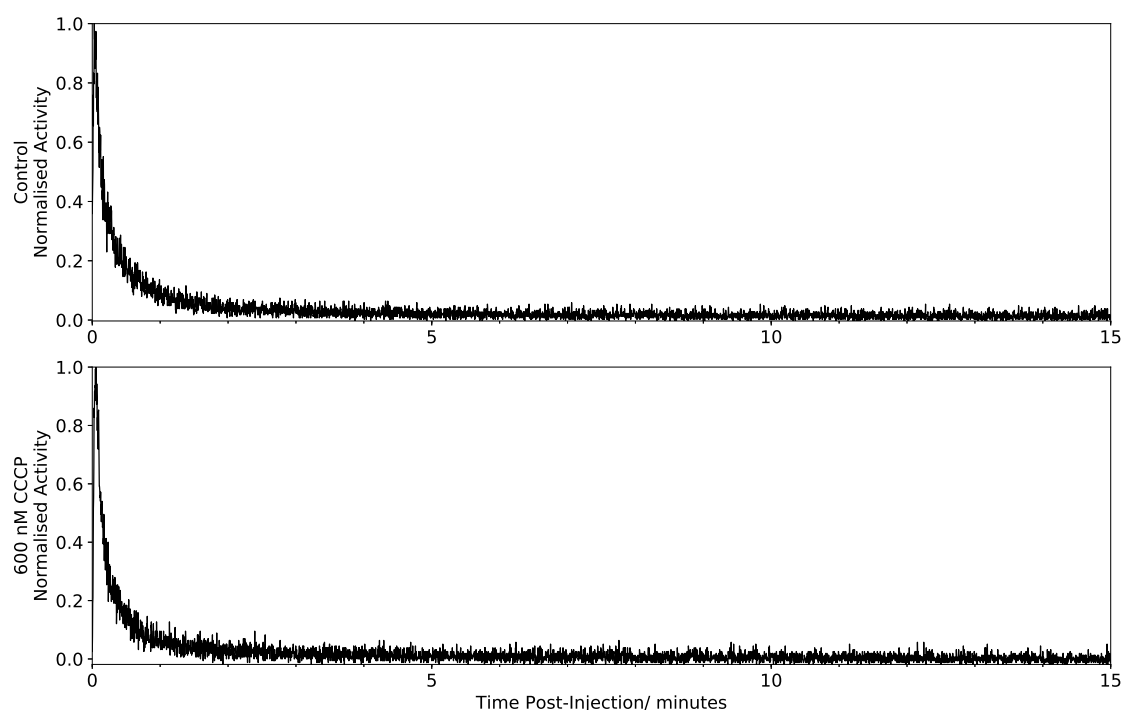


Figure 5.13: Representative time-activity curves demonstrating injections of [^{68}Ga]Ga-DO3A-xy-TXP into healthy hearts and hearts infused with 600 nM CCCP, as analysed by the Python Script in Appendix A.1.

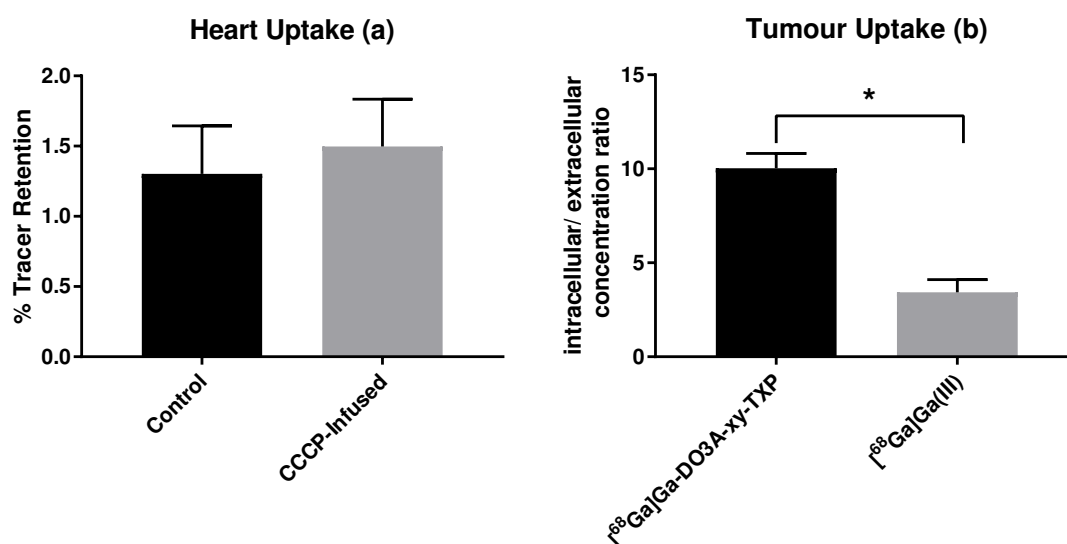


Figure 5.14: **a:** Retention values obtained using the two-injection protocol 15 minutes post-injection for [^{68}Ga]Ga-DO3A-xy-TXP. Control data were obtained from the injection before CCCP infusion, whilst CCCP data were obtained during 600 nM CCCP infusion. Data are mean $\pm$ SD ($n = 3$). **b:** Results of the *in vitro* tumour cell experiment for [^{68}Ga]Ga-DO3A-xy-TXP and unchelated [^{68}Ga]Ga(III) control. Measurements were taken in triplicate. Data are mean $\pm$ SD. * = $P < 0.05$.

increase in radiotracer uptake compared to free [^{68}Ga]Ga(III). An associated trypan blue cell viability study showed over 90% of cells used in both the [^{68}Ga]Ga-DO3A-xy-TXP and control experiments survived.

5.3.3 [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$

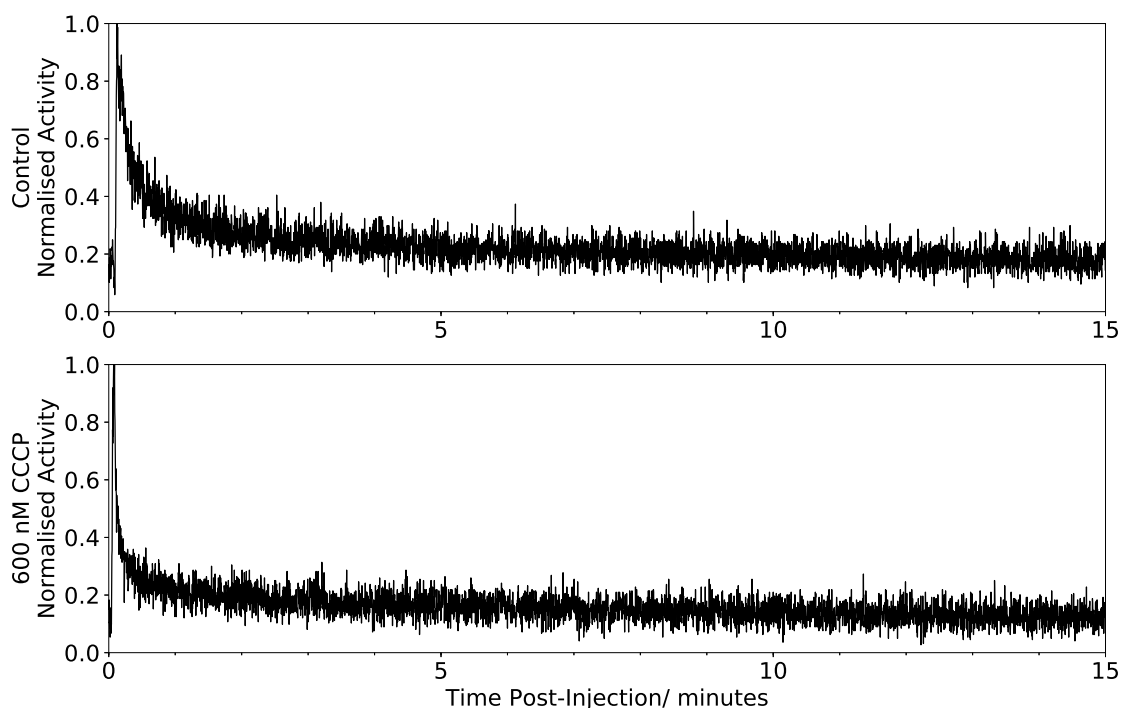


Figure 5.15: Representative time-activity curves demonstrating injections of [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$ into healthy hearts and hearts infused with 600 nM CCCP, as analysed by the Python Script in Appendix A.1.

The next tracer to be scrutinised in the perfused heart was [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$, following the protocol described in Section 7.3.4.1. As shown by the representative time-activity curves in Figure 5.15 and summarised in Figure 5.16, [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$ demonstrates $15.5 \pm 2.2\%$ ($n = 3$) retention after 15 minutes in hearts under normal conditions, and $10.8 \pm 1.4\%$ ($n = 3$, $P < 0.05$) retention after 15 minutes in hearts under depolarising conditions. The uptake in normal hearts is a ten-fold increase on that of [^{68}Ga]Ga-DO3A-xy-TXP, although still half that of MIBI. [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$ also shows appreciable uptake in hearts under depolarising conditions, an effect not seen for any radiotracer tested so far.

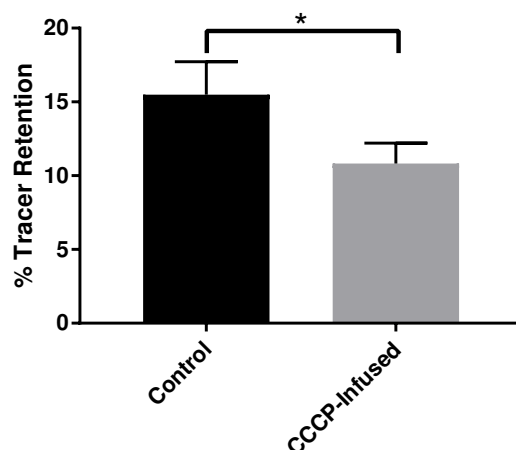


Figure 5.16: Retention values obtained using the two-injection protocol 15 minutes post-injection for $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TXP)}_2$. Control data were obtained from the injection before CCCP infusion, whilst CCCP data were obtained during 600 nM CCCP infusion. Data are mean $\pm$ SD ($n = 3$). * = $P < 0.05$.

5.3.4 $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$

The final radiotracer to be assessed in the perfused heart was $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$, following the protocol described in Section 7.3.4.1. As shown by the representative time-activity curves

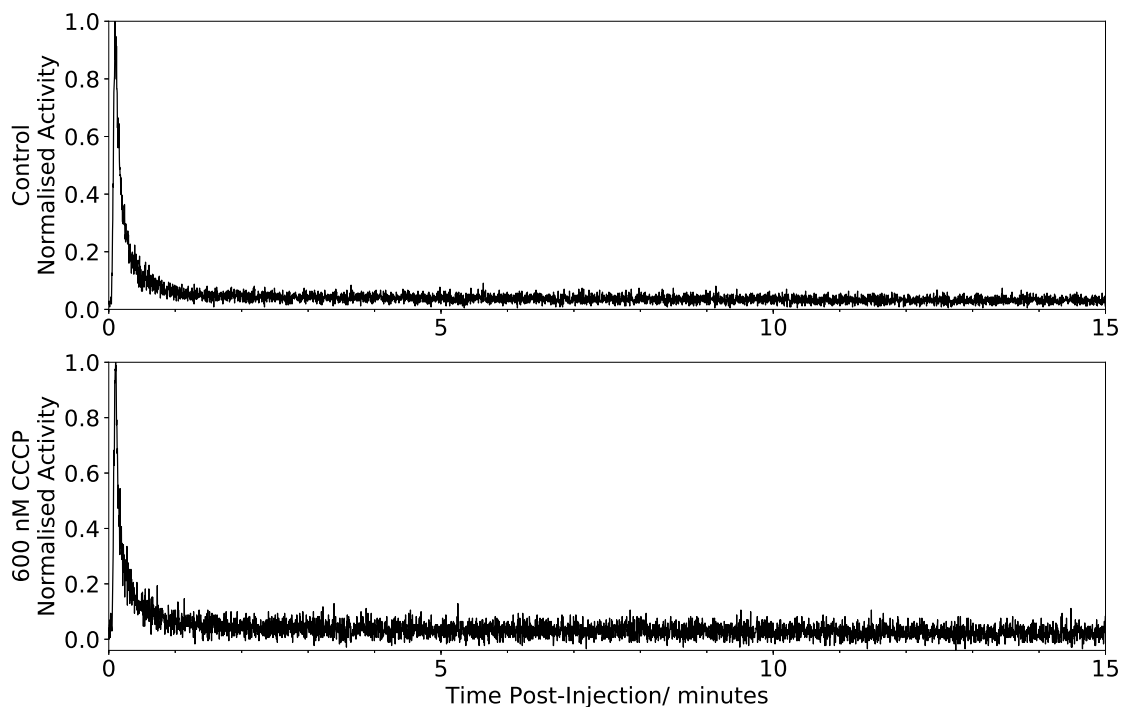


Figure 5.17: Representative time-activity curves demonstrating injections of $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ into healthy hearts and hearts infused with 600 nM CCCP, as analysed by the Python Script in Appendix A.1.

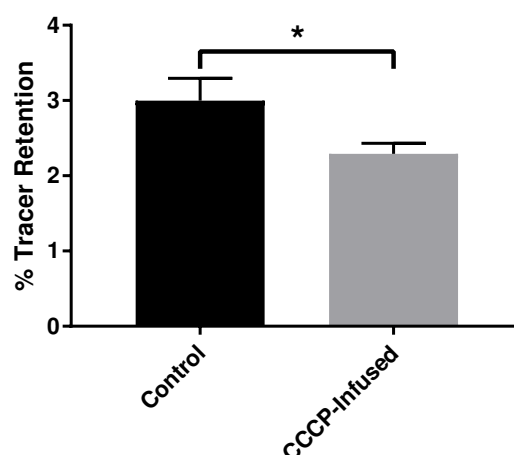


Figure 5.18: Retention values obtained using the two-injection protocol 15 minutes post-injection for [^{68}Ga]Ga-DO2A-Xy₂. Control data were obtained from the injection before CCCP infusion, whilst CCCP data were obtained during 600 nM CCCP infusion. Data are mean $\pm$ SD (n = 3). * = P < 0.05.

in Figure 5.17 and summarised in Figure 5.18, [^{68}Ga]Ga-DO2A-Xy₂ demonstrates $3.0 \pm 0.3\%$ (n = 3) retention after 15 minutes in hearts under normal conditions, and $2.3 \pm 0.14\%$ (n = 3, < 0.05) retention after 15 minutes in hearts under depolarising conditions. This tracer both shows less cardiac uptake in healthy and CCCP-infused cardiac tissue than [^{68}Ga]Ga-DO2A-(xy-TXP)₂DO2A-(xy-TXP)₂, although it is an improvement on [^{68}Ga]Ga-DO3A-xy-TXP.

5.4 Discussion

5.4.1 Analysis of the Two-Injection Protocol Using MIBI

Previous studies using the perfused heart model with triple-gamma detector to assess radiotracer uptake kinetics used three-injection protocols.^{4,6} The main drawback of these protocols is their lengthy 75 minute experiment time, especially notable when dealing with shorter half-life radioisotopes such as gallium-68. A reduction in experimental time was the main rationale behind the design of the two-injection protocol, which can be performed in under 45 minutes and give reliable values for myocardial uptake in three experiments. This model also in effect allows each heart to function as its own control experiment, as any defects present in the heart will affect both radiotracer injections. The two-injection protocol comes with a major limitation in the lack of a time-matched control experiment, in which two boluses of radiotracer are injected at the usual timepoints into a heart which does not receive a CCCP infusion, al-

lowing for more accurate comparisons between injections at the same time post-cannulation, as well as for establishing control measures in the pre-infusion injections. Nevertheless, the two-injection protocol was deemed a useful tool for the initial screening of novel lipophilic cations, before more rigorous testing such as CCCP dose-response can be performed on the most promising candidates.

Another reason for performing these initial experiments was to determine the effects of 600 nM CCCP infusion had on the heart, and the rate at which these effects manifest. The most dramatic changes in heart function are observed in LVDP and LVEDP. As the heart stops beating as a result of CCCP infusion, LVDP decreases rapidly within 5 minutes as the lack of contractile function means no pressure is developed within the heart. The rapid increase in LVEDP demonstrates the fact that CCCP infusion causes the myocardial muscles to contract, and thus the heart can no longer relax. The increase in perfusion pressure arises from the fact that the perfusate can only move through the coronary vasculature at diastole, since the coronary arteries are constricted when the heart contracts. Since the heart contracts irreversibly upon CCCP infusion, there is an increased resistance to buffer flow and thus a backpressure builds up in the arterial line. The heart rate is recorded as becoming erratic upon CCCP infusion, despite the fact that the heart ceases to beat even though it is still being externally paced. This is likely a result of the software attempting to fit a beat pattern between changes in LV pressure despite the LV pressure being effectively constant. As such, upon CCCP infusion heart rate is no longer seen as being a reliable marker for heart viability. The most important factor arising from the analysis of haemodynamic parameters is that a functional change is being observed within 5 minutes of CCCP infusion, meaning that the mitochondria have been depolarised and changes in radiotracer uptake will reflect these changes.

As expected from a clinically used gold-standard lipophilic cation, MIBI shows exemplary $\Delta\Psi_m$ -dependant retention in hearts. These experiments with MIBI show that the two-injection protocol is powerful enough to be used as a screen for the uptake of novel gallium-68 lipophilic cations in hearts. These experiments also allowed for the development of the analysis protocol on actual experimental data. As a result of the MIBI experiments, the analysis protocol was changed from normalising using the minimum value in a 15 minute block as a zero point to using the activity between 1–2 minutes before the injection. This was to allow cases where the original bolus of radiotracer begins to leach out of the mitochondria upon depolarisation.

5.4.2 Comparison of Cardiac and Tumour Uptake of [^{68}Ga]Ga-DO3A-xy-TXP

Having established the efficacy of the two-injection protocol with MIBI, the model could be used to screen novel gallium-68-labelled lipophilic cations, beginning with [^{68}Ga]Ga-DO3A-xy-TXP. Unfortunately, this tracer showed effectively no uptake in either normal hearts or hearts under depolarising conditions. This is likely a result of the low lipophilicity of [^{68}Ga]Ga-DO3A-xy-TXP, which has a log D of -1.81 ± 0.02 , especially when compared to the log D value of 1.29 ± 0.15 observed for MIBI.¹³ The unbound carboxylate arm present in Ga-DO3A complexes is the cause of this hydrophilicity, and prevents the complex from passing through any of the lipid bilayer membranes present in the myocardium.

Since [^{68}Ga]Ga-DO3A-xy-TXP did not show myocardial uptake, it was decided to assess the uptake of the radiotracer in cultured breast cancer cells. Analogous [^{64}Cu]Cu-DO3A-xy-TTP complexes were found by Wang *et al.* to display significant uptake in tumours both *in vitro* and *in vivo*, despite the fact that the Cu(II) complex is overall neutrally charged.¹⁴ The uptake of the copper complexes is likely driven by the lipophilicity and positive charge of the TPP cation driving the movement of the more hydrophilic Cu-DO3A moiety across the lipid bilayer membrane. As discussed in Chapter 1, $\Delta\Psi_m$ is elevated in tumour cells, providing additional electromotive force which was proposed to overcome the repulsions between the membrane and the hydrophilic complex.

Unchelated [^{68}Ga]Ga(III) was chosen as a comparison for cultured breast cancer uptake experiments due to the fact that the RCY values for the radiosynthesis of [^{68}Ga]Ga-DO3A-xy-TXP were not quantitative, and therefore unchelated Ga(III) will remain in the reaction mixture. Ga(III) also exhibits a tricationic charge and is therefore potentially subject to uptake by $\Delta\Psi_m$, whilst its small ionic radius also means that it may also be taken up through small ion transport mechanisms in the membrane. The complex displays a three-fold improvement in cellular uptake over the unchelated Ga(III), showing that the uptake is assisted by the increase in lipophilicity and the TXP functionalisation. Reporting the tracer uptake as an intracellular/extracellular concentration ratio takes the relative total volumes of the tumour cells and the cell medium, and functions effectively as an equilibrium constant since a ratio of 1 would equate to complete equilibration of the radiotracer between the cells and the medium, and any increase indicates a preference for the cancer cells.

5.4.3 Cardiac Uptake of [^{68}Ga]Ga-DO2A-(xy-TXP)₂

Unlike its DO3A-based analogue, [^{68}Ga]Ga-DO2A-(xy-TXP)₂ showed significant retention in hearts as a result of its increased lipophilicity. The log *D* measurements of the two complexes demonstrated the effect that the extra TXP group and removal of the pendant carboxylate arm has on the lipophilicity of the complex, and thus its ability to pass through the lipid bilayer membranes in the heart. Fears that the tricationic charge of the complex would hinder cellular uptake due to increased Born interactions and hydrophilicity are assuaged, and demonstrate the potential viability of multiply charged lipophilic cations.

An unexpected result, however, is the retention of the tracer in CCCP-infused hearts, especially when compared to the fact that MIBI, a more lipophilic complex, shows washout in CCCP-infused hearts. When a heart is infused with 600 nM CCCP, $\Delta\Psi_m$ is reduced to zero, and thus any radiotracer retention demonstrated is a result of lipophilic interactions with the membranes. It is believed that this result demonstrates the importance of charge distribution in potential lipophilic cations, as the highly lipophilic and cationic TXP moieties are capable of facilitating transport across the lipid bilayer membrane despite the overall lower lipophilicity of the complex, an effect also manifested in other mitochondrially targeting TPP-functionalised compounds.¹⁵ In contrast, the more symmetrical charge distribution present in MIBI means that it does not possess regions of higher lipophilicity, and as such can pass through lipid bilayer membranes freely, washing out of the membrane when no potential is present.

5.4.4 Cardiac Uptake of [^{68}Ga]Ga-DO2A-Xy₂

[^{68}Ga]Ga-DO2A-Xy₂ shows preferential uptake in healthy hearts compared to CCCP-infused hearts, which is reflected in its higher log *D* value than [^{68}Ga]Ga-DO3A-xy-TXP. It seems that the removal of the pendant carboxylate was enough to facilitate cardiac uptake, even without the assistance afforded by the TXP groups.

The retention of [^{68}Ga]Ga-DO2A-Xy₂ in both healthy and compromised hearts, however, is far lower than was observed for [^{68}Ga]Ga-DO2A-(xy-TXP)₂, despite the increased log *D* value and smaller size of the former indicating that it would likely show preferential uptake. The difference in uptake is attributable to two main factors; firstly the increased charge of [^{68}Ga]Ga-DO2A-(xy-TXP)₂ will facilitate uptake according to the Nernst Equation (Equation

3.1), especially since the $\log D$ values that differences in Born interactions have been mitigated. Secondly, these results highlight that $\log D$ alone is not a perfect indicator of cardiac uptake, since concepts such as charge distribution and shielding must be taken into account. It seems that the TXP functional groups both shield the Ga(III) centre from hydrophilic interactions through their steric bulk compared to the smaller xylyl groups, whilst also providing the complex with regions of higher lipophilicity that may aid its passage across the lipid bilayer membranes of the mitochondria. Despite showing a lower degree of uptake in cardiac tissue than $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TXP)}_2$, $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ shows a similar resolution between healthy and CCCP-infused hearts, with neither being comparable to the gold-standard radiotracer MIBI.

5.5 Conclusions

In this chapter, the cardiac uptake of the three novel gallium-68 complexes $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$, $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TXP)}_2$ and $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ were assessed using the Langendorff isolated perfused heart model, and compared to the gold-standard clinical radiotracer MIBI. $\log D$ values were used as an index for potential uptake in cardiac tissue, however as shown in Figure 5.19 there is no linear relationship between $\log D$ and effectiveness as a myocardial perfusion imaging agent. Indeed, the actual chemistry of lipophilic cations must be considered,

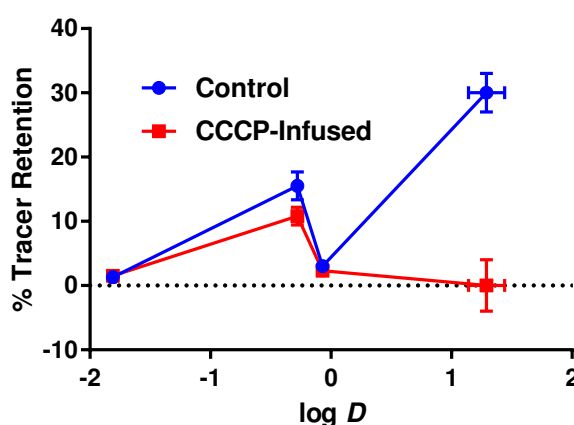


Figure 5.19: Comparison of the $\log D$ values of the four radiotracers studied in the perfused heart (MIBI, $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$, $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TXP)}_2$ and $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$) and their uptake in control hearts and hearts infused with 600 nM CCCP. $\log D$ data are mean $\pm$ SD ($n = 6$ for gallium-68-labelled tracers, $n = 3$ for MIBI)¹³; heart uptake data are mean $\pm$ SD ($n = 3$ for gallium-68-labelled tracers, $n = 4$ for MIBI).

as factors such as cationic charge, shielding of the cationic metal centre and charge distribution across the complex will affect the uptake kinetics drastically. It can be seen from Figure 5.19 that none of the novel gallium-68 complexes come close to the uptake and washout kinetics of MIBI, however both $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TXP)}_2$ and $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ showed a significant difference between uptake in healthy and CCCP-infused hearts, and as such could theoretically be used as PET-based myocardial perfusion imaging agents.

Only $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$ was tested in an *in vitro* breast cancer model, since its low lipophilicity essentially precluded its use in cardiac imaging. These results were promising, showing that the chelator aids uptake in tumour cells when compared to unchelated $[^{68}\text{Ga}]\text{Ga(III)}$. It would be interesting to determine the uptake of the radiotracer in cells with lower $\Delta\Psi_{\text{m}}$, such as non-cancerous cells, cancer cells depolarised by CCCP, or other cancer cell lines in order to determine the specificity of the tracers for cancer cells. In addition, the other gallium-68 tracers with higher $\log D$ values would be interesting to study as a comparison. Nevertheless, this initial experiment shows the potential of these radiotracers for tumour imaging, as well as the versatility of lipophilic and cationic radiotracers for mitochondrial imaging.

5.6 References for Chapter 5

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Chapter 6

Conclusions and Further Work

The primary aim of the work presented in this thesis was to synthesise and investigate a series of chelators to form lipophilic and cationic complexes with gallium-68 for mitochondrial imaging. This thesis was split into three results chapters, firstly describing the synthesis of the chelators and subsequent analysis of their radiochemistry and characterisation in biological models.

6.1 Synthesis of Lipophilic and Cationic Chelators for Gallium-68

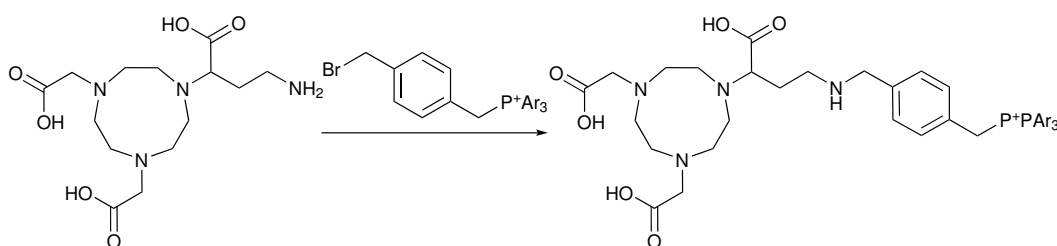
Chapter 3 begins with the synthesis of the DO3A-xy-TAP series of chelators. These syntheses allowed for the optimisation of triarylphosphine formation, as well as cation formation upon reaction with α,α' -dibromo-*m*-xylene. Initial attempts at functionalising DO3A with these cations were performed as one-pot reactions, however splitting this process into two steps aided purification of the compounds. The synthesis of non-aromatic phosphines and linking groups was also explored, albeit unsuccessfully, and this could be considered for finer tuning of complex lipophilicity in the future. Non-radioactive complexes were synthesised, and were found to exhibit one peak by HPLC.

The chapter continues with the synthesis of DO2A-based chelators, with a view to improving lipophilicity through removal of the pendant carboxylate arm in Ga-DO3A complexes. Two routes to the synthesis of DO2A were explored, with the Cbz-protection and hydrogen gas de-protection route proving to be the most effective. Synthesis of DO2A-(xy-TAP)₂ chelators was found to be entirely analogous to their DO3A counterparts, although in this case it was not possible to synthesise the non-radioactive reference complex. In order to study the speciation of the radiolabelled variant, future work should include the synthesis of these complexes. The production of DO2A-based chelators with monocationic charges was achieved by synthesising DO2A-Ar₂ compounds, even if an effective bis-functionalisation route without requiring the synthesis of DO2A diester could not be found.

Using intermediates from the syntheses of DO2A, it was possible to synthesise bridged cyclen variants, with the view that the increased rigidity of the macrocycle would allow for functionalisation with less hydrophilic groups. Less successful was the synthesis of HBED-based chelators, with the use of an aromatic backbone causing the chelator to decompose and TPP-functionalisation drastically lowering the solubility of the chelator. Instead of using an

aromatic backbone, as this seems to destabilise the chelator, cyclohexyl backbones could be explored as a means to instil rigidity upon the final complex.

These syntheses highlight the wide substrate scope of using TAP motifs to tune radiotracer lipophilicity, as the degree of functionalisation does not hinder the synthesis of the final chelators at all. This work also includes the second time that DO2A-based chelators have been used in the literature for gallium-68 chelation, and shows how DO2A can be potentially be used as a chelator for both gallium-68 and other radiometals when DO3A is inappropriate due to its large cavity.



Scheme 6.1: Proposed synthesis of a TAP-functionalised NODAGA derivative.

During the course of the project, work was also performed towards the synthesis of NOTA-based chelators due to its favourable radiolabelling kinetics with gallium-68. This was hindered due to difficulties with the synthesis of TACN, which prevented in-depth study and as such the work is not reported in this thesis. Nevertheless, should TACN be procured in the future through synthesis or commercially, future work could focus on TAP-functionalised NODAGA derivatives, a synthesis and example of which is proposed in Scheme 6.1, as this would allow for six-coordination whilst also providing a lipophilic and cationic portion to facilitate mitochondrial uptake. Other chelators which could be used in the future as a basis for synthesising lipophilic and cationic gallium-68 complexes include H₂dedpa (Figure 6.1a), which could be functionalised on the aromatic rings to tune lipophilicity, as well as on the

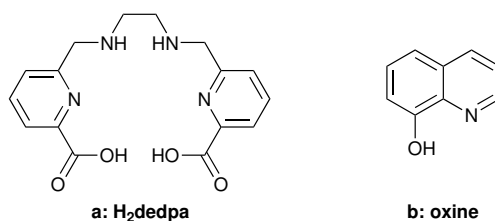


Figure 6.1: Examples of further chelators which could be used in the future to make lipophilic and cationic gallium-68 complexes.

diamine backbone to provide rigidity to the final complex. Whilst this thesis focuses on multi-dentate chelators, smaller ligands such as oxine (Figure 6.1b) could also be explored as long as the stability of their gallium-68 complexes was high enough. The aromatic rings of oxine make it a prime candidate due to its inherent lipophilicity, as well as the ease of its potential functionalisation.

6.2 Radiolabelling of Lipophilic Cations with Gallium-68

Chapter 4 then discusses the radiolabelling of these chelators with generator-produced gallium-68, and assessment of their potential suitability as lipophilic and cationic radiotracers. Work began by synthesising [^{68}Ga]Ga-DO3A-xy-TAP compounds, during which a fast iTLC method was developed for QC of lipophilic cations. Each radiolabelled compound exhibits a single peak by radioHPLC coeluting with the non-radioactive reference compound, demonstrating a lack of speciation and isomerism in the complexes. These radiotracers have low lipophilicities, with [^{68}Ga]Ga-DO3A-xy-TXP showing a $\log D$ value of -1.81 ± 0.02 , a result of the unbound carboxylate arm discussed previously.

The next part of the chapter discusses the radiolabelling of DO2A-(xy-TAP)₂ compounds, with an initial test demonstrating that the radiotracers have superior lipophilicities to their DO3A counterparts, with [^{68}Ga]Ga-DO2A-(xy-TXP)₂ showing a $\log D$ value of -0.28 ± 0.01 . It was discovered that the RCY values obtained could be optimised through alteration of the radiolabelling buffer, with sodium acetate providing better results than ammonium acetate. RadioHPLC analysis demonstrated the formation of kinetic and thermodynamic products in the reaction mixture, and experiments involving the alteration of reaction parameters found that whilst one species could be favoured through the use of thermodynamically forcing conditions, it was not possible to only form one species. This effect was most striking in the calculation of $\log D$ values for [^{68}Ga]Ga-DO2A-(xy-TXP)₂ over a prolonged period of heating, with the more lipophilic thermodynamic species being favoured over time. As discussed previously, future work in this area should focus on the synthesis of non-radioactive reference compounds, in order to determine if the speciation arises from *cis/trans* isomerism or sequential binding of carboxylate groups.

Despite the lower charge and steric bulk of the aryl-functionalisation seen in [^{68}Ga]Ga-

DO2A-Ar₂, they exhibited a similar degree of speciation upon radiolabelling to their TAP-functionalised counterparts. It is unclear whether the radiolabelling kinetics of [⁶⁸Ga]Ga-DO2A-Ar₂ were more or less favourable, with the RCY values of these compounds being less sensitive to ligand concentration but more sensitive to reaction time and temperature. Nevertheless, these complexes showed the most favourable log *D* values of any studied, with [⁶⁸Ga]Ga-DO2A-Xy₂ showing a log *D* of -0.07 ± 0.01 . Whilst a tolyl variant of these compounds (Figure 6.2) was not synthesised in this thesis, future work could explore its synthesis to determine whether it displays a similar speciation profile to its TTP-functionalised counterpart.

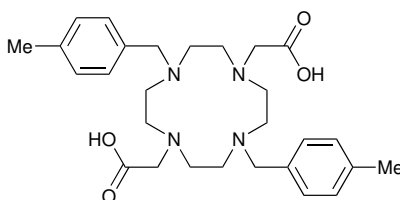


Figure 6.2: DO2A-Tol₂, the final variant of the DO2A-Ar₂ series to synthesise.

The final part of this chapter discusses the radiolabelling of the bridged cyclens. It was discovered that the dibenzyl-functionalised variant was not able to chelate gallium, indicating that bridged cyclens require additional Lewis acidic groups to bind the metal centre. The diacid variant was able to chelate gallium-68, with most effective results observed when the reaction was performed in 0.2 M NaOAc. Unfortunately, the radiotracer showed a log *D* value of -3.88 ± 0.22 , lower than the value for unchelated [⁶⁸Ga]Ga(III), and as such was not studied further. Indeed, screening of the chelators through the calculation of the log *D* values of their gallium-68 complexes was the main purpose of the radiolabelling experiments, to provide a metric for whether the complexes were worth studying biologically. Future development of the bridged cyclens could involve the use of larger macrocycles such as cyclam to try and bind the gallium without the need for additional Lewis acidic groups, or through the use of less hydrophilic Lewis acidic groups.

6.3 Biological Evaluation of Radiolabelled Lipophilic Cations

The final results chapter investigated the behaviour of the radiotracers in preclinical biological models. The first model used was the Langendorff isolated perfused heart model with

triple-gamma detector system. Using the gold standard lipophilic cationic myocardial perfusion imaging agent MIBI, a baseline for cardiac radiotracer uptake was established using a fast and efficient two-injection protocol for the screening of lipophilic cations. The most lipophilic examples of each gallium-68-labelled radiotracer series were screened using this model; firstly [^{68}Ga]Ga-DO3A-xy-TXP did not show appreciable uptake in accordance with $\Delta\Psi_m$. The other two radiotracers assessed using this model, [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$ and [^{68}Ga]Ga-DO2A-Xy $_2$, did show $\Delta\Psi_m$ -dependant retention in cardiac tissue, however the former showed a far higher degree of uptake in both healthy and mitochondrially-depolarised tissue. As such, these tracers could potentially be used for myocardial perfusion imaging, however neither tracer comes close to the quality of the imaging afforded by MIBI. During this project, it has been shown that despite our initial hypothesis, no clear linear relationship exists between $\log D$ values and cardiac uptake characteristics. As such, whilst $\log D$ is useful as a screen for radiotracers, chemical characteristics such as charge distribution must be carefully considered when designing novel lipophilic and cationic myocardial perfusion imaging agents.

Due to its low uptake in cardiac tissue, [^{68}Ga]Ga-DO3A-xy-TXP was also assessed in tumour cells *in vitro*, as this is the biological field for which DO3A-xy-TXP radiometal complexes have been previously used. [^{68}Ga]Ga-DO3A-xy-TXP follows in this vein, showing appreciable uptake in tumour cells when compared to unchelated [^{68}Ga]Ga(III). The low uptake of this radiotracer in cardiac tissue as explored previously may hint towards its potential use for tumour imaging *in vivo*, as it is likely to show low uptake in non-cancerous tissue and thus favourable biodistribution characteristics.

The future biological work for this class of tracers is extensive. Due to the positive result for tumour uptake *in vitro* for [^{68}Ga]Ga-DO3A-xy-TXP, extension of this study to the other two gallium-68 radiotracers with their higher lipophilicity could lead to the discovery of a novel tumour imaging agent. [^{68}Ga]Ga-DO2A-Xy $_2$ shows more promise in this regard due to its lower uptake in healthy cardiac tissue. It would also be useful to assess the uptake of the radiotracers in mitochondrially depolarised tumour cells in order to ensure that uptake is $\Delta\Psi_m$ dependant. Tracers demonstrating good uptake characteristics in these tests could potentially move on to *in vivo* tumour imaging studies.

Future work using the perfused heart model should include a full study of the two tracers which showed promise from the screening studies, namely [^{68}Ga]Ga-DO2A-(xy-TXP) $_2$ and

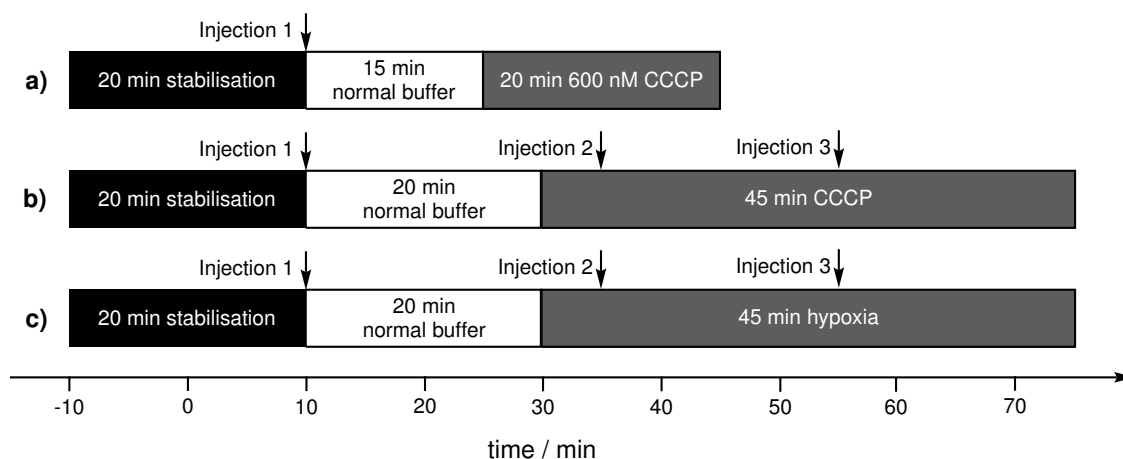


Figure 6.3: **a:** Timetable for the perfusion experiments performed in this thesis. **b and c:** Proposed future perfusion experiment timetables, for assessing the sensitivity of the radiotracers to mitochondrial depolarisation and cardiac hypoxia respectively.

[^{68}Ga]Ga-DO2A-Xy₂. Potential test timetables for the assessment of these tracers are shown in Figure 6.3. There should be a move towards the more traditional three-injection protocols, as these allow for the inclusion of time-matched controls as well as a more in-depth analysis of the behaviour of the radiotracers in cardiac tissue. Figure 6.3b outlines an experiment in which varying concentrations of CCCP are used to determine the sensitivity of the cardiac uptake of the radiotracer to the magnitude of mitochondrial depolarisation. Figure 6.3c outlines an experiment in which the effects of an alternate intervention, in this case the use of hypoxic buffer to induce hypoxic conditions on the heart, on the retention of radiotracers in the cardiac tissue.

Should any tracers show promise after this point, *in vivo* cardiac uptake studies could be performed. Nevertheless, the data and observations of the biological characteristics of the novel radiotracers contained in this thesis should feedback to the initial chemical design of future gallium-68-labelled lipophilic and cationic radiotracers.

Chapter 7

Experimental

7.1 Synthetic Chemistry (Chapter 3)

7.1.1 General

7.1.1.1 Conditions and Materials

All reactions were carried out at atmospheric pressure with stirring unless otherwise stated. No special precautions were taken to exclude air or moisture during reactions, work-ups or purifications unless otherwise stated. All solvents and reagents were obtained from commercial sources and used without further purification. Compounds **2c**, **2d**¹, tri-^tBu-DO3A², **6**³, **7**, DO2Adiester⁴, and cyclen glyoxal (**8**)⁵ were prepared by literature procedures from commercially available starting materials.

7.1.1.2 Chromatography

TLC was performed on Kieselgel 60 F₂₅₄ plates (Merck) and visualisation achieved under UV fluorescence (254 and 366 nm) or I₂ vapour. Silica was used for chromatographic separations. Semi-preparative reverse-phase HPLC was performed on an Agilent 1200 Series Liquid Chromatograph with UV using an Agilent Eclipse XDB-C18 column (21.2 × 150 mm, 5 μm) with a 5 mL min⁻¹ flow rate and UV spectroscopic detection at 214 nm. Reverse phase flash chromatography was performed on a Biotage Isolera Four with SNAP KP-C18-HS cartridges. For reverse-phase chromatography, mobile phase A contained water with 0.1% TFA, and mobile phase B contained MeCN with 0.1% TFA.

7.1.1.3 NMR

¹H, ¹³C{¹H}, ¹⁹F{¹H} and ³¹P{¹H} NMR spectra were recorded at ambient temperature on a Bruker 400 MHz spectrometer. ¹H, ¹³C{¹H} spectra were internally referenced to the residual solvent peaks of CDCl₃ at δ = 7.26 (¹H) and 77.2 ppm (¹³C{¹H}); MeOD at δ = 3.31 (¹H) and 49.2 ppm (¹³C{¹H}); D₂O at δ = 4.79 (¹H); DMSO-*d*₆ at δ = 2.50 (¹H) and 39.5 (¹³C{¹H});⁶ and external CFCl₃ and H₃PO₄ standards for ¹⁹F{¹H} and ³¹P{¹H} NMR spectra respectively. ¹³C{¹H} were assigned where possible using 2D correlation and Distortionless Enhancement by Polarisation Transfer (DEPT) spectroscopy. Additional NMR experiments were performed by Mr. P. Haycock, ¹H-³¹P{¹H} HMBC experiments were performed on a Bruker DRX 400

spectrometer. Spectra were processed and analysed using Mestrenova software. The signal multiplicities are indicated as: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), ddd (doublet of doublets of doublets), m (multiplet); defined as all multi-peak signals where overlap or complex coupling of signals makes definitive descriptions of peaks difficult. Coupling constants are defined as J given in Hz quoted to one decimal place.

7.1.1.4 Mass Spectrometry

High resolution ES+ mass spectrometry analyses were conducted by Dr. L. Haigh at the Mass Spectrometry Service, Imperial College London on a Waters LCT Premier (ES-ToF)/Acquity i-Class spectrometer.

7.1.1.5 Additional

pH measurements were carried out using a Jenway model 3510 pH/mV/temperature meter, calibrated against 'NIST' pH 10.00, pH 7.00 and pH 4.00 buffers. Elemental analyses were performed by Mr. Stephen Boyer at London Metropolitan University. Room temperature (rt) is defined as between 19–22 °C. 'Reduced pressure' is used to describe solvent removal by Büchi rotary evaporation between 20–50 °C at approximately 10 mmHg unless otherwise stated, '*in vacuo*' is used to describe solvent removal at approximately 0.75 mmHg. 'Degassed' refers to the process of removing O₂ from a solution by bubbling N₂ through the solution in the reaction vessel prior to use.

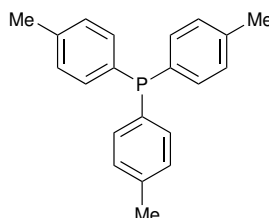
7.1.2 DO3A-xy-TAP (Section 3.1)

7.1.2.1 General Procedure 1 - Synthesis of Aryl-Functionalised Triphenylphosphines

The methodology was a modification of the procedure described by Haslop *et al.*¹ A solution of the aryl bromide (3 eqv) in anhydrous THF (10 mL) was added dropwise to magnesium turnings (3 eqv) under an N₂ atmosphere whilst maintaining reflux. The resulting suspension was stirred for 2 h before a solution of PCl₃ (1 eqv) in anhydrous THF (15 mL) was added, and the resulting mixture was stirred for a further 2 h. The reaction was quenched with water (10 mL), and the aqueous phase was removed using a separating funnel. Removal of the

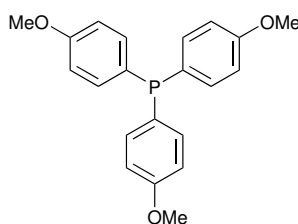
organic solvent under reduced pressure yielded the desired product.

Tri-*p*-tolylphosphine (2b)⁷



This compound was originally synthesised by Mann *et al.*⁷ Following **general procedure 1**, the title compound was prepared from 4-bromotoluene (2.89 mL, 23.5 mmol), magnesium turnings (0.57 g, 23.5 mmol) and PCl₃ (0.69 mL, 7.8 mmol) as a pale yellow solid of 94% purity (determined by ³¹P {¹H} NMR) (0.96 g, 40%). ¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 7.24 (6H, t, ³J_{HH} = 7.9 Hz), 7.17 (6H, d, ³J_{HH} = 7.5 Hz), 2.38 (9H, s, CH₃). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_C (ppm): 138.6 (*p*-Ar), 133.9 (d, ¹J_{CP} = 20.6 Hz, *i*-Ar), 132.2 (d, ²J_{CP} = 9.7 Hz, *o*-Ar), 129.4 (d, ³J_{CP} = 7.0 Hz, *m*-Ar), 21.4 (CH₃). ³¹P{¹H}-NMR (162 MHz, CDCl₃) δ_P (ppm): -7.9. HRMS-TOF+ MS (*m/z*) calcd for C₂₁H₂₂P ([M+H]⁺) 305.1459. found: 305.1445.

Tris(4-methoxyphenyl)phosphine (2e)⁷



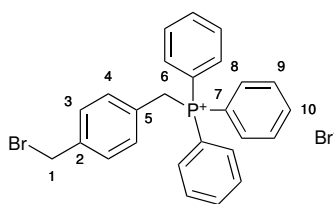
This compound was originally synthesised by Mann *et al.*⁷ Following **general procedure 1**, the title compound was prepared from 4-bromoanisole (2.94 mL, 23.5 mmol), magnesium turnings (0.57 g, 23.5 mmol) and PCl₃ (0.69 mL, 7.8 mmol) as a yellow solid (0.25 g, 9%). ¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 7.24–7.20 (6H, m, *o*-Ar), 6.89–6.85 (6H, m, *m*-Ar), 3.80 (9, s, OCH₃). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_C (ppm): 160.1 (*p*-Ar), 134.9 (d, ²J_{CP} = 20.9 Hz, *o*-Ar), 128.8 (*i*-Ar), 114.1 (d, ³J_{CP} = 7.8 Hz, *m*-Ar), 55.2 (CH₃). ³¹P{¹H}-NMR

(162 MHz, CDCl₃) δ_P (ppm): -10.2. HRMS-TOF+ MS (m/z) calcd for C₂₁H₂₂O₄P ([M+OH]⁺) 369.1250. found: 369.1246.

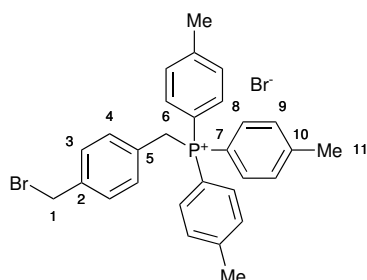
7.1.2.2 General Procedure 2 - Monosubstitution of α,α' -Dibromo-*p*-xylene with Aryl-Functionalised Triphenylphosphines

The methodology was a modification of the procedure described by Wang *et al.*⁸ α,α' -Dibromo-*p*-xylene (1 eqv) was added to toluene (5 mL) and heated to 60 °C until all the solid was dissolved. To this, a solution of the triarylphosphine (1 eqv) in toluene (6 mL) was added dropwise, before the resulting solution was stirred at 60 °C under an N₂ atmosphere overnight. The white precipitate was isolated by filtration, before being washed with toluene (5 mL) and Et₂O (2 × 10 mL), then dried *in vacuo* to yield the desired product.

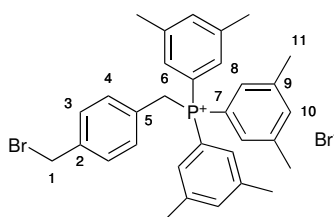
(4-(Bromomethyl)benzyl)triphenylphosphonium bromide (3a)⁸



This compound was originally synthesised by Wang *et al.*⁸ Following **general procedure 2**, the title compound was prepared from α,α' -dibromo-*p*-xylene (0.53 g, 2 mmol) and triphenylphosphine (0.54 g, 2 mmol) as a white solid (0.86 g, 82%). ¹H NMR (400 MHz, CDCl₃) δ_H (ppm): 7.73 (9H, m, H_{9/10}), 7.60 (6H, m, H₈), 7.11 (4H, d, ³J_{HH} = 1.3 Hz, H_{3/4}), 5.50 (2H, d, ²J_{HP} = 14.6 Hz, H₆), 4.37 (2H, d, ⁴J_{HH} = 1.3 Hz, H₁). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_C (ppm): 138.2 (d, ⁴J_{CP} = 4.4 Hz, C₁₀), 135.1 (d, ⁵J_{CP} = 2.8 Hz, C₂), 134.5 (d, ²J_{CP} = 9.3 Hz, C₈), 132.1 (d, ³J_{CP} = 6.1 Hz, C₄), 130.3 (d, ³J_{CP} = 6.1 Hz, C₉), 129.5 (d, ⁴J_{CP} = 3.5 Hz, C₃), 127.9 (d, ³J_{CP} = 8.8 Hz, C₂), 117.9 (d, ¹J_{CP} = 85.9 Hz, C₇), 33.0 (C₁), 30.5 (d, ¹J_{CP} = 47.2 Hz, C₆). ³¹P{¹H}-NMR (162 MHz, CDCl₃) δ_P (ppm): 23.5. HRMS (ES-TOF+): m/z calcd. for C₂₆H₂₃BrP ([M-Br]⁺) 445.0721, found 445.0737.

(4-(Bromomethyl)benzyl)tri-*p*-tolylphosphonium bromide (3b)

Following **general procedure 2**, the title compound was prepared from α,α' -dibromo-*p*-xylene (0.53 g, 2 mmol) and tri-*p*-tolylphosphine (0.72 g, 2.37 mmol) as a white solid (1.04 g, 92%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.54 (6H, m, H_9), 7.38 (6H, dd, $^3J_{\text{HP}} = 8.2$, $^3J_{\text{HH}} = 3.2$ Hz, H_8), 7.13 (2H, d, $^3J_{\text{HH}} = 8.1$ Hz, H_3), 7.08 (2H, dd, $^3J_{\text{HH}} = 8.4$, $^4J_{\text{HP}} = 2.4$ Hz, H_4), 5.24 (2H, d, $^2J_{\text{HP}} = 14.5$ Hz, H_6), 4.38 (2H, s, H_1), 2.44 (9H, s, H_{11}). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 146.3 (d, $^5J_{\text{CP}} = 2.6$ Hz, C_2), 138.1 (d, $^4J_{\text{CP}} = 3.9$ Hz, C_{10}), 134.3 (d, $^2J_{\text{CP}} = 10.4$ Hz, C_8), 132.0 (d, $^3J_{\text{CP}} = 5.7$ Hz, C_4), 130.4 (d, $^3J_{\text{CP}} = 3.4$ Hz, C_9), 129.5 (d, $^4J_{\text{CP}} = 3.6$ Hz, C_3), 128.1 (d, $^2J_{\text{CP}} = 8.6$ Hz, C_2), 114.6 (d, $^1J_{\text{CP}} = 88.7$ Hz, C_7), 33.1 (C_1), 30.9 (d, $^1J_{\text{CP}} = 48.1$ Hz, C_6), 22.0 (C_{11}). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 22.4. HRMS (ES-TOF+): m/z calcd. for $\text{C}_{29}\text{H}_{29}\text{PBr}$ ($[\text{M}-\text{Br}]^+$) 529.1660, found 529.1669. Anal. calcd. for $\text{C}_{29}\text{H}_{29}\text{PBr}_2$ (found): C 61.3 (61.2); H 5.1 (5.3)%.

(4-(Bromomethyl)benzyl)tris(3,5-dimethylphenyl)phosphonium bromide (3c)

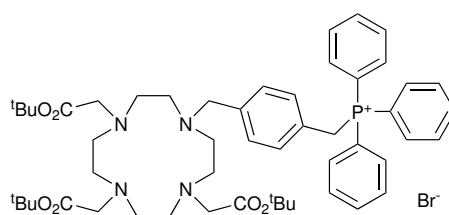
Following **general procedure 2**, the title compound was prepared from α,α' -dibromo-*p*-xylene (0.53 g, 2 mmol) and tris(3,5-dimethylphenyl)phosphine (0.87 g, 2 mmol) as a white solid (2.74 g, 88%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.34 (3H, s, H_{10}), 7.21 (6H, d, $^3J_{\text{HP}} = 13.0$ Hz, H_8), 7.15 (2H, d, $^3J_{\text{HH}} = 7.6$ Hz, H_3), 7.02 (2H, dd, $^3J_{\text{HH}} = 8.0$ Hz, $^4J_{\text{HP}} = 2.7$ Hz, H_4), 5.21 (2H, d, $^2J_{\text{HP}} = 14.3$ Hz, H_6), 4.40 (2H, s, H_1), 2.36 (18H, s, H_{11}).

$^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 140.3 (d, $^2J_{\text{CP}} = 12.3$ Hz, C_5), 138.0 (d, $^5J_{\text{CP}} = 3.5$ Hz, C_2), 136.8 (d, $^4J_{\text{CP}} = 3.5$ Hz, C_3), 132.1 (d, $^3J_{\text{CP}} = 5.1$ Hz, C_4), 131.7 (d, $^2J_{\text{CP}} = 10.1$ Hz, C_8), 129.3 (d, $^4J_{\text{CP}} = 3.5$ Hz, C_{10}), 128.5 (d, $^3J_{\text{CP}} = 8.7$ Hz, C_9), 117.8 (d, $^1J_{\text{CP}} = 84.5$ Hz, C_7), 33.1 (C_1), 30.9 (d, $^1J_{\text{CP}} = 46.6$ Hz, C_6), 21.5 (C_{11}). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 22.7. HRMS (ES-TOF+): m/z calcd. for $\text{C}_{32}\text{H}_{35}\text{PBr}$ ($[\text{M-Br}]^+$) 529.1660, found 529.1643. Anal. calcd. for $\text{C}_{32}\text{H}_{35}\text{PBr}_2$ (found): C, 62.97 (62.98); H, 5.78 (5.73)%.

7.1.2.3 General Procedure 3 - Coupling of Triarylphosphine-Substituted Bromoxylene to DO3Atriester Hydrobromide

The methodology was a modification of the procedure described by Kardashinsky *et al.*⁹ To a solution of DO3Atriester.HBr (1 eqv) and triarylphosphane-substituted bromoxylene (1 eqv) in MeCN (15 mL), Na_2CO_3 (5 eqv) was added, and the resulting suspension was heated to reflux and stirred under an N_2 atmosphere overnight. The inorganic base was removed by filtration and the solvent removed under reduced pressure. The resulting residue was recrystallised from acetone/ Et_2O to yield the desired product.

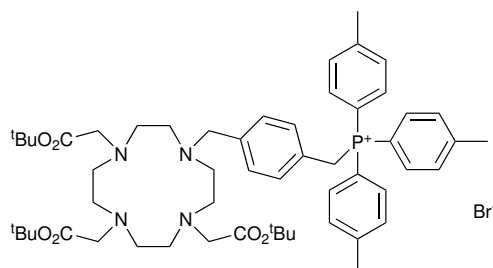
Triphenyl(4-((4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium bromide (4a)⁹



This compound was originally synthesised by Kardashinsky *et al.*⁹ Following **general procedure 3**, the title compound was prepared from DO3Atriester.HBr (0.30 g, 0.5 mmol), **3a** (0.26 g, 0.5 mmol) and Na_2CO_3 (0.26 g, 2.5 mmol) as a white solid (0.37 g, 76%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.78 (3H, tt, $^3J_{\text{HH}} = 6.9$ Hz, $^4J_{\text{HH}} = 1.7$ Hz, $p\text{-PAR}_3\text{H}$), 7.74–7.63 (12H, m, $o/m\text{-PAR}_3\text{H}$), 7.05–7.04 (4H, m, C_6H_4), 5.33 (2H, broad s, PCH_2), 3.64 (2H, s, $\text{C}_6\text{H}_4\text{CH}_2$), 3.41–2.11 (22H, m, ring $\text{H}/\text{CH}_3\text{CO}_2$), 1.47 (18H, s, *cis* $\text{C}(\text{CH}_3)_3$), 1.43 (9H, s, *trans* $\text{C}(\text{CH}_3)_3$). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 173.7 ($\text{C}=\text{O}$), 172.7 ($\text{C}=\text{O}$), 135.6 (d, $^5J_{\text{CP}} = 4.0$ Hz), 135.3 (d, $^4J_{\text{CP}} = 2.7$ Hz), 134.4 (d, $^3J_{\text{CP}} = 9.8$ Hz), 131.6 (d, $^3J_{\text{CP}} =$

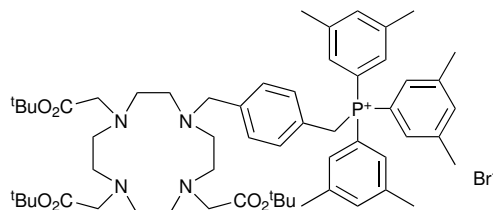
5.3 Hz), 130.9 (d, $^4J_{CP} = 1.8$ Hz), 130.4 (d, $^2J_{CP} = 12.8$ Hz), 129.9 (d, $^2J_{CP} = 8.7$ Hz), 117.1 (d, $^1J_{CP} = 86.0$ Hz), 83.1 (*cis* $\underline{C}(\text{CH}_3)_3$), 82.6 (*trans* $\underline{C}(\text{CH}_3)_3$), 57.9, 57.0, 50.9, 30.8 (d, $^1J_{CP} = 46.5$ Hz, $\underline{PCH}_2$), 28.2 ($\underline{C}(\text{CH}_3)_3$), 28.0 ($\underline{C}(\text{CH}_3)_3$). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 23.1. HRMS (ES-TOF+): m/z calcd. for $\text{C}_{52}\text{H}_{72}\text{N}_4\text{O}_6\text{P}$ ($[\text{M}-\text{Br}]^+$) 879.5190, $\text{C}_{52}\text{H}_{73}\text{N}_4\text{O}_6\text{P}$ ($[\text{M}-\text{Br}+\text{H}]^{2+}$) 440.2628, $\text{C}_{52}\text{H}_{74}\text{N}_4\text{O}_6\text{P}$ ($[\text{M}-\text{Br}+2\text{H}]^{3+}$) 293.8443. found: 879.5258, 440.2600, 293.8420.

Tri-*p*-tolyl(4-((4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium bromide (4b)



Following **general procedure 3**, the title compound was prepared from DO3Atriester.HBr (0.30 g, 0.5 mmol), **3b** (0.28 g, 0.5 mmol) and Na_2CO_3 (0.26 g, 2.5 mmol) as a white solid (0.23 g, 47%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.57–7.52 (6H, m, *o*-Ar), 7.43 (6H, dd, $^3J_{\text{HH}} = 8.3$, $^4J_{\text{HH}} = 3.3$ Hz, *m*-Ar), 7.07 (2H, dd, $^3J_{\text{HH}} = 8.3$, $^4J_{\text{HH}} = 2.3$ Hz, C_6H_4), 7.03 (2H, d, $^3J_{\text{HH}} = 8.1$ Hz, C_6H_4), 5.18 (2H, d, $^2J_{\text{HP}} = 14.4$ Hz, $\underline{\text{CH}_2\text{P}}$), 3.68 (2H, s, $\text{C}_6\text{H}_4\underline{\text{CH}_2}$), 3.43–2.18 (16H, m, macrocycle $\underline{\text{H}}$), 3.04–3.00 (6H, m, $\underline{\text{CH}_2\text{CO}_2}$), 2.47 (9H, s, ArCH_3), 1.48 (18H, s, *cis* $\underline{\text{C}}(\text{CH}_3)_3$), 1.43 (9H, s, *trans* $\underline{\text{C}}(\text{CH}_3)_3$). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 173.7 ($\underline{\text{C}}=\text{O}$), 172.7 ($\underline{\text{C}}=\text{O}$), 146.4 ($\underline{\text{C}}_6\text{H}_4$), 135.6 ($\underline{\text{C}}_6\text{H}_4$), 134.3 (d, $^2J_{CP} = 10.3$ Hz, *o*-Ar), 131.5 (d, $^4J_{CP} = 6.4$ Hz, $\underline{\text{C}}_6\text{H}_4$), 131.0 (d, $^3J_{CP} = 12.5$ Hz, $\underline{\text{C}}_6\text{H}_4$), 130.8 (*m*-Ar), 127.2 (d, $^4J_{CP} = 8.5$ Hz, *p*-Ar), 114.6 (d, $^1J_{CP} = 88.7$ Hz, *i*-Ar), 83.0 ($\underline{\text{C}}(\text{CH}_3)_3$), 82.6 ($\underline{\text{C}}(\text{CH}_3)_3$), 58.0 ($\text{C}_6\text{H}_4\underline{\text{CH}_2}$), 56.9 ($\underline{\text{CH}_2\text{CO}_2}$), 56.0 ($\underline{\text{CH}_2\text{CO}_2}$), 51.0 (broad, macrocycle $\underline{\text{C}}$), 49.4 (broad, macrocycle $\underline{\text{C}}$), 31.1 (d, $^1J_{CP}$, $\underline{\text{CH}_2\text{P}}$), 28.2 (*cis* $\underline{\text{C}}(\text{CH}_3)_3$), 28.0 (*trans* $\underline{\text{C}}(\text{CH}_3)_3$), 22.0 (Ar $\underline{\text{CH}_3}$). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 22.1. HRMS (ES-TOF+): m/z calcd. for $\text{C}_{55}\text{H}_{78}\text{N}_4\text{O}_6\text{P}$ ($[\text{M}-\text{Br}]^+$) 921.5659, $\text{C}_{55}\text{H}_{79}\text{N}_4\text{O}_6\text{P}$ ($[\text{M}-\text{Br}+\text{H}]^{2+}$) 461.2863. found: 921.5684, 461.2828.

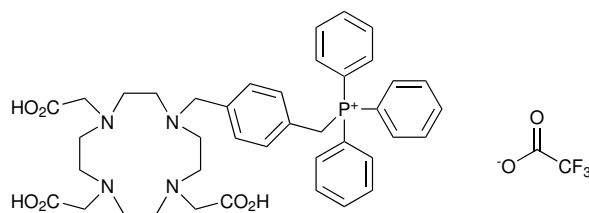
Tris(3,5-dimethylphenyl)(4-((4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium bromide (4c)



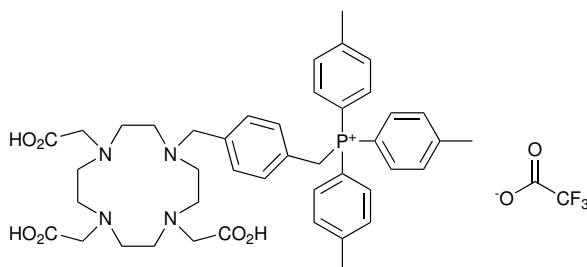
Following **general procedure 3**, the title compound was prepared from DO3Atriester.HBr (0.30 g, 0.5 mmol), **3c** (0.31 g, 0.5 mmol) and Na₂CO₃ (0.26 g, 2.5 mmol) as a white solid (0.36 g, 70%). ¹H NMR (400 MHz, CDCl₃) δ_H (ppm): 7.35 (3H, s, *p*-Ar), 7.21 (6H, d, ³*J*_{HP} = 13.1 Hz, *o*-Ar), 7.11 (2H, d, ³*J*_{HH} = 8.0 Hz), 7.05 (2H, dd, ³*J*_{HH} = 8.2, ⁴*J*_{HP} = 2.4 Hz), 5.15 (2H, d, ²*J*_{HP} = 14.2 Hz, CH₂P), 3.68 (2H, s), 3.05 (2H, s), 3.01 (4H, s), 2.97 - 2.16 (16H, m, macrocycle H), 2.36 (18H, s, Ar CH₃), 1.48 (18H, s, *cis* C(CH₃)₃), 1.44 (9H, s, *trans* C(CH₃)₃). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_C (ppm): 173.6 (C=O), 172.7 (C=O), 140.2 (d, ²*J*_{CP} = 12.6 Hz, C₆H₄), 136.8 (*p*-Ar), 135.9 (C₆H₄), 131.6 (*o*-Ar), 131.5 (C₆H₄), 130.7 (C₆H₄), 127.5 (d, ³*J*_{CP} = 8.3 Hz, *m*-Ar), 117.7 (d, ¹*J*_{CP} = 84.9 Hz, *i*-Ar), 82.9 (CH₂CO₂), 82.5 (CH₂CO₂), 57.9 (C₆H₄CH₂), 56.7 (CH₂CO₂), 55.9 (CH₂CO₂), 50.8 (broad s, macrocycle C), 49.2 (broad s, macrocycle C), 30.9 (d, ¹*J*_{CP} = 48.5 Hz, CH₂P), 28.1 (*cis* C(CH₃)₃), 27.9 (*trans* C(CH₃)₃), 21.5 (Ar CH₃). ³¹P{¹H}-NMR (162 MHz, CDCl₃) δ_P (ppm): 22.4. HRMS (ES-TOF+): *m/z* calcd. for C₅₈H₈₄N₄O₆P ([M-Br]⁺) 963.6129, C₅₈H₈₅N₄O₆P ([M-Br+H]²⁺) 482.3098. found: 963.6139, 482.2990.

7.1.2.4 General Procedure 4 - Trifluoroacetic Acid Deprotection of *Tert*-Butyl Protected DO3A-xy-TAP Compounds

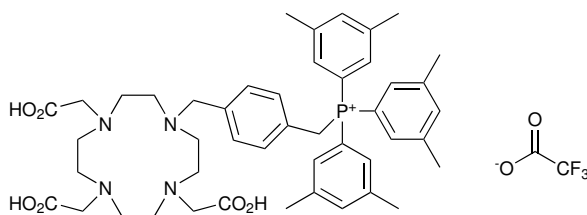
The methodology was a modification of the procedure described by Kardashinsky *et al.*⁹ *Tert*-butyl protected DO3A-xy-TAP compounds were dissolved in trifluoroacetic acid (reaction mixture concentration 0.1 g mL⁻¹) and stirred at rt under an N₂ atmosphere for 24 h. The acid was removed *in vacuo*, before the residue was taken up in H₂O (50 mL) and washed with CHCl₃ (3 × 10 mL). The aqueous solvent was removed under reduced pressure, before the residue was purified by HPLC using a C-18 reverse phase semi-preparative column.

DO3A-xy-TPPCF₃CO₂ (5a)⁹

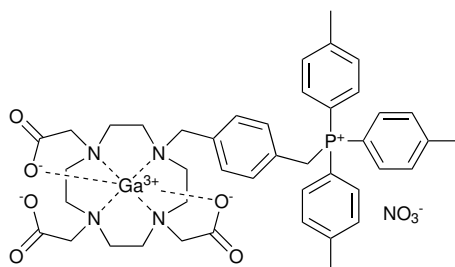
This compound was originally synthesised by Kardashinsky *et al.*⁹ Following **general procedure 4**, the title compound was prepared from **4a** (0.25 g, 0.26 mmol) and trifluoroacetic acid (2.5 mL) and purification by semi-preparative reverse-phase HPLC (Eluent gradient 0–100% B in A for 50 min, isocratic 100% B for 10 min, at a flow rate of 5 mL min⁻¹) as a white solid (0.16 g, 73%). ¹H NMR (400 MHz, MeOD) δ_{H} (ppm): 7.91 (3H, td, ³*J*_{HH} = 7.3, ⁴*J*_{HH} = 1.8 Hz, *p*-Ar), 7.78 - 7.62 (12H, m, *o,m*-Ar), 7.51 (2H, d, ³*J*_{HH} = 7.8 Hz, C₆H₄), 7.08 (2H, dd, ³*J*_{HH} = 8.0 Hz, ⁴*J*_{HP} = 2.7 Hz, C₆H₄), 4.95 (2H, d, ²*J*_{HP} = 15.1 Hz, CH₂P), 4.34 (2H, s), 3.97 (2H, s), 3.64 (2H, s), 3.39 (4H, s), 3.29 - 3.02 (16H, m). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_{C} (ppm): 162.8 (C=O), 162.5 (C=O), 136.6 (*p*-Ar), 135.3 (³*J*_{CP} = 9.1 Hz, *m*-Ar), 133.2 (³*J*_{CP} = 5.2 Hz, C₆H₄), 131.5 (²*J*_{CP} = 13.6 Hz, *o*-Ar), 119.6 (C₆H₄), 119.0 (¹*J*_{CP} = 86.9 Hz, *i*-Ar), 116.7 (C₆H₄), 58.5, 55.6, 54.1, 50.5 (broad s), 50.4 (broad s), 30.3 (¹*J*_{CP} = 48.6 Hz, CH₂P). ¹⁹F{¹H}-NMR (377 MHz, MeOD) δ_{F} (ppm): -73.0. ³¹P{¹H}-NMR (162 MHz, MeOD) δ_{P} (ppm): 26.8. HRMS (ES-TOF+): *m/z* calcd. for C₄₀H₄₈N₄O₆P ([M-CF₃CO₂]⁺) 711.3311, C₄₀H₄₉N₄O₆P ([M-CF₃CO₂+H]²⁺) 356.1689. found: 711.3320, 356.1727. Retention time: 20–22 min.

DO3A-xy-TTPCF₃CO₂ (5b)

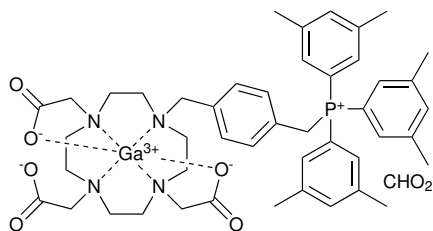
Following **general procedure 4**, the title compound was prepared from **4b** (0.21 g, 0.23 mmol) and trifluoroacetic acid (2.1 mL) and purification by semi-preparative reverse-phase HPLC (Eluent gradient 0–100% B in A for 100 min, isocratic 100% B for 20 min, at a flow rate of 5 mL min⁻¹) as a white solid (0.16 g, 73%). as a white solid (0.10 g, 57%). ¹H-NMR (400 MHz, MeOD) δ_{H} (ppm): 7.58–7.52 (12H, m, *o/m*-Ar), 7.47 (2H, d, ³*J*_{HH} = 8.2 Hz, C₆H₄), 7.09 (2H, broad s, C₆H₄), 4.83 (2H, d, ²*J*_{HP} = 15.3 Hz, CH₂P), 4.48 (2H, broad s), 4.15 (2H, broad s), 3.78–3.43 (4H, m, CH₂CO₂H), 3.29–2.90 (16H, m, macrocycle H). 2.50 (9H, s, CH₃). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_{C} (ppm): 174.2 (C=O), 171.7 (C=O), 148.2 (*p*-Ar), 135.2 (d, ³*J*_{CP} = 10.3 Hz, C₆H₄), 133.2 (d, ⁴*J*_{CP} = 5.4 Hz, C₆H₄), 132.0 (d, ²*J*_{CP} = 12.4 Hz, *o*-Ar), 121.9 (C₆H₄), 119.0 (*m*-Ar), 115.7 (d, ¹*J*_{CP} = 90.2 Hz, *i*-Ar), 113.2 (C₆H₄), 58.6, 55.4, 53.6 (CH₂CO₂H), 50.8 (broad, macrocycle C), 30.7 (d, ¹*J*_{CP} = 48.7 Hz, CH₂P), 21.8 (CH₃). ¹⁹F{¹H}-NMR (377 MHz, MeOD) δ_{F} (ppm): -77.3. ³¹P{¹H}-NMR (162 MHz, MeOD) δ_{P} (ppm): 22.0. HRMS (ES-TOF+): *m/z* calcd. for C₄₃H₅₄N₄O₆P ([M-CF₃CO₂]⁺) 753.3781, C₄₃H₅₅N₄O₆P ([M-CF₃CO₂+H]²⁺) 377.1924. found: 753.3786, 377.2102. Retention time: 39–41 min.

DO3A-xy-TXPCF₃CO₂ (**5c**)

Following **general procedure 4**, the title compound was prepared from **4c** (0.33 g, 0.32 mmol) and trifluoroacetic acid (3.3 mL) and purification by semi-preparative reverse-phase HPLC (Eluent gradient 0–100% B in A for 100 min, isocratic 100% B for 20 min, at a flow rate of 5 mL min⁻¹) as a white solid as a white solid (0.17 g, 57%). ¹H NMR (400 MHz, MeOD) δ_{H} (ppm): 7.52 (3H, s, *p*-Ar), 7.49 (2H, d, ³*J*_{HH} = 7.7 Hz, C₆H₄), 7.18 (6H, d, ³*J*_{HP} = 13.1 Hz, *o*-Ar), 7.00 (2H, d, ³*J*_{HH} = 6.7 Hz, C₆H₄), 4.68 (2H, d, ²*J*_{HP} = 15.0 Hz, CH₂P), 4.24 (2H, s), 3.83 (2H, s), 3.64 (2H, s), 3.42 - 3.01 (18H, m, macrocycle H), 2.36 (18H, s, CH₃). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_{C} (ppm): 161.6 (C=O), 161.3 (C=O), 140.4 (d, ²*J*_{CP} = 13.7 Hz, C₆H₄), 136.6 (d, ⁴*J*_{CP} = 3.7 Hz, *p*-Ar), 132.0 (d, ⁴*J*_{CP} = 5.5 Hz, C₆H₄), 131.5 (C₆H₄), 131.2 (d, ²*J*_{CP} = 10.4 Hz, *o*-Ar), 118.3 (C₆H₄), 117.5 (d, ¹*J*_{CP} = 84.7 Hz, *i*-Ar), 115.4 (*m*-Ar), 56.8, 54.4, 49.8 (broad s), 49.1 (broad s), 48.7 (broad s), 29.2 (d, ¹*J*_{CP} = 49.7 Hz, CH₂P), 19.9 (CH₃). ¹⁹F{¹H}-NMR (377 MHz, MeOD) δ_{F} (ppm): -76.8. ³¹P{¹H}-NMR (162 MHz, MeOD) δ_{P} (ppm): 22.3. HRMS (ES-TOF+): *m/z* calcd. for C₄₆H₆₀N₄O₆P ([M-CF₃CO₂]⁺) 795.4250, C₄₆H₆₁N₄O₆P ([M-CF₃CO₂+H]²⁺) 398.2159. found: 795.4326, 398.2164. Retention time: 39–41 min.

Ga-DO3A-xy-TTPNO₃ (Ga5b)

The methodology was a modification of the procedure described by Yang *et al.*¹⁰ To a solution of DO3A-xy-TTPCl (78.9 mg, 0.1 mmol) in 0.5 M NH₄OAc (200 μ L), Ga(NO₃)₃·H₂O (27.3 mg, 0.1 mmol) was added, and the resulting solution was heated at 100 °C for 30 min. The reaction mixture was cooled to rt, the insoluble impurities removed by centrifugation (1.3×10^4 rpm, 3 min), and acetone was added until a precipitate formed. The precipitate was isolated by centrifugation (1.3×10^4 rpm, 3 min) followed by removal of the supernatant liquid and drying *in vacuo* to yield the desired product as a white solid (27.7 mg, 31%). ¹H NMR (400 MHz, MeOD) δ_{H} (ppm): 7.55–7.49 (12H, m, ArH), 7.23 (2H, d, ³*J*_{HH} = 7.8 Hz, C₆H₄), 7.03 (2H, dd, ³*J*_{HH} = 8.3, ⁴*J*_{HP} = 2.5 Hz, C₆H₄), 4.95 (2H, d, ²*J*_{HP} = 15.0 Hz, CH₂P), 3.92 (2H, d, ³*J*_{HH} = 14.5 Hz), 3.82 (4H, s, *trans*-CH₂CO₂), 3.76 (2H, s), 3.66 (2H, s), 3.50–3.20 (12H, m), 2.90 (2H, s, ³*J*_{HH} = 13.4 Hz), 2.48 (9H, s, CH₃). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_{C} (ppm): 173.7 (C=O), 172.4 (C=O), 148.0 (d, ⁴*J*_{CP} = 3.5 Hz, *p*-Ar), 135.2 (d, ³*J*_{CP} = 10.4 Hz, *m*-Ar), 133.4 (C₆H₄), 132.6 (d, ³*J*_{CP} = 5.4 Hz, C₆H₄), 132.0 (d, ²*J*_{CP} = 13.8 Hz, *o*-Ar), 130.8, 130.7, 115.9 (d, ¹*J*_{CP} = 88.4 Hz, *i*-Ar), 64.5, 62.9, 61.0, 58.0, 57.8, 55.4, 54.3, 30.7 (d, ¹*J*_{CP} = 48.0 Hz, CH₂P), 21.8 (CH₃). ³¹P{¹H}-NMR (162 MHz, MeOD) δ_{P} (ppm): 22.1. HRMS (ES-TOF+): *m/z* calcd for C₄₃H₅₁N₄O₆P⁶⁹Ga ([M-NO₃]⁺) 819.2802, C₄₃H₅₂N₄O₆P⁶⁹Ga ([M-NO₃+H]²⁺) 410.1435. found: 819.2815, 410.1418.

Ga-DO3A-xy-TXPCHO₂ (Ga5c)

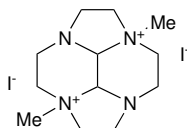
To a solution of DO3A-xy-TXP₂Cl (83.1 mg, 0.1 mmol) in 0.5 M NH₄OAc (200 μL), Ga(NO₃)₃·H₂O (27.4 mg, 0.1 mmol) was added, and the resulting solution was heated at 100 °C for 30 min. The reaction mixture was cooled to rt, and the insoluble impurities were removed by centrifugation (1.3 × 10⁴ rpm, 3 min) before the compound was purified by reverse-phase flash chromatography (C-18 SiO₂, 0–50% MeCN in H₂O (0.1% HCO₂H)) followed by lyophilisation to yield the desired product as a white solid (55.2 mg, 76%). ¹H NMR (400 MHz, MeOD) δ_H (ppm): 7.52 (3H, s, *p*-Ar), 7.39 (2H, d, ³H_{HH} = 7.7 Hz, C₆H₄), 7.21 (6H, d, ³H_{HH} = 13.2 Hz, *o*-Ar), 7.04 (2H, d, ³H_{HH} = 6.6 Hz, C₆H₄), 3.99 (2H, s), 3.87–3.69 (6H, m), 3.56 (2H, s), 3.50–3.11 (14H, m, macrocycle H), 2.85 (2H, d, ²H_{HP} = 12.0 Hz), 2.38 (18H, s, CH₃). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_C (ppm): 173.8 (C=O), 173.5 (C=O), 141.8 (d, ²J_{CP} = 13.6 Hz, C₆H₄), 137.9 (*p*-Ar), 133.2 (C₆H₄), 133.1 (C₆H₄), 132.7 (d, ³J_{CP} = 5.3 Hz, C₆H₄), 132.5 (d, ²J_{CP} = 10.2 Hz, *o*-Ar), 130.8 (d, ³J_{CP} = 8.7 Hz, *m*-Ar), 119.0 (d, ¹J_{CP} = 85.0 Hz, *i*-Ar), 63.8, 63.2, 60.9, 57.7, 57.3, 55.0, 53.7, 30.3 (d, ¹J_{CP} = 50.2 Hz, CH₂P), 21.3 (CH₃). ³¹P{¹H}-NMR (162 MHz, MeOD) δ_P (ppm): 22.3. HRMS (ES-TOF⁺): *m/z* calcd for C₄₆H₅₇N₄O₆P⁶⁹Ga (M⁺) 861.3272. found: 861.3285.

7.1.3 DO2A-(xy-TAP)₂ and DO2A-Bn₂ (Section 3.2)**7.1.3.1 General Procedure 5 - Trans-Disubstitution of Cyclen Glyoxal with Alkylating Agents**

The methodology was a modification of the procedure described by Rohovec *et al.* and Niu *et al.*^{11,12} To a solution of cyclen glyoxal in MeCN, the alkylating agent was added in 1 portion, and the resulting solution was stirred at rt under an N₂ atmosphere until a precipitate evolved. The precipitate was isolated by vacuum filtration, washed with MeCN (3 × 5 mL) and dried

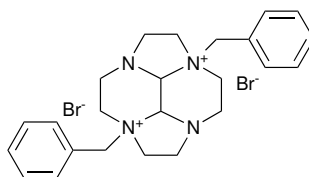
in vacuo to yield the desired product.

4a,8a-Dimethyldodecahydro-2a,4a,6a,8a-tetraazacyclopenta[fg]acenaphthylene-4a,8a-diiium diiodide (9a)¹¹



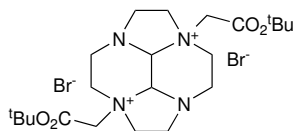
This compound was originally synthesised by Rohovec *et al.*¹¹ Following **general procedure 5**, the title compound was prepared from glyclen glyoxal (0.86 g, 4.42 mmol), methyl iodide (0.83 mL, 13.28 mmol) and MeCN (4.42 mL) as a white solid (1.36 g, 65%). ¹H-NMR (400 MHz, D₂O) δ_{H} (ppm): 4.52 (2H, s, CHN), 4.12–4.02 (4H, m), 3.92 (2H, ddd, $^3J_{\text{HH}} = 12.3$, 10.3 , $^2J_{\text{HH}} = 4.3$ Hz), 3.69 (2H, dt, $^3J_{\text{HH}} = 12.8$, $^2J_{\text{HH}} = 2.3$ Hz), 3.63 (2H, td, $^3J_{\text{HH}} = 8.7$, $^2J_{\text{HH}} = 4.2$ Hz), 3.46–3.39 (8H, m, CH_3), 3.21–3.09 (4H, m). ¹³C{¹H}-NMR (100 MHz, D₂O) δ_{C} (ppm): 78.1 (CHN), 65.0, 59.2, 46.7 (CH_3), 46.5, 43.1. HRMS (ES-TOF+): m/z calcd. for C₁₂H₂₄N₄ ([M-2I]²⁺) 112.1000. found: 112.0997.

4a,8a-Dibenzyl-dodecahydro-2a,4a,6a,8a-tetraazacyclopenta[fg]acenaphthylene-4a,8a-diiium dibromide (9b)¹²



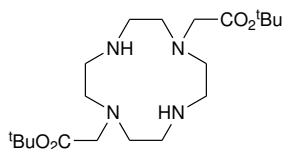
This compound was originally synthesised by Niu *et al.*¹² Following **general procedure 5**, the title compound was prepared from glyclen glyoxal (0.26 g, 1.34 mmol), benzyl bromide (1.00 mL, 8.41 mmol) and MeCN (10 mL) as a white solid in quantitative yield. ¹H-NMR (400 MHz, D₂O) δ_{H} (ppm): 7.65–7.56 (10H, m, C₆H₅), 4.96 (2H, d, $^3J_{\text{HH}} = 13.3$ Hz), 4.82 (2H, s, CHN), 4.31 (2H, td, $^3J_{\text{HH}} = 10.6$, $^2J_{\text{HH}} = 3.8$ Hz), 3.92–3.84 (2H, m), 3.70–3.65 (2H, m), 3.63–3.57 (4H, m), 3.48–3.40 (4H, m), 3.17–3.11 (2H, m). ¹³C{¹H}-NMR (100 MHz, D₂O) δ_{C} (ppm): 132.4, 131.4, 129.8, 126.2 (*i*-C₆H₅), 77.7 (CHN), 61.2, 61.0, 54.9, 46.1, 42.8. HRMS (ES-TOF+): m/z calcd. for C₂₄H₃₂N₄ ([M-2Br]²⁺) 188.1313. found: 188.1315.

4a,8a-Bis(2-(*tert*-butoxy)-2-oxoethyl)dodecahydro-2a,4a,6a,8a-tetraazacyclopenta-[fg]-acenaphthylene-4a,8a-diium bromide (9c)¹¹



This compound was originally synthesised by Rohovec *et al.*¹¹ Following **general procedure 5**, the title compound was prepared from cyclen glyoxal (3.05 g, 15.71 mmol), *tert*-butyl bromoacetate (9.19 mL, 47.12 mmol) and MeCN (15.7 mL) as a white solid (12.34 g, 79%). ¹H-NMR (400 MHz, MeOD) δ_{H} (ppm): 4.84 (2H, d, $^2J_{\text{HH}} = 16.9$ Hz), 4.77 (2H, s), 4.66 (2H, dd, $^2J_{\text{HH}} = 16.9$, $^3J_{\text{HH}} = 1.7$ Hz), 4.32–4.21 (2H, m), 4.21–4.10 (4H, m), 4.10–3.99 (2H, m), 3.60 (2H, td, $^3J_{\text{HH}} = 8.4$, 3.7 Hz), 3.41–3.20 (4H, m), 3.10 (2H, q, $^3J_{\text{HH}} = 8.8$ Hz), 1.56 (18H, s). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_{C} (ppm): 165.7 (C=O), 87.0 (C(CH₃)₃), 80.4 (NCH), 65.2, 58.3, 58.1, 48.3, 44.1, 28.3 (C(CH₃)₃). HRMS (ES-TOF+): m/z calcd. for C₂₂H₄₀N₄O₄Br ([M-Br]⁺) 503.2233, C₂₂H₄₀N₄O₄ ([M-2Br]²⁺) 212.1519. found: 503.2238, 212.1510.

Di-*tert*-butyl 2,2'-(1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate (DO2Adiester)¹³



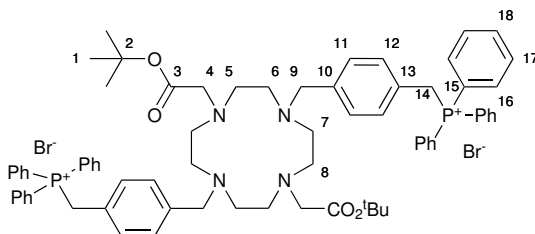
The methodology was a modification of the procedure described by Oukhatar *et al.*¹³ To a Schlenk flask charged with a suspension of **9c** (1.07 g, 1.84 mmol) in anhydrous THF (50 mL) under N₂, *o*-phenylenediamine (0.22 g, 2.02 mmol) was added, and the resulting mixture was stirred at rt overnight. The solvent was removed by vacuum filtration under a nitrogen funnel, before the reaction flask was washed with THF (50 mL) and added to the Büchner funnel. The solid was washed with further THF aliquots (2 × 20 mL) and dried under vacuum. This solid was dissolved in anhydrous DCM (50 mL) in a Schlenk flask under N₂. Cs₂CO₃ (1.33 g, 4.05 mmol) was added, and the resulting mixture was stirred at rt overnight. The inorganic

base was removed by filtration, the solvent removed under reduced pressure and the residue dried *in vacuo* to yield the title compound as a white foam (0.42 g, 57%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 3.38 (4H, s, CH_2CO_2), 2.93 (8H, broad s), 2.75 (8H, broad s), 1.45 (18H, s, $\text{C}(\text{CH}_3)_3$). ^{13}C NMR (100 MHz, MeOD) δ_{C} (ppm): 171.25 ($\text{C}=\text{O}$), 81.46 ($\text{C}(\text{CH}_3)_3$), 57.15, 52.12, 47.17, 28.34 ($\text{C}(\text{CH}_3)_3$). HRMS (ES-TOF+): m/z calcd. for $\text{C}_{20}\text{H}_{41}\text{N}_4\text{O}_4$ ($[\text{M}+\text{H}]^{\text{H}}$) 401.3128. found: 401.3139.

7.1.3.2 General Procedure 6 - Synthesis of *Tert*-Butyl Protected DO2A-(xy-TAP)₂ Compounds

To a solution of DO2A diester (1 eqv) and Na_2CO_3 (10 eqv) in MeCN (20 mL), **3a-c** (2 eqv) was added, and the resulting suspension was stirred at 70 °C for 48 h. The inorganic solids were removed by gravity filtration and the solvent was removed under reduced pressure, before the residue was recrystallised from acetone/ Et_2O to yield the desired product.

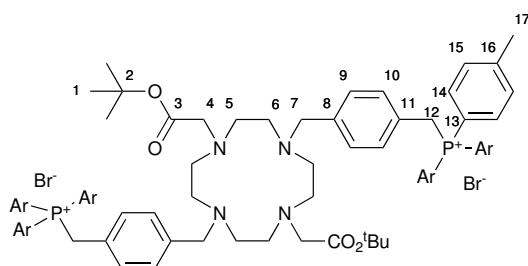
Bis-triphenyl(4-((4,10-bis(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1,7-diyl)methyl)benzyl)phosphonium dibromide (**10a**)



Following **general procedure 6**, the title compound was prepared from DO2A diester (0.16 g, 0.4 mmol), **3a** (0.42 g, 0.8 mmol) and Na_2CO_3 (0.42 g, 4 mmol) as a white solid (0.27 g, 53%). ^1H -NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.77 (8H, tdd, $^3J_{\text{HH}} = 9.7$ Hz, $^4J_{\text{HH}} = 4.8$ Hz, H_{16-18}), 7.70 (10H, td, $^3J_{\text{HH}} = 6.3$ Hz, $^4J_{\text{HH}} = 4.4$ Hz, H_{16-18}), 7.68–7.61 (10H, m, H_{16-18}), 7.59–7.53 (2H, m, H_{16-18}), 7.10 (4H, dd, $^3J_{\text{HH}} = 8.3$ Hz, $^4J_{\text{HH}} = 2.6$ Hz, $\text{H}_{11/12}$), 7.03 (4H, d, $^3J_{\text{HH}} = 7.8$ Hz, $\text{H}_{11/12}$), 5.32 (4H, broad s, H_{14}), 3.41 (4H, broad s, H_9), 2.35 (16H, broad s, $\text{H}_{5/6}$), 2.15 (4H, s, H_4), 1.43 (18H, s, H_1). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 174.3 (C_3), 136.5 (C_{10}), 135.5 ($\text{C}_{11/12}$), 134.5 (d, $^3J_{\text{CP}} = 10.4$ Hz, C_{17}), 132.0 (d, $^4J_{\text{CP}} = 4.3$ Hz, C_{18}), 130.6 ($\text{C}_{11/12}$), 130.4 (d, $^2J_{\text{CP}} = 13.4$ Hz, C_{16}), 127.1 (d, $^2J_{\text{CP}} = 8.7$ Hz, C_{13}),

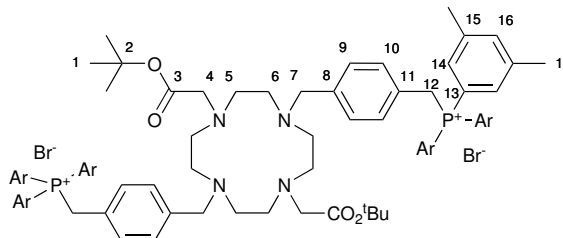
117.6 (d, $^1J_{\text{CP}} = 85.1$ Hz, C₁₅), 84.0 (C₂), 58.7, 57.1, 49.4, 49.6, 31.0, 30.7 (d, $^1J_{\text{CP}} = 47.1$ Hz, C₁₄), 28.4, 28.3. $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl₃) δ_{P} (ppm): 23.2. MS (ES-TOF+): m/z calcd. for C₇₂H₈₄N₄O₄P₂ ([M-2Br]²⁺) 565.30, C₇₂H₈₅N₄O₄P₂ ([M-2Br+H]³⁺) 377.20. found: 565.30, 377.20.

Bis-triphenyl(4-((4,10-bis(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1,7-diyl)methyl)4-methylphenyl)phosphonium dibromide (10b)



Following **general procedure 6**, the title compound was prepared from DO2Adiester (0.16 g, 0.4 mmol), **3b** (0.45 g, 0.8 mmol) and Na₂CO₃ (0.42 g, 4 mmol) as a white solid (0.24 g, 42%). ^1H -NMR (400 MHz, CDCl₃) δ_{H} (ppm): 7.54–7.45 (12H, m, H_{14/15}), 7.43–7.32 (12H, m, H_{14/15}), 7.09 (4H, dd, $^3J_{\text{HH}} = 8.3$, $^3J_{\text{HP}} = 2.4$ Hz, H₁₀), 7.05 (4H, d, $^3J_{\text{HH}} = 8.3$ Hz, H₉), 5.09–5.06 (4H, m, H₁₂), 2.93 (4H, s, H_{4/7}), 2.46 (18H, s, H₁₇), 2.43–2.36 (16H, m, H_{5/6}), 2.15 (4H, s, H_{4/7}), 1.44 (18H, s, H₁). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl₃) δ_{C} (ppm): 174.2 (C₃), 146.4 (d, $^2J_{\text{CP}} = 3.5$ Hz, C₁₁), 146.2 (d, $^4J_{\text{CP}} = 3.4$ Hz, C₁₆), 136.2 (C₈), 134.3 (d, $^3J_{\text{CP}} = 10.5$ Hz, C₁₅), 131.8 (d, $^4J_{\text{CP}} = 5.1$ Hz, C₉), 131.0 (d, $^3J_{\text{CP}} = 13.8$ Hz, C₁₀), 130.8 (d, $^2J_{\text{CP}} = 18.2$ Hz, C₁₄), 114.5 (d, $^1J_{\text{CP}} = 88.6$ Hz, C₁₃), 83.8 (C₂), 65.9, 57.9 (d, $^1J_{\text{CP}} = 119.9$ Hz, C₁₂), 49.5, 31.0, 28.4 (C₁), 22.0 (C₁₇). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl₃) δ_{P} (ppm): 22.1. HRMS (ES-TOF+): m/z calcd. for C₇₈H₉₆N₄O₄P₂ ([M-2Br]²⁺) 607.3432. found: 607.3304.

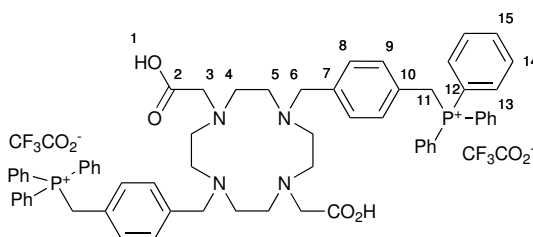
Bis-triphenyl(4-((4,10-bis(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1,7-diyl)methyl)3,5-dimethylphenyl)phosphonium dibromide (10c)



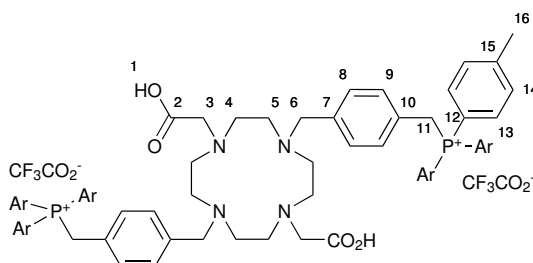
Following **general procedure 6**, the title compound was prepared from DO2Adiester (0.14 g, 0.36 mmol), **3c** (0.44 g, 0.72 mmol) and Na₂CO₃ (0.38 g, 3.60 mmol) as a white solid (0.39 g, 75%). ¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 7.35 (4H, s, H₉), 7.27 (6H, s, H₁₆), 7.19 (12H, d, ²J_{HP} = 12.9 Hz, H₁₄), 7.10 (4H, s, H₁₀), 5.14 (4H, broad s, H_{4/7}), 3.49 (4H, broad s, H_{4/7}), 2.96 (4H, s, ₁₂), 2.81 (8H, broad s, H_{5/6}), 2.35 (36H, s, H₁₇), 1.71 (8s, s, H_{5/6}), 1.47 (18 H, s, H₁). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_C (ppm): 174.3 (C₃), 140.3 (d, ²J_{CP} = 12.2 Hz, C₁₁), 136.9 (C₉), 136.1 (C_{8/15}), 132.0 (d, ³J_{CP} = 5.3 Hz, C₁₀), 131.9 (C_{8/15}), 131.7 (d, ²J_{CP} = 9.0 Hz, C₁₄), 130.7 (C₁₆), 117.8 (d, ¹J_{CP} = 85.0 Hz, C₁₃), 83.7 (C₂), 57.8 (d, ¹J_{CP} = 90.1 Hz), 57.1 (C_{4/7}), 31.0 (C_{4/7}), 29.4 (C₁), 21.6 (C₁₇). ³¹P{¹H}-NMR (162 MHz, CDCl₃) δ_P (ppm): 22.6. HRMS (ES-TOF+): *m/z* calcd. for C₈₄H₁₀₈N₄O₄P₂ ([M-2Br]²⁺) 649.3923. found: 649.3918.

7.1.3.3 General Procedure 7 - Deprotection of *Tert*-butyl Protected DO2A-(xy-TAP)₂ using Trifluoroacetic Acid

Compounds **10a-c** were dissolved in trifluoroacetic acid (reaction mixture concentration 0.1 g mL⁻¹) and stirred at rt under an N₂ atmosphere for 24 h. The acid was removed *in vacuo*, before the residue was taken up in H₂O (50 mL) and washed with CHCl₃ (3 × 10 mL). The aqueous solvent was removed under reduced pressure, before the residue was purified by reverse-phase flash chromatography (C-18 SiO₂, 0–100% B in A) to yield the desired product.

DO2A-(xy-TPP)₂.2CF₃CO₂ (11a)

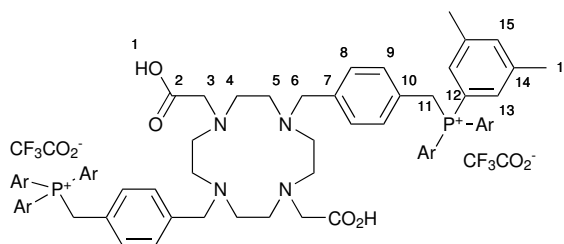
Following **general procedure 7**, the title compound was prepared from **10a** (0.25 g, 0.19 mmol) and TFA (2.5 mL) as a colourless oil (0.13 g, 57%). ¹H-NMR (400 MHz, MeOD) δ_{H} (ppm): 7.91 (6H, t, $^3J_{\text{HH}} = 7.4$ Hz, H₁₅), 7.79–7.63 (24H, m, H_{13/14}), 7.57 (4H, d, $^3J_{\text{HH}} = 7.8$ Hz, H₈), 7.17 (4H, dd, $^3J_{\text{HH}} = 7.8$, $^4J_{\text{HP}} = 2.5$ Hz, H₉), 5.20 (4H, s, H_{3/6}), 5.02 (4H, d, $^2J_{\text{HP}} = 15.5$ Hz, H₁₁), 4.55 (4H, s, H_{3/6}), 3.49–3.37 (4H, m, H_{4/5}), 3.37–3.25 (4H, m, H_{4/5}), 3.19–3.10 (4H, m, H_{4/5}), 3.10–2.93 (4H, m, H_{1/4/5}). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_{C} (ppm): 175.1 (C₂), 136.6 (C₁₅), 135.3 (d, $^3J_{\text{CP}} = 9.1$ Hz, C₁₄), 133.5 (C₈), 133.3 (d, $^3J_{\text{CP}} = 5.3$ Hz, C₉), 131.8 (d, $^2J_{\text{CP}} = 8.5$ Hz, C₁₀), 131.5 (d, $^2J_{\text{CP}} = 13.6$ Hz, C₁₃), 130.8 (d, $^5J_{\text{CP}} = 3.4$ Hz, C₇), 118.9 (d, $^1J_{\text{CP}} = 86.9$ Hz, C₁₂), 58.8 (C_{3/6}), 53.6 (C_{4/5}), 51.4 (C_{4/5}), 49.6 (C_{3/6}), 30.3 (d, $^1J_{\text{CP}} = 48.6$ Hz, C₁₁). ¹⁹F{¹H}-NMR (377 MHz, MeOD) δ_{F} (ppm): -77.1. ³¹P{¹H}-NMR (162 MHz, MeOD) δ_{P} (ppm): 23.0. HRMS (ES-TOF+): m/z calcd. for C₆₄H₆₈N₄O₄P₂ ([M-2CF₃CO₂]²⁺) 509.2358, C₆₄H₆₉N₄O₄P₂ ([M-2CF₃CO₂+H]³⁺) 339.8259. found: 509.2363, 339.8252.

DO2A-(xy-TTP)₂.2CF₃CO₂ (11b)

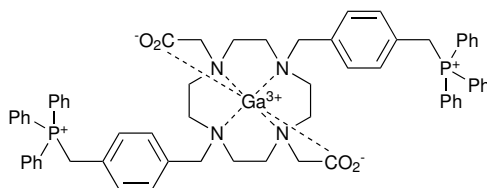
Following **general procedure 7**, the title compound was prepared from **10b** (0.21 g, 0.15 mmol) and TFA (2.1 mL) as a pale yellow oil (51 mg, 25%). ¹H-NMR (400 MHz, MeOD) δ_{H} (ppm): 7.57–7.49 (28H, m, H_{8/13/14}), 7.16 (4H, dd, $^3J_{\text{HH}} = 8.1$, $^4J_{\text{HP}} = 2.5$ Hz, H₉),

4.88 (2H, d, $^2J_{\text{HP}} = 15.4$ Hz, H_{11}), 4.55 (4H, s, $\text{H}_{3/6}$), 3.48–3.38 (4H, m, $\text{H}_{4/5}$), 3.36–3.26 (4H, m, $\text{H}_{4/5}$), 3.20–3.09 (4H, m, $\text{H}_{3/6}$ and $\text{H}_{4/5}$), 3.07–2.96 (4H, m, $\text{H}_{4/5}$), 2.51 (18H, s, H_{16}). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, MeOD) δ_{C} (ppm): 175.2 (C_2), 148.2 (d, $^4J_{\text{CP}} = 3.5$ Hz, C_{15}), 135.2 (d, $^3J_{\text{CP}} = 9.2$ Hz, C_{14}), 133.2 (d, $^4J_{\text{CP}} = 3.4$ Hz, C_8), 133.3 (d, $^3J_{\text{CP}} = 5.1$ Hz, C_9), 131.6 (d, $^2J_{\text{CP}} = 13.2$ Hz, C_{13}), 130.6 (d, $^2J_{\text{CP}} = 3.5$ Hz, C_{10}), 119.2 (C_7), 115.7 (d, $^1J_{\text{CP}} = 88.4$ Hz, C_{12}), 58.8 ($\text{C}_{3/6}$), 53.6 ($\text{C}_{3/6}$), 51.4 ($\text{C}_{4/5}$), 49.6 ($\text{C}_{4/5}$), 30.7 (d, $^1J_{\text{CP}} = 49.2$ Hz, C_{11}), 21.8 (C_{16}). $^{19}\text{F}\{^1\text{H}\}$ -NMR (377 MHz, MeOD) δ_{F} (ppm): -77.1. $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, MeOD) δ_{P} (ppm): 22.1. HRMS (ES-TOF+): m/z calcd. for $\text{C}_{70}\text{H}_{80}\text{N}_4\text{O}_4\text{P}_2$ ($[\text{M}-2\text{CF}_3\text{CO}_2]^{2+}$) 551.2827, $\text{C}_{70}\text{H}_{81}\text{N}_4\text{O}_4\text{P}_2$ ($[\text{M}-2\text{CF}_3\text{CO}_2+\text{H}]^{3+}$) 367.8572. found: 551.2809, 367.8524.

DO2A-(xy-TXP) $_2$.2CF $_3$ CO $_2$ (**11c**)



Following **general procedure 7**, the title compound was prepared from **10c** (0.38 g, 0.26 mmol) and TFA (3.8 mL) as a pale yellow oil (28.3 mg, 8%). ^1H -NMR (400 MHz, MeOD) δ_{H} (ppm): 7.58–7.51 (12H, m, H_{15}), 7.21 (6H, d, $^3J_{\text{HP}} = 13.1$ Hz, H_{13}), 7.12–7.09 (4H, m, $\text{H}_{8/9}$), 4.87 (4H, d, $^2J_{\text{HP}} = 15.2$ Hz, H_{11}), 4.55 (4H, s), 3.44–3.25 (8H, m, macrocycle H), 3.13 (4H, s), 3.12–2.96 (8H, m, macrocycle H), 2.36 (36H, s, H_{16}), 2.03 (2H, s, H_1). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, MeOD) δ_{C} (ppm): 175.2 (C_2), 141.8 (d, $^2J_{\text{CP}} = 13.4$ Hz, C_{10}), 138.0, 133.4, 132.6, 132.5, 132.4, 130.4, 118.9 (d, $^1J_{\text{CP}} = 86.5$ Hz, C_{12}), 58.6, 53.7, 51.3, 49.7, 30.5 (d, $^2J_{\text{CP}} = 49.3$ Hz, C_{11}), 21.3 (C_{16}). $^{19}\text{F}\{^1\text{H}\}$ -NMR (377 MHz, MeOD) δ_{F} (ppm): -77.3. $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, MeOD) δ_{P} (ppm): 22.5. HRMS (ES-TOF+): m/z calcd. for $\text{C}_{76}\text{H}_{92}\text{N}_4\text{O}_4\text{P}_2$ ($[\text{M}-2\text{CF}_3\text{CO}_2]^{2+}$) 593.3297, $\text{C}_{76}\text{H}_{93}\text{N}_4\text{O}_4\text{P}_2$ ($[\text{M}-2\text{CF}_3\text{CO}_2+\text{H}]^{3+}$) 395.8885. found: 593.3289, 395.8867.

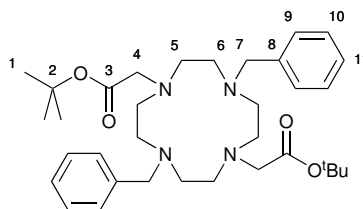
Ga-DO2A-(xy-TPP)₂ (Ga11a) - Attempted Synthesis

To a solution of **11a** (80.4 mg, 73.8 μ mol) in 0.5 M NH_4OAc (150 μ L), $\text{Ga}(\text{NO}_3)_3 \cdot \text{H}_2\text{O}$ (27.2 mg, 73.8 μ mol) was added, and the resulting solution was heated at 100 $^\circ\text{C}$ for 30 min. The reaction mixture was cooled to rt, the insoluble impurities removed by centrifugation (1.3×10^4 rpm, 3 min), before recrystallisation was attempted through addition of acetone into the reaction mixture. Purification was then attempted by purification by reverse-phase flash chromatography (C-18 SiO_2 , 0–100% MeCN in H_2O (0.1% HCO_2H)).

7.1.3.4 General Procedure 8 - Aryl Functionalisation of DO2Adiester

To a solution of DO2Adiester (1 eqv) and N_2CO_3 (10 eqv) in MeCN, aryl bromide (2 eqv) was added dropwise before the resulting solution was stirred at 70 $^\circ\text{C}$ under an N_2 atmosphere for 48 h. The inorganic base was removed by filtration, before the solvent was removed under reduced pressure to yield the desired product.

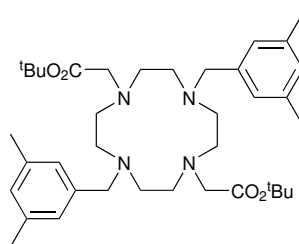
Di-tert-butyl 2,2'-(4,10-dibenzyl-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate (13a)



Following **general procedure 8**, the title compound was prepared from DO2Adiester (0.46 g, 1.16 mmol), benzyl bromide (0.28 mL, 2.32 mmol), Na_2CO_3 (1.23 g, 11.60 mmol) and MeCN (60 mL) as an orange oil (0.54 g, 79%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.41 (4H, d, $^3J_{\text{HH}} = 7.5$ Hz, H_9), 7.31 (4H, t, $^3J_{\text{HH}} = 7.4$ Hz, H_{10}), 7.23 (2H, t, $^3J_{\text{HH}} = 7.3$ Hz, H_{11}), 3.57

(4H, s, H_{4/7}), 3.14 (4H, s, H_{4/7}), 2.86 (8H, broad t, $^3J_{\text{HH}} = 5.3$ Hz, H_{5/6}), 2.63 (8H, broad t, $^3J_{\text{HH}} = 5.4$ Hz, H_{5/6}), 1.41 (18H, s, H₁). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl₃) δ_{C} (ppm): 171.3 (C₃), 140.2 (C₈), 129.2 (C₉), 128.2 (C₁₀), 126.8 (C₁₁), 80.6 (C₂), 60.3 (C_{4/7}), 56.5 (C_{4/7}), 52.7 (C_{5/6}), 52.4 (C_{5/6}), 28.3 (C₁). HRMS (ES-TOF+): m/z calcd for C₃₄H₅₃N₄O₄ ([M+H]⁺) 581.4067, C₃₄H₅₂N₄O₄Na ([M+Na]⁺) 603.3881. found: 581.4081, 603.3890.

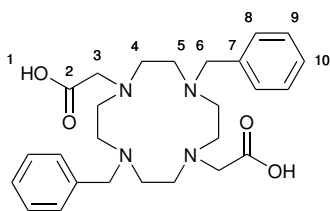
Di-tert-butyl 2,2'-(4,10-bis(3,5-dimethylbenzyl)-1,4,7,10-tetraazacyclododecane-1,7-di-yl)di-acetate (13b)



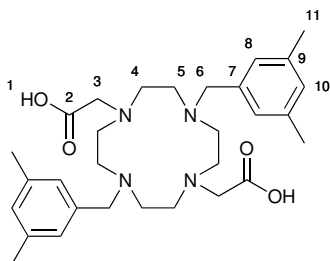
Following **general procedure 8**, the title compound was prepared from DO2Adiester (0.16 g, 0.4 mmol), 1-(bromomethyl)-3,5-dimethylbenzene (0.16 g, 0.8 mmol), Na₂CO₃ (0.42 g, 4.0 mmol) and MeCN (20 mL) as a colourless foam, which was used in the next step without further purification or characterisation.

7.1.3.5 General Procedure 9 - Deprotection of *Tert*-Butyl Protected Di-Aryl DO2A

Compounds **13a** and **13b** were dissolved in conc. HCl (reaction mixture concentration 0.1 g mL⁻¹) and stirred at rt under an N₂ atmosphere for 24 h. The acid was removed *in vacuo*, before the residue was taken up in H₂O (50 mL) and washed with CHCl₃ (3 × 10 mL). The aqueous solvent was removed under reduced pressure, before the residue was purified by reverse-phase flash chromatography (C-18 SiO₂, 0–100% B in A) to yield the desired product.

2,2'-(4,10-Dibenzyl-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetic acid (14a)

Following **general procedure 9**, the title compound was prepared from **13a** (0.52 g, 0.90 mmol) and conc. HCl (5.2 mL) as a white solid (0.27 g, 64%). ^1H NMR (400 MHz, MeOD) δ_{H} (ppm): 7.75–7.69 (4H, m, H_9), 7.57–7.48 (6H, m, $\text{H}_{8/10}$), 4.61 (4H, s, $\text{H}_{3/6}$), 3.56 (4H, ddd, $^3J_{\text{HH}} = 14.3, 6.9$, $^4J_{\text{HH}} = 3.4$ Hz, $\text{H}_{4/5}$), 3.40 (4H, ddd, $^3J_{\text{HH}} = 14.3, 7.6$, $^4J_{\text{HH}} = 3.4$ Hz, $\text{H}_{4/5}$), 3.21 (4H, ddd, $^3J_{\text{HH}} = 15.2, 7.6$, $^4J_{\text{HH}} = 3.6$ Hz, $\text{H}_{4/5}$), 3.08–2.94 (8H, m, $\text{H}_{3/6}$ and $\text{H}_{4/5}$). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, MeOD) δ_{C} (ppm): 174.8 (C_2), 132.6 (C_9), 131.6 (C_8), 130.8 (C_{10}), 130.4 (C_7), 59.6, 53.9 ($\text{C}_{3/6}$), 51.7, 49.7 ($\text{C}_{4/5}$). HRMS (ES-TOF+): m/z calcd for $\text{C}_{26}\text{H}_{37}\text{N}_4\text{O}_4$ ($[\text{M}+\text{H}]^+$) 469.2815. found: 469.2816.

2,2'-(4,10-Bis(3,5-dimethylbenzyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetic acid (14b)

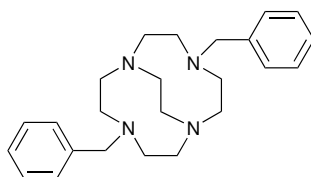
Following **general procedure 9**, the title compound was prepared from **13b** (0.39 g, 0.61 mmol) and conc. HCl (3.9 mL) as a white solid (0.12 g, 38%). ^1H NMR (400 MHz, MeOD) δ_{H} (ppm): 7.21 (4H, s, H_8), 7.08 (2H, s, H_{10}), 4.20 (4H, s, $\text{H}_{3/6}$), 3.18 (16H, s, $\text{H}_{4/5}$), 3.16 (4H, s, $\text{H}_{3/6}$), 2.36 (12H, s, H_{11}). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, MeOD) δ_{C} (ppm): 174.3 (C_2), 140.3 ($\text{C}_{7/10}$), 132.4 (C_{11}), 131.7 ($\text{C}_{7/10}$), 130.9 (C_8), 59.2 ($\text{C}_{3/6}$), 56.5 ($\text{C}_{3/6}$), 51.1 (broad, $\text{C}_{4/5}$), 21.4 (C_{11}). HRMS (ES-TOF+): m/z calcd for $\text{C}_{30}\text{H}_{45}\text{N}_4\text{O}_4$ ($[\text{M}+\text{H}]^+$) 525.3441, $\text{C}_{30}\text{H}_{46}\text{N}_4\text{O}_4$ ($[\text{M}+2\text{H}]^{2+}$) 263.1754. found: 525.3450, 263.1954.

7.1.4 Bridged Cyclens (Section 3.3)

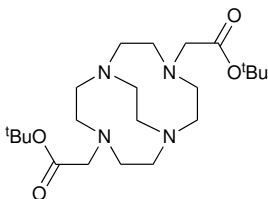
7.1.4.1 General Procedure 10 - Formation of Bisfunctionalised Tetraazabicycles using Sodium Borohydride

The methodology was a modification of the procedure described by Niu *et al.*¹² To a solution of *trans*-disubstituted cyclen glyoxal (1 eqv) in EtOH, NaBH₄ (37 eqv) was added portionwise over 20 min, before the reaction was stirred at rt for 4 days. Excess NaBH₄ was decomposed through the slow addition of 3 M HCl, before the solvent was removed under reduced pressure. The residue was dissolved in H₂O, adjusted to pH 14 through portionwise addition of solid KOH and allowed to cool before the product was extracted with toluene (6 × 10 mL). The organic extracts were combined, dried over MgSO₄ and the solvent removed under reduced pressure to yield the desired product.

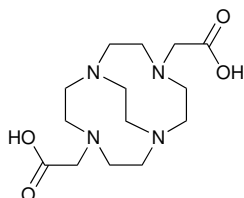
4,10-Dibenzyl-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane (15b)¹²



This compound was originally synthesised by Niu *et al.*¹² Following **general procedure 10**, the title compound was prepared from **9b** (0.78 g, 1.45 mmol), NaBH₄ (2.05 g, 54.22 mmol), HCl (3M, 20 mL) and H₂O (8 mL) as a yellow oil (0.29 g, 52%). ¹H-NMR (400 MHz, CDCl₃) δ_{H} (ppm): 7.35–7.27 (10H, m, C₆H₅), 3.73 (4H, s), 3.13 (4H, s), 2.94–2.81 (8H, m, macrocycle H), 2.78–2.66 (8H, m, macrocycle H). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_{C} (ppm): 140.6 (*i*-C₆H₅), 129.0, 128.3, 126.8, 61.3, 57.8 (macrocycle C), 57.6 (macrocycle C), 56.5. HRMS (ES-TOF+): *m/z* calcd. for C₂₄H₃₅N₄ ([M+H]⁺) 379.2862. found: 379.2870.

Di-*tert*-butyl 2,2'-(1,4,7,10-tetraazabicyclo[5.5.2]tetradecane-4,10-diyl)diacetate (15c)

Following **general procedure 10**, the title compound was prepared from **9c** (0.55 g, 0.94 mmol) NaBH₄ (1.32 g, 34.78 mmol), HCl (3M, 14 mL) and H₂O (5 mL) as a white solid in quantitative yield. ¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 3.26 (4H, s), 3.00–2.93 (4H, m), 2.82–2.71 (16H, m, macrocycle H), 1.45 (18H, s, CH₃). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_C (ppm): 172.3 (C=O), 80.7 (C(CH₃)₃), 58.2 (macrocycle C), 57.7, 57.2 (macrocycle C), 56.6, 28.4 (CH₃). HRMS (ES-TOF+): *m/z* calcd. for C₂₂H₄₃N₄O₄ ([M+H]⁺) 427.3284. found: 427.3280.

2,2'-(1,4,7,10-Tetraazabicyclo[5.5.2]tetradecane-4,10-diyl)diacetic acid (16)

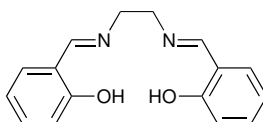
15c (0.46 g, 1.10 mmol) was dissolved in TFA (4.6 mL) and stirred at rt under an N₂ atmosphere overnight. The TFA was removed under reduced pressure, and the resulting residue was purified by reverse-phase flash chromatography (C-18 SiO₂, 0–100% B in A) to yield the desired product as a white solid in quantitative yield. ¹H-NMR (400 MHz, MeOD) δ_H (ppm): 3.60 (4H, s, CH₂CO₂H), 3.16–3.05 (16H, m, macrocycle H), 2.98–2.91 (4H, bridge H). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_C (ppm): 175.4 (C=O), 56.7, 55.3, 53.0, 47.8. HRMS (ES-TOF+): *m/z* calcd. for C₁₄H₂₇N₄O₄ ([M+H]⁺) 315.2032. found: 315.2028.

7.1.5 HBED Derivatives (Section 3.4)

7.1.5.1 General Procedure 11 - Synthesis of H₂salen Diimine Derivatives

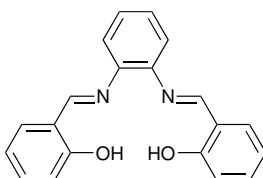
The methodology was a modification of the procedure described by Schmitt *et al.*¹⁴ To a solution of diamine (1 eqv) in EtOH, salicylaldehyde (2 eqv) was added, and the resulting solution was stirred at reflux under an N₂ atmosphere for 1 h. The reaction mixture was cooled to -78 °C, and the resulting precipitate was isolated by vacuum filtration and washed with cold EtOH to yield the desired compound.

2,2'-((1*E*,1'*E*)-(Ethane-1,2-diylbis(azaneylylidene))bis(methaneylylidene))diphenol (H₂salen)¹⁴



This compound was originally synthesised by Schmitt *et al.*¹⁴ Following **general procedure 11**, the title compound was prepared from ethylenediamine (1.67 mL, 25 mmol), *o*-salicylaldehyde (5.33 mL, 50 mmol) and EtOH as a yellow solid (7.08 g, quantitative yield). ¹H NMR (400 MHz, CDCl₃) δ_H (ppm): 13.21 (2H, s, N=CH), 8.36 (2H, s, OH), 7.29 (2H, ddd, ³J_{HH} = 8.9, 7.3, ³J_{HH} = 1.9 Hz), 7.23 (2H, dd, ³J_{HH} = 7.6, ⁴J_{HH} = 1.7 Hz), 6.95 (2H, d, ³J_{HH} = 8.0 Hz), 6.86 (2H, t, ³J_{HH} = 7.6 Hz), 3.94 (4H, s, CH₂CH₂). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_C (ppm): 166.6, 161.1, 132.5, 131.6, 118.8, 118.8, 117.1, 59.9. HRMS (ES-TOF): *m/z* calcd. for C₁₆H₁₆N₂O₂ ([M+H]⁺) 269.1290. found: 269.1288.

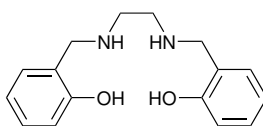
2,2'-((1*E*,1'*E*)-(1,2-Phenylenebis(azanylylidene))bis(methanylylidene))diphenol (20)¹⁵



This compound was originally synthesised by Groysman *et al.*¹⁵ Following **general procedure 11**, the title compound was prepared from *o*-phenylenediamine (2.70 g, 25 mmol), *o*-

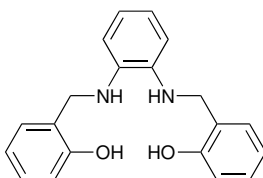
salicylaldehyde (5.33 mL, 50 mmol) and EtOH (75 mL) as a yellow solid (7.36 g, 93%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 13.04 (2H, s, OH), 8.64 (2H, s, $\text{N}=\text{CH}$), 7.40–7.33 (6H, m), 7.24 (2H, dd, $^3J_{\text{HH}} = 5.9, 3.4$ Hz), 7.05 (2H, d, $^3J_{\text{HH}} = 8.1$ Hz, $^4J_{\text{HH}} = 1.1$ Hz), 6.92 (2H, td, $^3J_{\text{HH}} = 7.5$ Hz, $^4J_{\text{HH}} = 1.1$ Hz). ^{13}C NMR (100 MHz, CDCl_3) δ_{C} (ppm): 163.9, 161.5, 142.7, 133.5, 132.5, 127.8, 119.9, 119.4, 119.1, 117.7.

2,2'-((Ethane-1,2-diylbis(azanediyl))bis(methylene))diphenol (H_4salen)¹⁴



The methodology was performed according to the synthesis described by Schmitt *et al.*¹⁴ To a suspension of H_2salen (5.37 g, 20 mmol) in MeOH (100 mL), NaBH_4 (1.51 g, 40 mmol) was added portionwise and the resulting solution was stirred at rt for 30 min. The reaction mixture was cooled to -78°C , and the resulting precipitate was isolated by vacuum filtration, washed with cold MeOH (4×50 mL) and dried *in vacuo* to yield the title compound as a white solid (2.99 g, 55%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.18 (2H, td, $^3J_{\text{HH}} = 7.8$, $^4J_{\text{HH}} = 1.8$ Hz), 6.98 (2H, dd, $^3J_{\text{HH}} = 7.4$, $^4J_{\text{HH}} = 1.7$ Hz), 6.84 (2H, dd, $^3J_{\text{HH}} = 7.9$, $^4J_{\text{HH}} = 1.3$ Hz), 6.79 (2H, td, $^3J_{\text{HH}} = 7.3$, $^4J_{\text{HH}} = 1.2$ Hz), 3.99 (4H, s), 2.84 (4H, s). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 158.1, 129.0, 128.6, 122.3, 119.3, 116.5, 52.7, 48.0. HRMS (ES-TOF): m/z calcd. for $\text{C}_{16}\text{H}_{21}\text{N}_2\text{O}_2$ ($[\text{M}+\text{H}]^+$) 273.1603. found: 273.1605.

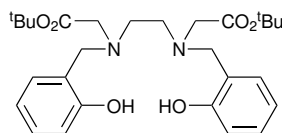
2,2'-((1,2-phenylenebis(azanediyl))bis(methylene))diphenol (21)¹⁵



The methodology was a modification of the procedure described by Schmitt *et al.* and Groysman *et al.*^{14,15} NaBH_4 (1.89 g, 50 mmol) was slowly added to a stirred suspension of **20** (3.16 g, 10 mmol) in MeOH (50 mL), and was left to stir for 30 min. The methanolic solution was poured into water (40 mL) and left to stand for several hours whilst a white precipitate

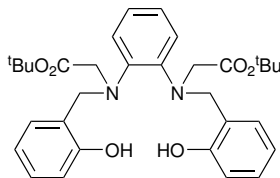
formed. The precipitate was isolated by filtration, washed with water, and dried *in vacuo* at 50 °C to yield the title compound as a white solid (2.31 g, 72%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.85 (2H, broad s, NH), 7.25–7.19 (4H, m, Ar H), 6.97–6.85 (8H, m, Ar H), 4.41 (4H, s, NCH_2), 3.73 (2H, s, OH). ^{13}C NMR (100 MHz, CDCl_3) δ_{C} (ppm): 156.5, 136.8, 129.5, 129.4, 123.0, 121.8, 120.5, 116.6, 114.4, 48.0. HRMS (ES-TOF): calcd. for $\text{C}_{20}\text{H}_{21}\text{N}_2\text{O}_2$ ($[\text{M}+\text{H}]^+$) 321.1603. found: 321.1602. Anal. calcd. for $\text{C}_{20}\text{H}_{20}\text{N}_2\text{O}_2$ (found): C 75.0 (75.0); H 6.3 (6.3); N 8.7 (8.8).

Di-*tert*-butyl 2,2'-(ethane-1,2-diylbis((2-hydroxybenzyl)azanediyl))diacetate (18)



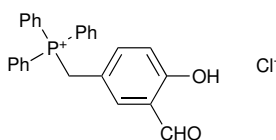
To a suspension of H_4salen (1.36 g, 5 mmol) and NaHCO_3 (0.92 g, 11 mmol) in MeCN (50 mL) previously cooled to 0 °C, *tert*-butyl bromoacetate (1.62 mL, 11 mmol) was added over 30 minutes. The reaction mixture was allowed to warm to rt and was stirred under an N_2 atmosphere for 48 h. The inorganic base was removed by gravity filtration and the solvent removed under reduced pressure. Purification by column chromatography (SiO_2 , CH_2Cl_2) yielded the title compound as colourless crystals (0.98 g, 39%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 9.66 (2H, s, OH), 7.17 (2H, td, $^3J_{\text{HH}} = 7.8$, $^4J_{\text{HH}} = 1.7$ Hz), 6.89 (2H, dd, $^3J_{\text{HH}} = 7.7$, $^4J_{\text{HH}} = 1.7$ Hz), 6.84 (2H, dd, $^3J_{\text{HH}} = 8.2$, $^4J_{\text{HH}} = 1.1$ Hz), 6.75 (2H, td, $^3J_{\text{HH}} = 7.3$, $^4J_{\text{HH}} = 1.4$ Hz), 3.72 (4H, s), 3.16 (4H, s), 2.68 (4H, s), 1.45 (18H, s, CH_3). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 170.2, 157.6, 129.4, 129.3, 121.8, 119.3, 116.5, 82.2, 58.1, 55.6, 50.3, 28.2. HRMS (ES-TOF): m/z calcd. for $\text{C}_{28}\text{H}_{41}\text{N}_2\text{O}_6$ ($[\text{M}+\text{H}]^+$) 501.2965, $\text{C}_{28}\text{H}_{40}\text{N}_2\text{O}_6\text{Na}$ ($[\text{M}+\text{Na}]^+$) 523.2779. found: 501.2790, 523.2787.

Di-*tert*-butyl 2,2'-(1,2-phenylenebis((2-hydroxybenzyl)azanediyl))diacetate - Attempted Synthesis



To a stirred mixture of **21** (0.60 g, 1.87 mmol) and NaHCO₃ (0.35 g, 4.11 mmol) in MeCN (20 mL) under N₂ cooled to 0 °C, *tert*-butyl bromoacetate (0.61 mL, 4.11 mmol) was added dropwise. The resulting mixture was allowed to warm to rt and stirred for 48 h under N₂. The inorganic base was removed by filtration, and the solvent removed under reduced pressure to yield an oil. This oil was triturated in MeCN and Et₂O to yield the title compound as a white solid, which turned pink overnight. This solid was recrystallised from hot toluene to yield a red solution.

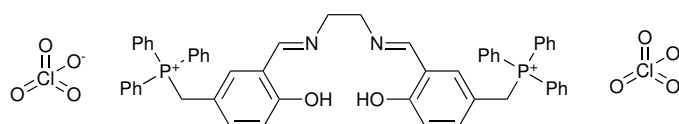
(3-Formyl-4-hydroxybenzyl)triphenylphosphonium chloride (**24**)¹⁶



The methodology was a modification of the procedure described by Asadi *et al.*¹⁶ To a solution of *o*-salicylaldehyde **17** (3.41 mL, 32 mmol) in conc. HCl (20 mL), paraformaldehyde (0.60 g, 20 mmol) was added portionwise, and the resulting solution was stirred at rt under an N₂ atmosphere for 48 h. The precipitate formed was isolated by vacuum filtration, washed with aqueous NaHCO₃ (0.5%, 2 × 10 mL) and water (2 × 10 mL) before being dried *in vacuo* to yield the intermediate as a pink solid (3.12 g, 18.3 mmol). The intermediate was taken up in MeCN (61 mL), before triphenylphosphine (4.79 g, 18.3 mmol) was added and the resulting solution was stirred at reflux under an N₂ atmosphere for 6 h. The reaction mixture was cooled to rt, the precipitate was isolated by vacuum filtration and washed with Et₂O (3 × 30 mL) before being dried *in vacuo* to yield the desired product as a white solid (5.04 g, 58%). ¹H NMR (400 MHz, MeOD) δ_H (ppm): 7.93–7.89 (3H, m, C₆H₅), 7.77–7.69 (6H, m, C₆H₅),

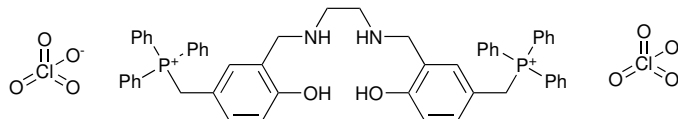
7.68–7.63 (6H, m, C₆H₅), 7.07 (1H, t, $^4J_{\text{HP}} = 2.6$ Hz), 6.81 (1H, dt, $^3J_{\text{HH}} = 8.3$, $^4J_{\text{HP}} = 2.5$ Hz), 6.68 (1H, d, $^3J_{\text{HH}} = 8.4$ Hz), 5.47 (1H, s, OH), 4.84 (2H, d, $^2J_{\text{HP}} = 14.1$ Hz, CH₂). ¹³C{¹H}-NMR (100 MHz, MeOD) δ_{C} (ppm): 157.2 (C=O), 136.4 (d, $^4J_{\text{CP}} = 3.5$ Hz), 135.4 (d, $^2J_{\text{CP}} = 10.0$ Hz), 133.5 (d, $^3J_{\text{CP}} = 5.2$ Hz), 131.6 (d, $^3J_{\text{CP}} = 5.4$ Hz), 131.3 (d, $^2J_{\text{CP}} = 13.0$ Hz), 125.9 (d, $^4J_{\text{CP}} = 3.5$ Hz), 119.3 (d, $^1J_{\text{CP}} = 86.5$ Hz, *i*-C₆H₅), 118.2 (d, $^3J_{\text{CP}} = 8.7$ Hz), 117.1, 100.9, 30.3 (d, $^1J_{\text{CP}} = 47.0$ Hz). ³¹P{¹H}-NMR (162 MHz, MeOD) δ_{P} (ppm): 21.9. HRMS (ES-TOF+): m/z calcd. for C₂₆H₂₃O₂P ([M+H]⁺) 397.1357. found: 397.1373.

(((1*E*,1'*E*)-(Ethane-1,2-diylbis(azaneylylidene))bis(methaneylylidene))bis(4-hydroxy-3,1-phenylene))bis(methylene))bis(triphenylphosphonium) bisperchlorate (25.2ClO₄⁻)



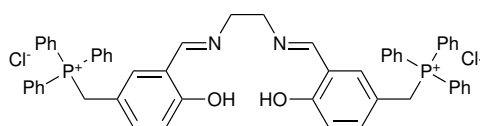
The methodology was a modification of the procedure described by Asadi *et al.*¹⁶ To a solution of **24** (0.54 g, 1.25 mmol) in H₂O (50 mL), a solution of ethylenediamine (41.4 μ L, 0.62 mmol) in EtOH (20 mL) was added dropwise, and the resulting solution was stirred at reflux under an N₂ atmosphere for 3 h. The reaction mixture was allowed to cool to rt, and a solution of NaClO₄ (0.15 g, 1.25 mmol) in H₂O (5 mL) was added. The precipitate was isolated by vacuum filtration, washed with cold EtOH (3 $\times$ 10 mL) and dried *in vacuo* to yield the desired product as a yellow solid (0.20 g, 33%). ¹H NMR (400 MHz, DMSO-*d*₆) δ_{H} (ppm): 13.50 (2H, s, OH), 8.28 (2H, s, N=CH), 7.88 (6H, td, $^3J_{\text{HH}} = 7.2$, $^4J_{\text{HH}} = 1.8$ Hz, *p*-Ar), 7.75–7.60 (24H, m, *o*/*m*-Ar), 6.96 (2H, t, $^4J_{\text{HP}} = 2.6$ Hz), 6.88 (2H, dt, $^3J_{\text{HH}} = 8.2$, $^4J_{\text{HP}} = 2.3$ Hz), 6.74 (2H, d, $^3J_{\text{HH}} = 8.8$ Hz), 5.07 (4H, d, $^2J_{\text{HP}} = 15.3$ Hz, CH₂P), 3.87 (4H, s, CH₂CH₂). ¹³C{¹H}-NMR (100 MHz, DMSO-*d*₆) δ_{C} (ppm): 165.8 (CH=N), 160.8 (CH=N), 135.1, 134.6 (d, $^3J_{\text{CP}} = 3.6$ Hz), 134.0 (d, $^2J_{\text{CP}} = 9.5$ Hz), 133.8, 130.1 (d, $^2J_{\text{CP}} = 12.1$ Hz), 129.8, 118.4 (d, $^3J_{\text{CP}} = 3.2$ Hz), 117.7 (d, $^1J_{\text{CP}} = 85.0$ Hz, *i*-Ar), 117.0 (d, $^3J_{\text{CP}} = 8.7$ Hz), 58.2 (CH₂CH₂), 27.4 (d, $^1J_{\text{CP}} = 46.0$ Hz, CH₂P). ³¹P{¹H}-NMR (162 MHz, DMSO-*d*₆) δ_{P} (ppm): 22.3. HRMS (ES-TOF+): m/z calcd. for C₅₄H₄₈N₂O₂P₂ ([M]²⁺) 409.1596. found: 409.1607.

(((Ethane-1,2-diylbis(azanediyl))bis(methylene))bis(4-hydroxy-3,1-phenylene))-bis(methylene))bis(triphenylphosphonium) bisperchlorate (26.2ClO_4^-) - Attempted Synthesis



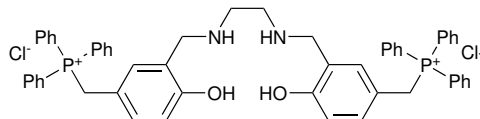
To a solution of 25.2ClO_4^- (0.15 g, 0.15 mmol) in MeOH (10 mL) NaBH_4 (11.3 mg, 0.3 mmol) was added portionwise and the resulting mixture left to stir at rt for 1.5 h. Two additional portions of NaBH_4 (25 mg, 0.66 mg) were added 30 mins and 1 h after the start of the reaction, before the reaction mixture was poured into H_2O (16 mL) and cooled to -18°C overnight. The precipitate evolved was isolated by centrifugation.

(((1E,1'E)-(Ethane-1,2-diylbis(azaneylylidene))bis(methaneylylidene))bis(4-hydroxy-3,1-phenylene))bis(methylene))bis(triphenylphosphonium) dichloride (25.2Cl_2^-)



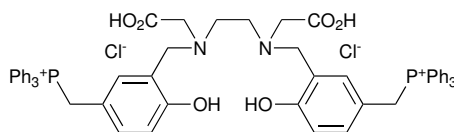
To a solution of **24** (0.54 g, 1.25 mmol) in H_2O (50 mL), a solution of ethylenediamine (41.4 μL , 0.62 mmol) in EtOH (20 mL) was added dropwise, and the resulting solution was stirred at reflux under an N_2 atmosphere for 3 h. The solvent was removed under reduced pressure, and the residue was recrystallised from $\text{CHCl}_3/\text{Et}_2\text{O}$ to yield the title compound as a yellow solid (0.47 g, 85%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 13.27 (2H, s, $\text{N}=\text{CH}$), 8.04 (2H, s, OH), 7.79-7.69 (18H, m, *o/p*-Ar), 7.64-7.52 (12H, m, *m*-Ar), 7.12 (2H, t, $^4J_{\text{HH}} = 2.3$ Hz), 6.94 (2H, dt, $^3J_{\text{HH}} = 8.4$, $^4J_{\text{HH}} = 2.3$ Hz), 6.59 (2H, d, $^3J_{\text{HH}} = 8.3$ Hz), 5.44 (4H, d, $^2J_{\text{HH}} = 14.0$ Hz, CH_2P), 3.75 (4H, s, CH_2CH_2). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 166.1 ($\text{N}=\text{CH}$), 161.4, 135.3 (d, $^3J_{\text{CP}} = 5.2$ Hz), 135.1 (d, $^3J_{\text{CP}} = 6.9$ Hz), 135.0, 134.6 (d, $^2J_{\text{CP}} = 9.2$ Hz), 130.2 (d, $^2J_{\text{CP}} = 12.4$ Hz), 118.7, 118.1 (d, $^1J_{\text{CP}} = 88.6$ Hz, *i*-Ar), 117.5, 117.0 (d, $^2J_{\text{CP}} = 7.7$ Hz), 59.4 (CH_2CH_2), 29.7 (CH_2P). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CHCl_3), δ_{P} (ppm): 22.7.

(((Ethane-1,2-diylbis(azanediyl))bis(methylene))bis(4-hydroxy-3,1-phenylene))-bis(methylene))bis(triphenylphosphonium) dichloride (26.2Cl⁻) - Attempted Synthesis



To a solution of the **25.2Cl⁻** (0.36 g, 0.4 mmol) in MeOH (10 mL), NaBH₄ (30 mg, 0.8 mmol) was added portionwise, and the resulting solution was stirred at rt for 30 min. The solvent was removed under reduced pressure to yield a mix of yellow and colourless residues.

(((Ethane-1,2-diylbis((carboxymethyl)azanediyl))bis(methylene))bis(4-hydroxy-3,1-phenylene))-bis(methylene))bis(triphenylphosphonium) dichloride (28) - Attempted Synthesis



To a solution of **18** (0.63 g, 1.25 mmol) in conc. HCl (37% v/v, 2 mL), paraformaldehyde (48.6 μL, 1.56 mmol) was added and the resulting solution was stirred at rt under an N₂ atmosphere for 48 h. The evolved precipitate was isolated by vacuum filtration, before being washed with aqueous NaHCO₃ (0.5% w/v, 2 × 10 mL) and water (2 × 10 mL) before being dried *in vacuo* to yield the intermediate as a grey solid (0.43 g). The intermediate was dissolved in MeCN (5 mL) and PPh₃ (0.47 g, 1.78 g) was added. The resulting mixture was stirred at reflux under an N₂ atmosphere for 8 h. The reaction mixture was cooled to -18 °C, before the evolved precipitate was isolated by vacuum filtration.

7.2 Radiochemistry (Chapter 4)

7.2.1 General

All general experimental procedures listed in Section 7.1.1 apply.

7.2.1.1 Procurement of Gallium-68 Trichloride

Gallium-68 was eluted as [^{68}Ga] GaCl_3 from an Eckert & Ziegler gallium-68 generator using 5 mL of a 0.1 M solution of clinical-grade hydrochloric acid.

7.2.1.2 iTLC

iTLC was performed using Agilent iTLC-SG Chromatography Paper. Aliquots of radiolabelled compound (2 μL , approx 1 kBq) was spotted 15 mm from the baseline of the iTLC plate, before the iTLC was run in 0.1 M disodium EDTA to a solvent front height of 70 mm. iTLC plates were visualised using a LabLogic Flow-Count detector with a sodium-iodide probe (B-FC-3200).

7.2.1.3 RadioHPLC

Analytical reverse-phase radioHPLC was performed on an Agilent 1200 Series Liquid Chromatograph with UV and) using an Agilent Eclipse XDB-C18 column (4.6 $\times$ 150 mm, 5 μm) with a 1 mL min $^{-1}$ flow rate and UV spectroscopic detection at 250 nm.

Method 1 Isocratic 100% A for 5 min; 0–80% B in A for 20 min; isocratic 100% A for 5 min.

Method 2 Isocratic 100% A for 5 min; 0–100% B in A for 20 min; isocratic 100% B for 5 min.

7.2.1.4 Determination of Log *D* Values

Aliquots of the radiolabelled compound (10 μL , approx. 4 kBq) were added to vials containing a layered mixture of *n*-octanol saturated with PBS (500 μL) and PBS saturated with *n*-octanol (pH 7.4, 500 μL). After vortex mixing for 1 min, the mixture was centrifuged to separate the octanol/PBS phases (10 ^{4} rpm, 2 min), before aliquots of the two phases (100 μL) were taken and transferred into vials for counting. Relative amounts of test compound in each phase were

determined by automatic gamma counter. The log D value was reported as the mean $\pm$ SD of data obtained in 6 independent experiments.

7.2.2 Generic Radiolabelling Procedure

To a solution of chelator in acetate buffer (500 μ L), an aliquot of [^{68}Ga]GaCl₃ in 0.1 M HCl (approx. 50–100 MBq, 500 μ L) was added, and the resulting mixture was heated to yield the desired product. QC was performed using the iTLC method described previously.

7.2.3 [^{68}Ga]Ga-DO3A-xy-TAP (Section 4.1)

[^{68}Ga]Ga-DO3A-xy-TAP compounds were prepared according to the generic radiolabelling procedure, using DO3A-xy-TAP (approx. 100 μ g) in 0.1 M NH₄OAc and heating at 100 °C for 30 min. QC was performed using the iTLC method described previously. Products were analysed by analytical reverse-phase HPLC (Method 1). Retention times were 15, 18 and 20 min for the TPP, TTP and TXP-functionalised variants respectively.

7.2.4 [^{68}Ga]Ga-DO2A-(xy-TAP)₂ (Section 4.2)

7.2.4.1 Initial Radiolabelling

[^{68}Ga]Ga-DO2A-(xy-TAP)₂ compounds were first prepared according to the generic radiolabelling procedure, using DO2A-(xy-TAP)₂ (approx. 100 μ g) in 0.1 M NH₄OAc and heating at 100 °C for 30 min. QC was performed using the iTLC method described previously.

7.2.4.2 Buffer Alteration

Radiolabelling of DO2A-(xy-TAP)₂ was optimised by following the generic radiolabelling procedure, using DO2A-(xy-TAP)₂ (500 μ M) and heating at 100 °C for 30 min. Buffers assessed were 0.05 M, 0.1 M and 0.2 M solutions of NH₄OAc and NaOAc, the pH values of which upon addition to an equivoluminal aliquot of 0.1 M HCl were assessed by pH meter. Experiments were performed in triplicate.

7.2.4.3 Optimised Radiolabelling

$[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TAP)}_2$ compounds were prepared according to the generic radiolabelling procedure, using DO2A-(xy-TAP)_2 (500 μM) in 0.2 M NaOAc and heating at 100 °C for 30 min. Products were analysed by analytical reverse-phase HPLC (Method 2).

7.2.4.4 Analysis of Radiolabelling and Speciation in $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$

Ligand Concentration Alteration

$[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ was prepared by following the generic radiolabelling procedure, using DO2A-(xy-TPP)_2 (500, 400, 300, 200, 100, 50, 5, 0.5 μM) in 0.2 M NaOAc and heating at 100 °C for 30 min. Products were analysed by analytical reverse-phase HPLC (Method 2). Unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ retention time: 2 min; $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ retention times: 14 and 16 mins.

Varying Reaction Times

$[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ was prepared by following the generic radiolabelling procedure, using DO2A-(xy-TPP)_2 (500 μM), in 0.2 M NaOAc and heating at 100 °C. These reactions were performed for 5, 10, 15 and 20 minutes before being analysed by analytical reverse-phase HPLC (Method 2). Unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ retention time: 2 min; $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ retention times: 14 and 16 mins.

An analogous experiment was performed as above, but at room temperature.

Varying Reaction Temperatures

$[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ was prepared by following the generic radiolabelling procedure, using DO2A-(xy-TPP)_2 (500 μM), in 0.2 M NaOAc and heating for 30 minutes. These reactions were performed at 25, 40, 60 and 80 °C before being analysed by analytical reverse-phase HPLC (Method 2). Unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ retention time: 2 min; $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ retention times: 14 and 16 mins.

Assessment of Long-Term Stability

$[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ was prepared by following the optimised radiolabelling procedure (Section 7.2.4.3), before being analysed by analytical reverse-phase HPLC (Method 2). The rest of the reaction mixture was allowed to stand at room temperature, with further HPLC analyses being performed post-reaction. Unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ retention time: 2 min; $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ retention times: 14 and 16 mins.

Varying Sodium Acetate Buffer Concentration

$[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ was prepared by following the generic radiolabelling procedure, using DO2A-(xy-TPP)_2 (500 μM) and heating at 100 °C for 30 min. These reactions were performed in 0.05, 0.1, 0.2 and 0.5 M NaOAc, before being analysed by analytical reverse-phase HPLC (Method 2). Unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ retention time: 2 min; $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TPP)}_2$ retention times: 14 and 16 mins.

7.2.4.5 Prolonged Heating of $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TTP/TXP)}_2$

$[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TTP/TXP)}_2$ compounds were prepared by following the generic radiolabelling procedure, using $\text{DO2A-(xy-TTP/TXP)}_2$ (500 μM), in 0.2 M NaOAc and heating at 100 °C. At various timepoints throughout the heating process, the products were analysed by analytical reverse-phase HPLC (Method 2). Unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ retention time: 2 min; $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TTP)}_2$ retention times: 16 and 20 mins; $[^{68}\text{Ga}]\text{Ga-DO2A-(xy-TXP)}_2$ retention times: 19 and 22 mins.

7.2.5 $[^{68}\text{Ga}]\text{Ga-DO2A-Ar}_2$ (Section 4.3)

7.2.5.1 Initial Radiolabelling

$[^{68}\text{Ga}]\text{Ga-DO2A-Ar}_2$ compounds were prepared by following the generic radiolabelling procedure, using DO2A-Ar_2 (500 μM) in 0.2 M NaOAc and heating at 100 °C for 30 min. Products were analysed by analytical reverse-phase HPLC (Method 2). Unchelated $[^{68}\text{Ga}]\text{Ga(III)}$ retention time: 2 min; $[^{68}\text{Ga}]\text{Ga-DO2A-Bn}_2$ retention times: 12 and 13 mins; $[^{68}\text{Ga}]\text{Ga-DO2A-Xy}_2$ retention times: 14 and 15 mins.

7.2.5.2 Analysis of [^{68}Ga]Ga-DO2A-Ar₂ Speciation

Concentration Alteration Experiments

[^{68}Ga]Ga-DO2A-Ar₂ compounds were prepared by following the generic radiolabelling procedure, using DO2A-Ar₂ (500, 400, 300, 200, 100, 50, 5, 0.5 μM) in 0.2 M NaOAc and heating at 100 °C for 30 min.

Reaction Temperature Alteration

[^{68}Ga]Ga-DO2A-Xy₂ was prepared by following the generic radiolabelling procedure, using DO2A-Ar₂ (500 μM) in 0.2 M NaOAc and heating for 30 minutes. These reactions were performed at 25, 40, 60 and 80 °C before being analysed by analytical reverse-phase HPLC (Method 2). Unchelated [^{68}Ga]Ga(III) retention time: 2 min; [^{68}Ga]Ga-DO2A-Xy₂ retention times: 14 and 15 mins.

Reaction Time Alteration

[^{68}Ga]Ga-DO2A-Xy₂ was prepared by following the generic radiolabelling procedure, using DO2A-Ar₂ (500 μM) in 0.2 M NaOAc and heating at 100 °C. These reactions were performed for 5, 10, 15 and 20 minutes before being analysed by analytical reverse-phase HPLC (Method 2). Unchelated [^{68}Ga]Ga(III) retention time: 2 min; [^{68}Ga]Ga-DO2A-Xy₂ retention times: 14 and 15 mins.

7.2.6 Bridged Cyclens (Section 4.4)

7.2.6.1 Dibenzyl-Functionalised Bridged Cyclen ([^{68}Ga]Ga15b) - Attempted Synthesis

[^{68}Ga]Ga15b was attempted to be prepared according to the generic radiolabelling procedure, using DO2A-(xy-TAP)₂ (500 μM) in 0.1 M NH₄OAc and 0.1 M NaOAc and heating at 100 °C for 30 min. QC was performed using the iTLC method described previously.

7.2.6.2 Dibenzyl-Functionalised Bridged Cyclen ([^{68}Ga]Ga16)

[^{68}Ga]Ga16 was prepared according to the generic radiolabelling procedure, using DO2A-(xy-TAP)₂ (500 μM) in 0.1 M NH₄OAc and 0.1 M NaOAc and heating at 100 °C for 30 min. QC

was performed using the iTLC method described previously.

Further optimisation of this process was performed by using 0.05 M, 0.1 M and 0.2 M solutions of NH_4OAc and NaOAc as radiolabelling buffers.

7.3 Biological Evaluations (Chapter 5)

7.3.1 Isolated Perfused Heart Apparatus

A schematic representation of the isolated perfused heart apparatus is shown in Figure 5.5, with the triple γ -detector system highlighted in red. All parts of the glassware, including the buffer reservoir and the outflow collection cup were jacketed with warm-circulating water at approximately 40 °C in order to maintain a heart temperature between 37.0–37.4 °C. The system was run in constant flow mode rather than constant pressure mode in a manner controlled by the peristaltic pump (Gilson, USA). Buffer outflow was removed using a second peristaltic pump connected to the outflow collection cup.

7.3.1.1 Krebs-Henseleit Buffer and Gas Mixture

Whilst it is possible to use fresh oxygenated blood in perfusion experiments, it is more technically challenging and rarely advantageous when compared to synthetic solutions such as Krebs-Henseleit Buffer (KHB). The perfusate fluid used in these experiments was a phosphorus-free modified Krebs-Henseleit buffer (mKHB) containing NaCl (118 mM), KCl (5.9 mM), MgSO_4 (2.3 mM), NaHCO_3 (25 mM), EDTA (0.65 mM), glucose (11.1 mM) and CaCl_2 (2.5 mM), freshly prepared and filtered before use. The buffer was heated in the reservoir at approximately 40 °C such that the temperature of the perfusate at the cannula was 37.0–37.4 °C, and gassed with 95% O_2 /5% CO_2 .

7.3.1.2 Measurement of Temperature, Flow Rate and Pressure

The temperature of the mKHB at the cannula was verified by inserting a thermocouple thermometer into the cannula, and adjusting the circulator temperature if necessary. Constant flow delivery of the perfusion buffer was controlled through the speed of the peristaltic pump in order to standardise the rate of the radiotracer delivery and to simplify the pharmacokinetic

modelling. A constant flow rate of mKHB through the cannula of 14 mL min^{-1} was obtained through manual calibration of the peristaltic pump speed before starting experiments. Pressure measurements were obtained using pressure transducers connected to a PowerLab/4SP data acquisition system running LabChart 7 (version 7.3.3, AD Instruments, UK.) and calibrated by sphygmomanometer before starting experiments.

7.3.1.3 Acquisition of Functional Data

Perfusion pressure (PP) was recorded with a pressure transducer inserted into the arterial line near the cannula. Cardiac contractile function was monitored using a liquid-filled intraventricular balloon. The left atrial appendage was removed, and a deflated PVC balloon connected to a pressure transducer was carefully inserted through the hole into the left ventricle. The balloon was then inflated with water until an end-diastolic pressure of 4–8 mmHg as measured by LabChart was achieved. From the measurement of the left ventricular pressure, Left Ventricular End Diastolic Pressure (LVEDP) was calculated as the lowest pressure recorded in each heartbeat cycle, Left Ventricular Developed Pressure (LVDP) was calculated as the maximum pressure developed over the LVEDP in each heartbeat cycle, and heart rate (HR) as the frequency of heartbeat cycles.

7.3.1.4 Heart Preparation and Perfusion Protocol

Adult male Wistar rats (250–300 g) were anaesthetised with sodium pentobarbital and heparin sodium (200 mg kg^{-1} and 200 IU kg^{-1} respectively, i.p.) Hearts were rapidly excised and placed in ice-cold mKHB. After trimming the lungs, trachea, thymus and connective tissue from the heart, the heart was then cannulated *via* the aorta with a very low flow of buffer (1–2 drops per second). The aorta was then securely tied with suture, and a small incision into the base of the pulmonary artery to allow outflow of buffer. The buffer flow rate was then increased to 14 mL min^{-1} . After insertion of the left ventricular balloon as described previously, the heart was then externally paced using a Harvard student stimulator pacing unit. One terminal was connected to the heart through the right atrium using a silver wire, the other terminal was connected to the metal cannula. In order to achieve a HR of 340 bpm, the frequency was set to 5.7 Hz. After the heart rate dropped consistently below 320 bpm, the voltage of the stimulator was slowly increased until the heart rate increased to 340 bpm.

7.3.2 Measurement of Radiotracer Kinetics in Isolated Perfused Hearts

7.3.2.1 The Gina StarTM Data Acquisition System and Na/I γ -Detector Array

Radiotracer trapping and washout through the isolated perfused heart was monitored using a custom-built triple detector system incorporated into the perfusion apparatus. Three Na/I γ -detectors were placed as shown in Figure 5.5: in the arterial line above the heart cannula (i), directly opposite the perfused heart (ii) and in the coronary effluent line (iii). This detection system was controlled by a modified Gina Star radioHPLC radiodetection apparatus controlled by Gina Star data acquisition software.

7.3.2.2 Analysis of Tracer Retention using Python

In order to calculate a reproducible and transparent value for the cardiac retention of radiotracers in perfusion experiments, the Python script in Appendix A.1 was designed. This script inputs the data shown in Figure 5.9b as a .csv file, before it is then decay corrected. The resulting time-activity curve is split into two 15-minute portions with the radiotracer injection as close to zero minutes as possible, then each time-activity curve is normalised such that 1 is the peak of the radiotracer bolus and 0 is the average of the activity values between 1–2 minutes before the injection. The script then reports the retention between 14–15 minutes post-injection as a fraction of these normalised values. The two time-activity curves are then plotted as two separate graphs to ensure that the script is functioning correctly and for ease of visualisation. An example of the graphical output of this script, having analysed the time-activity curve in Figure 5.9b is shown in Figure 5.10.

7.3.3 Radiopharmaceutical Preparations

MIBI was prepared and supplied by the Radiopharmacy at St. Thomas' Hospital. The gallium-68 radiolabelled radiotracers [⁶⁸Ga]Ga-DO3A-xy-TXP, [⁶⁸Ga]Ga-DO2A-(xy-TXP)₂ and [⁶⁸Ga]-Ga-DO2A-Xy₂ were prepared as described in the previous chapter.

For experiments in which aliquots of consistent activity were required, the Python script in Appendix A.2 was used. Upon being given a start time, the half-life of the radiotracer being studied as well as the initial volume and activity of a radiopharmaceutical preparation, the script prints the aliquot volume needed to obtain a particular activity of radiotracer. This

value increases throughout the day as the radioisotope decays; using this method allows for, for example, consistent 1 MBq boluses of radiotracer to be administered over the course of a day of perfusion experiments.

7.3.4 Experimental Protocols for Biological Studies

7.3.4.1 *Ex Vivo* Heart Uptake Study Using a Two-Injection Protocol

Rat hearts were excised, cannulated and perfused according to the methods described in Section 7.3.1.4. The hearts were then perfused with mKHB buffer gassed with 95% O₂ and 5% CO₂, and allowed to stabilise for 20 min. During this time, cardiac haemodynamic parameters (heart rate, LVDP and LVEDP) and perfusion pressure were monitored to confirm that surpass the exclusion criteria (Figure 5.6). After this 20 min stabilisation period the first bolus of radiotracer (1 MBq) was injected into the arterial line. The tracer kinetics were monitored for 15 min, before a 6 μ M solution of CCCP in mKHB heated to 37 °C was infused *via* the arterial line at 1.4 mL min⁻¹ (i.e. 10% of the coronary flow), whilst the flow rate at the peristaltic pump was reduced by 10% in order to maintain the same perfusate flow rate to the heart. A further 1 MBq radiotracer bolus was administered 5 min after the commencement of CCCP infusion. This experimental protocol is summarised in Figure 5.8. The radiotracer uptake after both injections was then calculated as discussed in Section 7.3.2.2. Statistical significance between the two values was established using Student's t-test.

7.3.4.2 *In Vitro* Tumour Uptake Study

For radiotracers which did not display myocardial uptake in the perfused heart, a parallel *in vitro* tumour uptake study was performed in collaboration with Mr. Peter Gawne (KCL). For the tumour uptake study, MDA-MB 231 breast cancer cells were used. The radiotracers were dissolved in serum-free cell medium to give an activity concentration of 1 MBq mL⁻¹. 100 kBq aliquots of radiotracer were added to cell samples (2 mL, 5 × 10⁵ cells mL⁻¹) and incubated for 30 min. The cell supernatant was transferred to tubes for gamma counting, before the cells were washed with PBS which was subsequently added to the cell supernatant. The cells were harvested by addition of trypsin (300 μ L) and washing with a serum-free cell medium (700 μ L). The supernatant and the harvested cells were assayed in an automatic

gamma counter to determine percentage tracer uptake. The experiments were performed in triplicate. An associated trypan blue assay was used to assess cell viability.

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Appendix A

Python Scripts

A.1 Cardiac Retention Calculator and Graph Plotter

```

from pandas import read_csv
from numpy import arange
import matplotlib.pyplot as plt

"""Input parameters here. Times are in minutes."""
list_title = 'Cnt c-2, CPS'
half_life = 68
file_title = '180607 D02AxyTXP2 Run 4.csv'
start_delay = 0.
fontsize = 18

injection_1_time = 10.
injection_2_time = 30.

graph_label = ["Control", "600 nM CCCP"]

def time_gen(csv):
    """
    Generates a time domain the same length as the input list.
    :param csv: List of activity values.
    :return: List of times, incrementing in 1/5 second intervals as
    recorded by GinaStar.
    """
    return arange(0, len(csv) * 1/300., 1/300.)

def heart_activity(csv, title, hl):
    """
    Finds a list of activity values located in a csv file output by
    GinaStar, then decay corrects them according to the half-life of the
    radioisotope.
    :param csv: Location of the csv file containing the activity values.
    :param title: Heading of the list containing the desired list of
    activity values.
    :param hl: Half-life of the radioisotope in minutes.
    :return: Decay corrected list of activity values.
    """
    a = read_csv(csv)[title].values
    t = time_gen(a)
    return [float(i) * (2. ** (t[i] / float(hl))) for i in a]

"""Generate the entire list of activity values and decay correct."""
whole_heart = heart_activity(file_title, list_title, half_life)

injection_1_time -= start_delay
injection_2_time -= start_delay

def fifteen_min_a(a, inj_t):
    """
    Takes a 15 minute portion of the activity curve after the injection
    time specified. The heart activity is then normalised by the
    activity 1 minute before injection, and divided by the max value.
    :param a: List of activity values.
    :param inj_t: Injection time of the radiotracer in minutes.
    :print: Activity between 14 - 15 minutes post-injection.
    :return: Normalised list of activity values.
    """
    pre_a = []
    post_a = []
    report = []
    t = time_gen(a)
    for i in range(len(a)):
        if float(inj_t - 2) <= t[i] < float(inj_t - 1):
            pre_a.append(a[i])
        elif float(inj_t) <= t[i] < float(inj_t + 15):

```

```

        post_a.append(a[i])
    pre_average = sum(pre_a) / float(len(pre_a))
    post_a = [i - pre_average for i in post_a]
    post_a = [i / max(post_a) for i in post_a]
    part_t = time_gen(post_a)
    for i in range(len(post_a)):
        if 14. <= part_t[i] < 15:
            report.append(post_a[i])
    print "Injection at " + str(int(inj_t)) + " minutes, 15 minute " \
          "retention = : " + \
          str(sum(report) / float(len(report)))
    return post_a

"""Initialises the graph plotting procedure."""
fig = plt.figure(num=None, figsize=(12.54, 8), tight_layout=True)
kwargs = {'linewidth': 1, 'color': 'k'}

def graph_plot(a, p):
    """
    Plots the graph of the normalised activity over a 15-minute period.
    Also has the capability to plot the two graphs one on top of the
    other.
    :param a: List of activity values.
    :param p: Whether this graph should be on top (0) or on bottom (1).
    Generally control experiments are 0 and CCCP-infused experiments
    are 1.
    :return: Graph of the two time/activity curves.
    """
    ax = fig.add_subplot(211 + p)
    plt.plot(time_gen(a), a, **kwargs)
    plt.ylabel(str(graph_label[p]) + '\nNormalised Activity', fontsize=
               fontsize)
    if min(a) >= 0.:
        plt.axis([0, 15, 0, 1])
    else:
        plt.axis([0, 15, min(a), 1])
    ax.set_xticks(arange(0, 15.1, 5))
    ax.set_xticks(arange(0, 15.1, 1), minor=True)
    plt.xticks(fontsize=fontsize)
    plt.yticks(fontsize=fontsize)
    if p == 1:
        plt.xlabel('Time Post-Injection/ minutes', fontsize=fontsize)

"""Plot the two graphs of the two normalised and measured activity
curves."""
graph_plot(fifteen_min_a(whole_heart, injection_1_time), 0)
graph_plot(fifteen_min_a(whole_heart, injection_2_time), 1)

plt.show()

```

A.2 Live Calculator for Aliquots of Consistent Radioactivity

```
import time

"""Input values here. Time_string in format "%d %m %Y %H %M %S", half-
life in minutes, all other values in consistent units."""
start_time = "03 09 2018 13 05 00"
initial_activity = 141.5
half_life = 68
initial_volume = 1000
required_activity = 1

def elapsed(t):
    """
    Calculates elapsed time since start time.
    :param t: Start time, in format %d %m %Y %H %M %S.
    :return: Elapsed time in seconds as an integer.
    """
    init_time = time.mktime(time.strptime(t, "%d %m %Y %H %M %S"))
    local_time = time.mktime(time.localtime(time.time()))
    return int(local_time - init_time)

"""Quits program if start time is set in the future."""
if elapsed(start_time) < 0:
    print "Error: Start time is set in the future!"
    quit()

def aliquot_vol(a0, th, t, init_vol, req_act):
    """
    Calculates volume of stock solution needed to obtain a desired
    activity value.
    :param a0: Initial activity.
    :param th: Radioisotope half-life in minutes.
    :param t: Current time.
    :param init_vol: Initial volume of the sample of radiotracer.
    :param req_act: Amount of activity required; for perfusion
    experiments this is usually 1 MBq.
    :return: Volume of radiotracer sample (in same units as init_vol)
    required to obtain the desired activity.
    """
    th = float(th) * 60.
    conc = float(a0) * (2. ** (- float(t) / th) / float(init_vol))
    return float(req_act) / conc

def time_print(v):
    """
    Prints current time and a volume.
    :param v: Volume to be printed.
    :return: Prints a string, with current time as %H:%M, followed by
    the volume. uL is assumed as units, although the actual units are
    the same as those specified by the initial volume.
    """
    print str(time.strftime('%H:%M', time.localtime(time.time()))) + " - " + \
          str(v) + " uL"

"""Once the script starts, the initial aliquot volume is checked. If
this is greater than the initial volume, an error message is printed
and the script terminates."""
current_vol = aliquot_vol(initial_activity, half_life, float(elapsed(
    start_time)), initial_volume, required_activity)
if current_vol > initial_volume:
    print "Error: Not enough initial activity for desired volume"
    quit()
else:
```

```
previous_vol = current_vol
time_print(current_vol)

"""Main program loop: every minute, an update aliquot volume is printed.
The program quits once the activity in the sample is too low to yield
the desired activity. Occasionally two values are printed in the same
minute, but these usually differ by less than 0.001 units and as such
either value can be used."""
while True:
    if elapsed(start_time) % 60 == 0:
        current_vol = aliquot_vol(initial_activity, half_life, float(elapsed(
            start_time)), initial_volume, required_activity)
    if current_vol > initial_volume:
        print "Not enough activity for desired volume"
        quit()
    else:
        if current_vol != previous_vol:
            previous_vol = current_vol
            time_print(current_vol)
```

Appendix B

Publications Arising From This Thesis



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Synthesis, gallium-68 radiolabelling and biological evaluation of a series of triarylphosphonium-functionalized DO3A chelators†

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Radiolabelled lipophilic cations that accumulate in mitochondria according to the magnitude of the mitochondrial membrane potential can be used to report non-invasively on mitochondrial dysfunction in cardiovascular disease, cardiotoxicity, and cancer. While several such cations are already commercially available for SPECT imaging, PET offers greater promise in terms of sensitivity, resolution, and capacity for dynamic imaging and pharmacokinetic modelling. We have therefore synthesised a series of three triarylphosphonium-functionalised DO3A chelators for positron emitter gallium-68, with differing alkyl-functionalisation motifs to provide opportunities for tunable lipophilicity as a means of optimising their pharmacokinetics. To assess their capacity to report on mitochondrial membrane potential, we assessed their pharmacokinetic profiles in isolated tumour cells and isolated perfused rat hearts before and after mitochondrial depolarisation with the ionophore CCCP. All three compounds radiolabelled with over 97% RCY and exhibited log *D* values of between −3.12 and −1.81. *In vitro* assessment of the uptake of the radiotracers in cultured tumour cells showed a three-fold increase in uptake compared to unchelated [⁶⁸Ga]Ga(III). However, each complex exhibited less than 1% retention in healthy hearts, which was not significantly diminished by mitochondrial depolarisation with CCCP. This preliminary work suggests that while this approach is promising, the lipophilicity of this class of tracers must be increased in order for them to be useful as cardiac or cancer imaging agents.

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Introduction

Lipophilic cations are widely used to map myocardial perfusion by means of SPECT, and to a lesser extent, PET imaging. They can accumulate in the mitochondrial matrix in a manner dependent on mitochondrial membrane potential ($\Delta\Psi_m$), which in normally functioning mitochondria is between 150–180 mV.^{1–4} In lipophilic cations that have been successfully used as probes of $\Delta\Psi_m$, the charge is delocalised over a large molecular surface area and as such, the tracers can pass easily through the lipid bilayer membrane.² Application of the Nernst equation to monocations predicts 100- to 500-fold accumulation compared to the extracellular space, and at 37 °C, mitochondrial uptake of lipophilic cations

increases 10-fold for every 61.5 mV increase in $\Delta\Psi_m$.^{1,2} This means that the degree of uptake of lipophilic cations can be related to mitochondrial function or dysfunction; for example, compared to healthy mitochondria, $\Delta\Psi_m$ is reduced to below 100 mV in myocardial tissue recovering from reperfusion injury, and at least 60 mV higher in tumour cells, and the uptake of lipophilic cations varies accordingly.^{5,6}

This basic concept has long been used experimentally to report on $\Delta\Psi_m$ through the use of fluorescent lipophilic cations such as tetramethylrhodamine (TMRE), BODIPY and Rhodamine-123.^{7–9} These tracers show great utility in cell culture and isolated tissue preparations, however the poor depth penetration and lack of whole body scanning inherent to fluorescent tracers limits their overall utility. On the other hand, nuclear medicine imaging methodologies such as SPECT and PET provide unparalleled depth penetration, allowing whole body imaging; clinically, SPECT-based lipophilic cations such as [^{99m}Tc]Tc-sestaMIBI (Fig. 1) and [^{99m}Tc]Tc-tetrofosmin (*t*_{1/2} = 6 h) are used.^{10–13} A clinical PET “equivalent” to the SPECT radiopharmaceuticals [^{99m}Tc]Tc-sestaMIBI and [^{99m}Tc]Tc-tetrofosmin would enable higher resolution imaging, and potential detection of anatomically smaller

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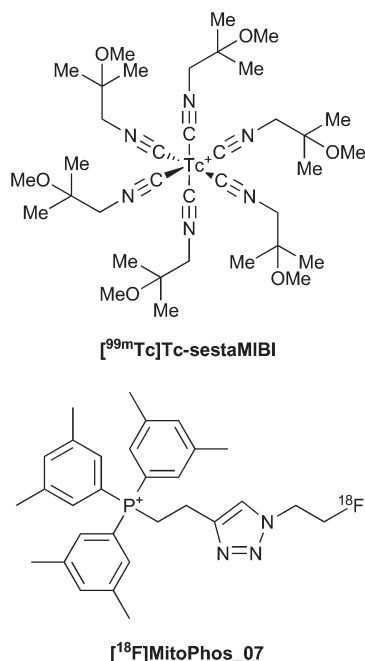


Fig. 1 [^{99m}Tc]Tc-sestaMIBI and [¹⁸F]MitoPhos_07, two examples of lipophilic and cationic radiotracers.^{10,27}

tissue defects with aberrant $\Delta\Psi_m$. In addition, PET radiotracers allow for dynamic imaging, so that the kinetics of influx and efflux can be easily followed. It should also be noted that there exist examples of multimodal optical/PET imaging agents, which consist of a fluorophore functionalised with a PET radioisotope.^{14–16} These compounds allow for the combination of the high resolution of optical imaging with the depth penetration of PET imaging.

PET radioisotopes such as carbon-11 and fluorine-18 ($t_{1/2}$ = 20 and 109.7 min respectively) have previously been incorporated into lipophilic triphenylphosphonium (TPP) cations. TPP is widely known to target mitochondria since the cationic charge nominally residing on the phosphorus atom can be delocalised around the aromatic rings.^{3,4,17,18} The first PET tracer to utilise TPP was designed by Fukuda *et al.* and consisted of TPP methylated with carbon-11 (methylTPP).¹⁹ Since then, there have been many different types of TPP-functionalised PET tracers for mitochondrial imaging, most commonly using fluorine-18 since its longer half-life facilitates clinical imaging.^{20–25} Recently, our group has synthesised the MitoPhos series of tracers, which are fluorine-18 radiolabelled alkyl-functionalised triarylphosphonium (TAP) cations, in order to provide radiotracers with tunable lipophilicity. One example of this series is [¹⁸F]MitoPhos_07, shown in Fig. 1.^{26,27}

Metallic PET radioisotopes, such as copper-64 and gallium-68 ($t_{1/2}$ = 12.7 h and 68 min respectively) have the advantage that once the chelator has been synthesised, the radiometal can be added *via* a simple final synthetic step. Chelators such as DO3A-xy-TPP, which consist of a DO3A chelating moiety, a xylyl linking group (xy) and a TPP cation, have been shown to chelate copper-64 to form effective tumour imaging

agents.^{28–30} Unlike cyclotron-produced copper-64, gallium-68 is generator-produced, and so has the potential to be more widely used as generators become clinically widespread.^{31–34} There has been some preliminary work on synthesising gallium-labelled lipophilic cations for mitochondrial imaging *e.g.* Kardashinsky *et al.* have synthesised DO3A-xy-TAP chelators for gallium-67, in which the TAP groups exhibit methyl and pyridyl functionalisation.^{35–37} However, no mitochondrial uptake studies have been performed on these chelators, and their utility as gallium-68 based imaging agents has yet to be explored.

In this study, we present a series of DO3A chelators for gallium-68 complexation, functionalised with triphenylphosphonium, tritolyphosphonium (TTP) and trixylylphosphonium (TXP) (Fig. 2). These chelators are designed to combine the rationale behind the DO3A-xy-TPP chelators previously used for copper-64 and gallium-67 chelation, with tunable lipophilicity afforded by alkyl-functionalised triarylphosphonium cations in order to form the novel chelators DO3A-xy-TTP and DO3A-xy-TXP.^{27,29,37} After initial synthesis and characterisation of the ligands, their radiolabelling characteristics and log *D* values were analysed to give an indication of their potential use as mitochondrial imaging agents. The tracers

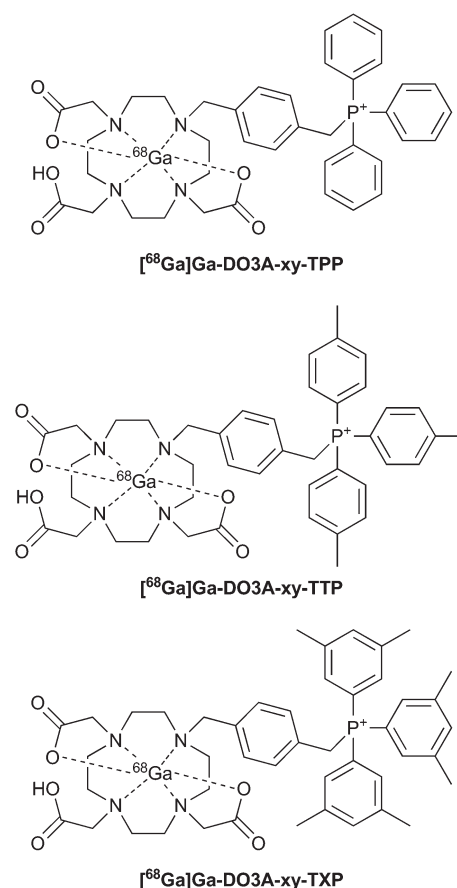


Fig. 2 The three gallium-68 chelate complexes synthesised in this study: [⁶⁸Ga]Ga-DO3A-xy-TPP, [⁶⁸Ga]Ga-DO3A-xy-TTP and [⁶⁸Ga]Ga-DO3A-xy-TXP.

were biologically evaluated in cultured tumour cells and using the *ex vivo* Langendorff isolated perfused heart model with a unique triple gamma detector system, which has been used previously in our group to assess radiotracer pharmacokinetics.^{38–41}

Results

Synthesis of the DO3A-xy-TAP chelators

DO3A-xy-TAP chelators were synthesised as outlined in Scheme 1. The synthesis combined methodologies used by Haslop *et al.*, Wang *et al.* and Kardashinsky *et al.*^{27,28,37} Firstly, an alkyl-functionalised aryl bromide was converted into a Grignard reagent before being reacted with phosphorus trichloride to form a triarylphosphine (1). This strategy allowed tuning of the final tracer lipophilicity by altering the alkyl functionalisation, in accordance with the work performed by Haslop *et al.*²⁷ Whilst anaerobic and anhydrous conditions were maintained as far as possible, some triarylphosphine was found by ³¹P{¹H}-NMR to be oxidised to its phosphine oxide. The phosphine oxide impurity did not require additional purification, since it was inert during the subsequent nucleophilic substitution step due to the lack of a phosphorus-centred lone pair.

Secondly, the triarylphosphine was reacted with α,α' -dibromo-*m*-xylene to form the phosphonium compound and incorporate the xylyl linking group (2). Wang *et al.* performed this reaction at 100 °C in toluene, forming a mixture of the desired monosubstituted product as well as starting material and disubstituted impurities.²⁸ Instead, we performed the reaction at the lowest temperature at which the dibromoxylene fully dissolves in toluene, *i.e.* 60 °C. At this temperature, as 2 forms, it precipitates out of the non-polar toluene solvent, and thus can be readily isolated by vacuum filtration.

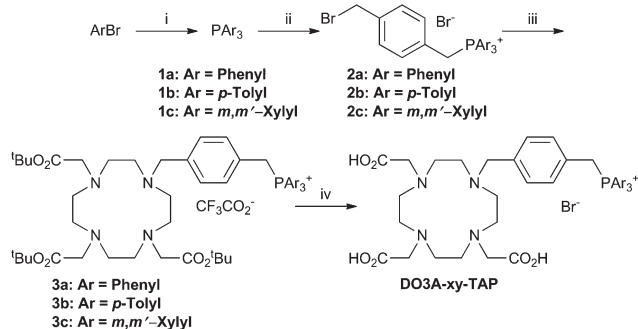
The monocationic bromoxylene phosphonium derivative 2 was then reacted with tris-*tert*-butyl-protected DO3A hydrobromide

to form TAP-functionalised DO3A derivatives (3).⁴² Using sodium carbonate as an inorganic base allowed for the purification by recrystallisation of the tris-*tert*-butyl protected intermediate, as well as its characterisation by NMR. The final step was deprotection of the *tert*-butyl protected carboxylates (3) using trifluoroacetic acid, followed by purification by semipreparative reverse-phase HPLC.

Radiolabelling studies of DO3A-xy-TAP with [⁶⁸Ga]GaCl₃ eluate

All three DO3A-xy-TAP compounds were radiolabelled and analysed using the same protocol. The ligand was dissolved in aqueous ammonium acetate solution in order to partially neutralise the acidic generator eluate containing [⁶⁸Ga]Ga(III). Next, the [⁶⁸Ga]Ga(III) eluate was added, and the resulting mixture was heated at 100 °C for 30 minutes. As a quality control (QC) method, instant thin-layer chromatography (iTLC) was employed, using a mobile phase of 0.1 M EDTA, in which unchelated [⁶⁸Ga]Ga(III) migrated to the solvent front, and left the cationic complex, which resisted transchelation to EDTA, on the baseline. iTLC analysis gave RCY values of 98, 100 and 97% for 100 μ M concentrations of [⁶⁸Ga]Ga-DO3A-xy-TTP, TTP and TXP respectively. Due to the high RCY of the three radiolabelling processes, no post-purification was undertaken.

Finally, the reaction mixture was analysed by radioHPLC to determine whether a single complex had formed and to give an initial indication of its lipophilicity. The radioHPLC traces obtained for the three compounds are shown in normalised form in Fig. 3. In each trace, a small peak can be seen at approximately 2 minutes, which corresponds to unreacted/non-chelated [⁶⁸Ga]Ga(III) species, and a single major peak corresponding to the complex, showing no evidence of isomerism. The relative retention times of the three compounds provide an insight into the lipophilicities of the three compounds. As can be seen from Fig. 2, increased TAP alkylation results in a longer retention time on a reverse-phase C-18 column, and indicates relatively greater lipophilicity. This trend is also seen in analogous HPLC traces of the unchelated



Scheme 1 Synthesis of DO3A-xy-TAP chelators. DO3A tris-*tert*-Bu-HBr was synthesised according to previously reported procedures.⁴² Reaction conditions: (i) Mg then PCl₃, dry THF, anaerobic conditions; (ii) α,α' -dibromo-*p*-xylene, toluene, 60 °C; (iii) DO3A tris-*tert*-Bu-HBr, Na₂CO₃, MeCN, reflux; (iv) TFA, semi-prep HPLC. The choice of the aryl bromide determines which DO3A-xy-TAP variant is synthesised: Ar = phenyl, DO3A-xy-TTP; Ar = tolyl, DO3A-xy-TTP; Ar = *m*-xylyl, DO3A-xy-TXP.

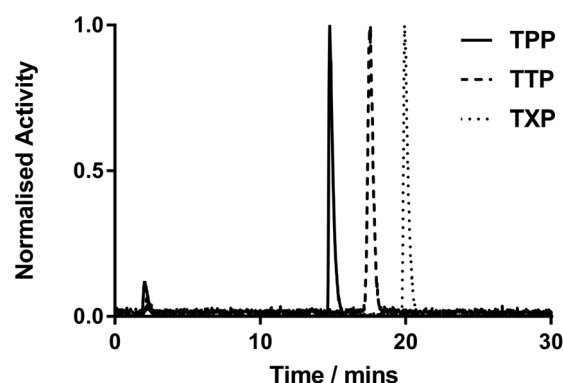


Fig. 3 RadioHPLC traces of the three labelled [⁶⁸Ga]Ga-DO3A-xy-TAP compounds (eluent gradient: 100% A for 5 min; 0–80% B in A for 20 min; 100% A for 5 min; flow rate 1 mL min^{−1}). All traces have been normalised such that the maximum activity measured for each compound is equal to 1.

Table 1 Retention times (in minutes) of the unchelated DO3A-xy-TAP ligands, along with their gallium-68-radiolabelled analogues as measured by HPLC. HPLC conditions are identical to those described in Fig. 3

Ar=	Unchelated ligand	[⁶⁸ Ga]Ga complex
TPP	14.3	14.8
TTP	17.2	17.6
TXP	18.5	19.9

ligands; the retention times of which are summarised in Table 1. The unchelated ligands also show shorter HPLC retention times by HPLC than their gallium-68-labelled counterparts, which indicates an increase in lipophilicity upon chelation, a result of the hydrophilicity of the carboxylate arms decreasing upon binding to the metal centre.

Lipophilicity measurements of [⁶⁸Ga]Ga-DO3A-xy-TAP compounds

To measure the lipophilicities of the radiotracers, their octanol/PBS partition coefficients at pH 7.4 (*log D*) were determined using a conventional 'shake-flask' method. The *log D* values, shown in Fig. 4, were determined to be -3.12 ± 0.17 , -2.66 ± 0.09 and -1.81 ± 0.02 for [⁶⁸Ga]Ga-DO3A-xy-TPP, TTP and TXP respectively ($n = 6$ for all compounds).

In vitro quantification of [⁶⁸Ga]Ga-DO3A-xy-TXP uptake in tumour cells

Intracellular/extracellular concentration ratios for [⁶⁸Ga]Ga-DO3A-xy-TXP and unchelated [⁶⁸Ga]Ga(III) in breast cancer cells (MDA-MB 231) *in vitro* were calculated, and are summarised in Fig. 5. [⁶⁸Ga]Ga-DO3A-xy-TXP shows a ratio of 10.03 ± 0.45 , indicating that the tracers localise inside the tumour cells. In comparison, [⁶⁸Ga]Ga(III) shows a ratio of 3.43 ± 0.39 , indicating that the chelator facilitates a three-fold increase in radiotracer uptake compared to unchelated [⁶⁸Ga]Ga(III). A trypan blue cell viability study showed over 90% of cells used in both the [⁶⁸Ga]Ga-DO3A-xy-TXP and control experiments survived.

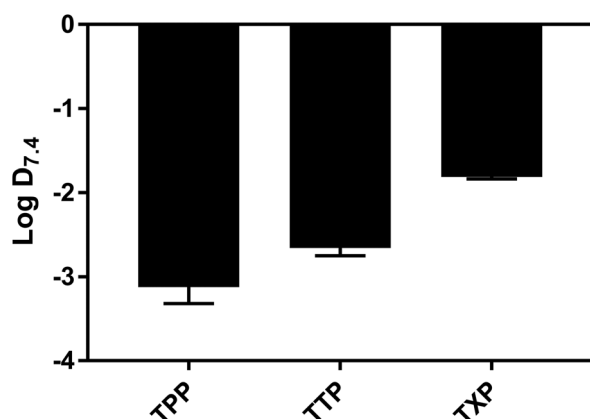


Fig. 4 *Log D* values obtained of the three labelled [⁶⁸Ga]Ga-DO3A-xy-TAP compounds. Data are mean ± SD ($n = 6$).

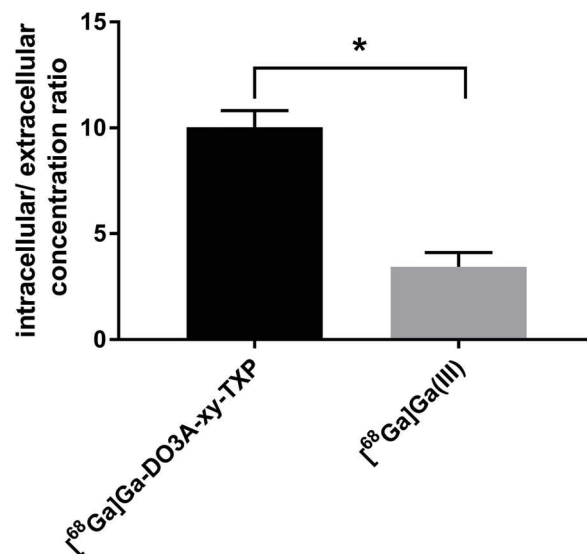


Fig. 5 Results of the *in vitro* tumour cell uptake experiment for [⁶⁸Ga]Ga-DO3A-xy-TXP and unchelated [⁶⁸Ga]Ga(III) control. Measurements were taken in triplicate. Data are mean ± SD. * = $P < 0.05$.

Quantification of [⁶⁸Ga]Ga-DO3A-xy-TXP myocardial uptake using the Langendorff isolated perfused heart model

We have previously made use of the Langendorff isolated perfused heart model to provide an *ex vivo* assessment of myocardial radiotracer uptake, with a unique triple radiodetector system which allows for accurate determination of time-activity curves.^{38–41} The time-activity curves for each of the three radiodetectors quantify radiotracer entering the heart (arterial detector), radiotracer retention in the heart (heart detector) and radiotracer exiting the heart (venous detector). The *ex vivo* nature of the experiment means that the behaviour of the tracer in the myocardium can be evaluated accurately and reproducibly without the complications of the *in vivo* setting such as aberrant metabolism or regional changes in perfusion, and haemodynamic parameters can be easily monitored, whilst maintaining constant perfusion to determine the specific contribution of mitochondrial membrane potential to tracer pharmacokinetics.

To assess radiotracer uptake under conditions in which $\Delta\Psi_m$ is reduced, the heart was infused with a 600 nM solution of the ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP).⁴³ We have previously found that this concentration is optimal to effect complete $\Delta\Psi_m$ depolarisation, validated using the clinical SPECT radiopharmaceutical, [^{99m}Tc]Tc-sestaMIBI (Fig. 1) to ensure validity of the model for this set of experiments.⁴⁴ As shown in Fig. 6A and summarised in Fig. 6B, [^{99m}Tc]Tc-sestaMIBI demonstrates $30 \pm 0.03\%$ ($n = 4$) radiotracer retention after 15 minutes in hearts under normal conditions, but $0 \pm 0.04\%$ ($n = 4$) retention after 15 minutes in hearts under depolarising conditions.¹²

[⁶⁸Ga]Ga-DO3A-xy-TXP (1 MBq) was injected as a bolus into the flow of Krebs buffer. Arterial influx, myocardial retention and venous efflux of radiotracer were measured using each

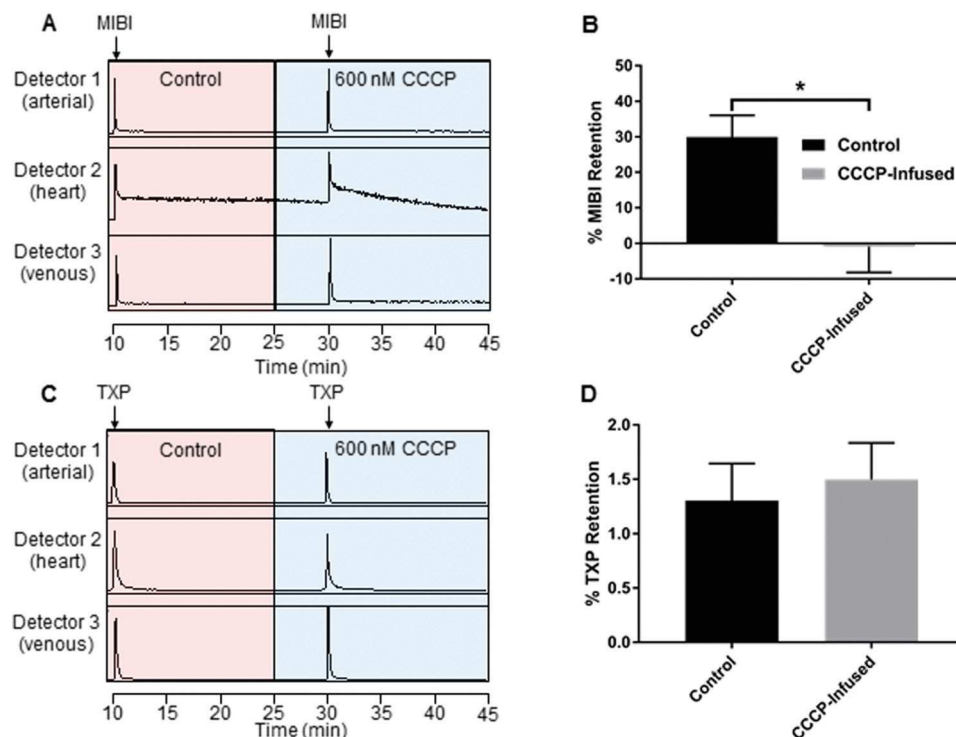


Fig. 6 (A and C) Representative example of data output of the triple-gamma detector system for $[^{99m}\text{Tc}]\text{Tc-sestaMIBI}$ and $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$ respectively. (B and D) Retention values obtained using the two-injection protocol 15 minutes post-injection for $[^{99m}\text{Tc}]\text{Tc-sestaMIBI}$ and $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$ respectively. Control data were obtained from the injection before CCCP infusion, whilst CCCP data were obtained during 600 nM CCCP infusion. Data for $[^{99m}\text{Tc}]\text{Tc-sestaMIBI}$ are mean $\pm$ SD ($n = 4$). * = $P < 0.05$. Data for $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$ are mean $\pm$ SD ($n = 3$).

respective γ -detector (Fig. 6A and C) for 15 min. Then, to assess radiotracer uptake under depolarising conditions, the same heart was infused with CCCP. After 5 min of infusion with CCCP, the second bolus of $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$ was injected, and the passage of radioactivity through the perfusion system was monitored using the three γ -detectors. As shown by Fig. 6C, most of the injected $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$ washed out of the heart within 3 minutes of bolus administration, irrespective of whether the mitochondria were depolarised. This stands in contrast to the control data for mitochondrial depolarisation dependant uptake of $[^{99m}\text{Tc}]\text{Tc-sestaMIBI}$ discussed previously. The average retained gallium-68 activity between 14–15 minutes post-injection over 3 experiments is reported in Fig. 6D. $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$ showed 1% retention in both control and CCCP-infused hearts. Over 3 experiments, there was no statistically significant difference between $[^{68}\text{Ga}]\text{Ga-DO3A-xy-TXP}$ retention in control and CCCP infused hearts.

Discussion

The series of DO3A-xy-TAP compounds was designed to be simple to synthesise and purify, whilst also being easy to modify in order to generate a series of compounds with tunable lipophilicity. Unlike other syntheses of TAP-functionalised DO3A compounds, this work utilises a highly modular

approach, which allows a library of compounds to be synthesised using identical reaction protocols. Use of a lower reaction temperature during formation of the phosphonium cation allows for the facile isolation of the pure target compound, which is especially useful given the difficulties in purifying phosphonium cations using column chromatography.

Upon radiolabelling, the three gallium-68 radiolabelled compounds synthesised all show high RCY values above 97%, despite the size mismatch between DO3A and Ga(III) cations. Whilst DO3A is not an ideal chelator for Ga(III) cations since radiolabelling requires heating and lengthy reaction times, it was chosen for the simplicity of its functionalisation chemistry. A useful part of this work is the method for the QC of gallium-68 labelled lipophilic cations. Basing this method on the facile chelation of Ga(III) by EDTA means that this method should be easily applicable to future syntheses of all gallium-68 labelled lipophilic cations.

Functionalisation of the TAP moiety with alkyl groups has been shown to dramatically affect the lipophilicity of the radiotracers with a 0.5 unit difference between the TPP and TTP-functionalised compounds, and a 0.8 unit difference between the TTP and TXP-functionalised compounds. It is interesting that even when conjugated to the bulky and hydrophilic DO3A chelator, such a small change as the addition of three methyl groups to the TPP group has such a dramatic effect on the log D values. The log D values of the compounds are low when compared to other lipophilic cationic compounds such as

[^{99m}Tc]Tc-sestaMIBI, which has a log *D* value of 1.29.⁴⁵ Nevertheless, it was decided to assess the tracer *ex vivo* as there is precedent of cations with low log *D* values crossing lipid bilayer membranes.^{25,46}

Uptake experiments with [⁶⁸Ga]Ga-DO3A-xy-TXP in tumour cells were undertaken *in vitro*, due to the high ΔΨ_m values often found in tumour cells.^{1,2,6,47–49} In this study, [⁶⁸Ga]Ga-DO3A-xy-TXP showed a three-fold increase in uptake in the cancer cell line when compared to unchelated [⁶⁸Ga]Ga(III), indicating that the chelator facilitates uptake of gallium-68 into tumour cells.

Analysis using the perfused heart model showed no statistically significant difference in [⁶⁸Ga]Ga-DO3A-xy-TXP retention between healthy and CCCP-infused hearts. In addition, the cardiac uptake of our compounds was low when compared to the parallel study performed using [^{99m}Tc]Tc-sestaMIBI. This is likely a result of the lower lipophilicity of [⁶⁸Ga]Ga-DO3A-xy-TXP hindering passage through the lipid bilayer membrane. Since the membrane potential in a normally functioning heart does not facilitate uptake of these tracers in cardiac cells, there will then be no difference in uptake once the mitochondria have been depolarized by CCCP infusion. This preliminary work suggests that while this approach is promising from a synthetic strategy perspective, the lipophilicity of this class of tracers must be increased in order for them to be useful as cardiac or cancer imaging agents.

Conclusions

We have synthesised a series of TAP-functionalised DO3A ligands for gallium-68 chelation with a view to synthesising lipophilic cationic radiotracers. The synthetic methodology developed is effective and entirely modular, and can be the basis of a strategy for synthesising libraries of tracers with tunable lipophilicities, by varying the chelator, linker and cation. The results encourage the development of future gallium-68 labelled lipophilic cations with chelators more appropriate for the efficient formation of lipophilic and positively charged complexes of gallium-68.

Experimental procedures

Materials and instrumentation

Commercially available reagent grade solvents and chemicals were used without further purification. All gas mixtures were purchased from BOC Industrial Gases. ¹H, ¹³C{¹H}, ¹⁹F{¹H}, ³¹P{¹H}, COSY, HSQC, and HMBC NMR spectra were obtained using a Bruker AVIII 400 spectrometer. High Resolution Electrospray Mass Spectrometry was carried out on a Waters LCT Premier (ES-ToF)/Acquity i-Class spectrometer. HPLC was performed on an Agilent 1200 Series Liquid Chromatograph with UV and LabLogic Flow-Count detector with a sodium-iodide probe (B-FC-3200). Mobile phase A contained water with 0.1% TFA, and mobile phase B contained MeCN with

0.1% TFA. Semi-preparative reverse phase HPLC was conducted using an Agilent Eclipse XDB-C18 column (21.2 × 150 mm, 5 μm) with a 5 mL min^{−1} flow rate and UV spectroscopic detection at 214 nm. Analytical reverse phase HPLC was conducted using an Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm) with a 1 mL min^{−1} flow rate and UV spectroscopic detection at 250 nm. Gallium-68 was eluted as [⁶⁸Ga]GaCl₃ from an Eckert and Ziegler gallium-68 generator using a 0.1 M solution of hydrochloric acid.

Synthesis

Synthesis of tris(3,5-dimethylphenyl)phosphane (**1c**) was performed according to previously reported literature procedures by Haslop *et al.*²⁷ Synthesis of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane hydrobromide (DO3Atriestate-HBr) was performed according to previously reported literature procedures by Jagadish *et al.*⁴²

Tri-*p*-tolylphosphane (1b**).** This compound was originally synthesised by Mann *et al.*⁵⁰ The methodology used here was a modification of the procedure described by Haslop *et al.*²⁷ A solution of 4-bromotoluene (2.89 mL, 23.5 mmol) in anhydrous THF (10 mL) was added dropwise whilst maintaining reflux to magnesium turnings (0.57 g, 23.5 mmol) in a Schlenk flask under N₂. The resulting suspension was stirred for 2 h before a solution of PCl₃ (0.69 mL, 7.8 mmol) in anhydrous THF (15 mL) was added dropwise whilst maintaining reflux, and the resulting mixture was stirred for a further 2 h. The reaction was quenched with water (10 mL), and the organic phase was isolated. The organic solvent was removed under reduced pressure to yield the title compound as a pale yellow solid (0.96 g, 40%). ¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 7.24 (6H, m), 7.17 (6H, m), 2.38 (9H, s, CH₃). ¹³C{¹H}-NMR (100 MHz, CDCl₃) δ_C (ppm): 138.6, 133.8, 129.4, 129.3, 21.4. ³¹P{¹H}-NMR (162 MHz, CDCl₃) δ_P (ppm): −7.9. HRMS (ES-TOF+): *m/z* calcd for C₂₁H₂₂P ([M + H]⁺) 305.1459, found 305.1445.

General procedure 1: monosubstitution of α,α'-dibromo-*p*-xylene with aryl-functionalized triarylphosphines (2)

The methodology used was a modification of the procedure described by Wang *et al.*²⁸ α,α'-Dibromo-*p*-xylene (1 eq.) was added to toluene (5 mL) and heated to 60 °C until all the solid had dissolved. To this, a solution of the triarylphosphine (1 eq.) in toluene (6 mL) was added dropwise, before the resulting solution was stirred at 60 °C under an N₂ atmosphere overnight. The white precipitate was isolated by filtration, before being washed with toluene (5 mL) and Et₂O (2 × 10 mL), then dried *in vacuo* to yield the desired product.

(4-(Bromomethyl)benzyl)triphenylphosphonium bromide (2a). This compound was originally synthesised by Wang *et al.*²⁸ Following **general procedure 1**, the title compound was prepared from α,α'-dibromo-*p*-xylene (0.53 g, 2 mmol) and triphenylphosphine (0.54 g, 2 mmol) as a white solid (0.86 g, 82%). ¹H NMR (400 MHz, CDCl₃) δ_H (ppm): 7.73 (9H, m, *m/p*-Ar), 7.60 (6H, m, *o*-Ar), 7.11 (4H, d, ³J_{HH} = 1.3 Hz, C₆H₄), 5.50 (2H, d, ²J_{HP} = 14.6 Hz, CH₂P), 4.37 (2H, d, ⁴J_{HH} = 1.3 Hz,

CH_2Br). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 138.2 (d, $^4J_{\text{CP}} = 4.4$ Hz, *p*-Ar), 135.1 (d, $^5J_{\text{CP}} = 2.8$ Hz), 134.5 (d, $^2J_{\text{CP}} = 9.3$ Hz, *o*-Ar), 132.1 (d, $^3J_{\text{CP}} = 6.1$ Hz, C_6H_4), 130.3 (d, $^3J_{\text{CP}} = 6.1$ Hz, *m*-Ar), 129.5 (d, $^4J_{\text{CP}} = 3.5$ Hz, C_6H_4), 127.9 (d, $^3J_{\text{CP}} = 8.8$ Hz), 117.9 (d, $^1J_{\text{CP}} = 85.9$ Hz, *i*-Ar), 33.0 (CH_2Br), 30.5 (d, $^1J_{\text{CP}} = 47.2$ Hz, CH_2P). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 23.5. HRMS (ES-TOF+): m/z calcd for $\text{C}_{26}\text{H}_{23}\text{BrP}$ ($[\text{M} - \text{Br}]^+$) 445.0721, found 445.0737.

(4-(Bromomethyl)benzyl)tri-*p*-tolylphosphonium bromide (2b). Following general procedure 1, the title compound was prepared from α,α' -dibromo-*p*-xylene (0.53 g, 2 mmol) and tri-*p*-tolylphosphane (0.72 g, 2 mmol) as a white solid (1.04 g, 92%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.54 (6H, m, *m*-Ar), 7.38 (6H, dd, $^3J_{\text{HP}} = 8.2$, $^3J_{\text{HH}} = 3.2$ Hz, *o*-Ar), 7.13 (2H, d, $^3J_{\text{HH}} = 8.1$ Hz, C_6H_4), 7.08 (2H, dd, $^3J_{\text{HH}} = 8.4$, $^4J_{\text{HP}} = 2.4$ Hz, C_6H_4), 5.24 (2H, d, $^2J_{\text{HP}} = 14.5$ Hz, CH_2P), 4.38 (2H, s, CH_2Br), 2.44 (9H, s, CH_3). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 146.3 (d, $^5J_{\text{CP}} = 2.6$ Hz, C_6H_4), 138.1 (d, $^4J_{\text{CP}} = 3.9$ Hz, *o*-Ar), 134.3 (d, $^2J_{\text{CP}} = 10.4$ Hz, *p*-Ar), 132.0 (d, $^3J_{\text{CP}} = 5.7$ Hz, C_6H_4), 130.4 (d, $^3J_{\text{CP}} = 3.4$ Hz, *m*-Ar), 129.5 (d, $^4J_{\text{CP}} = 3.6$ Hz, C_6H_4), 128.1 (d, $^2J_{\text{CP}} = 8.6$ Hz, C_6H_4), 114.6 (d, $^1J_{\text{CP}} = 88.7$ Hz, *i*-Ar), 33.1 (CH_2Br), 30.9 (d, $^1J_{\text{CP}} = 48.1$ Hz, CH_2P), 22.0 (CH_3). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 22.4. HRMS (ES-TOF+): m/z calcd for $\text{C}_{29}\text{H}_{29}\text{BrP}$ ($[\text{M} - \text{Br}]^+$) 529.1660, found 529.1669. Anal. calcd for $\text{C}_{29}\text{H}_{29}\text{PBr}_2$ (found): C, 61.29 (61.22); H 5.14 (5.26).

(4-(Bromomethyl)benzyl)tris(3,5-dimethylphenyl)phosphonium bromide (2c). Following general procedure 1, the title compound was prepared from α,α' -dibromo-*p*-xylene (0.53 g, 2 mmol) and tris(3,5-dimethylphenyl)phosphane (0.87 g, 2 mmol) as a white solid (2.74 g, 88%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.34 (3H, s, *p*-Ar), 7.21 (6H, d, $^3J_{\text{HP}} = 13.0$ Hz, *o*-Ar), 7.15 (2H, d, $^3J_{\text{HH}} = 7.6$ Hz, C_6H_4), 7.02 (2H, dd, $^3J_{\text{HH}} = 8.0$, $^4J_{\text{HP}} = 2.7$ Hz, C_6H_4), 5.21 (2H, d, $^2J_{\text{HP}} = 14.3$ Hz, CH_2P), 4.40 (2H, s, CH_2Br), 2.36 (18H, s, CH_3). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 140.3 (d, $^2J_{\text{CP}} = 12.3$ Hz, C_6H_4), 138.0 (d, $^5J_{\text{CP}} = 3.5$ Hz, C_6H_4), 136.8 (d, $^4J_{\text{CP}} = 3.5$ Hz, C_6H_4), 132.1 (d, $^3J_{\text{CP}} = 5.1$ Hz, C_6H_4), 131.7 (d, $^2J_{\text{CP}} = 10.1$ Hz, *o*-Ar), 129.3 (d, $^4J_{\text{CP}} = 3.5$ Hz, *p*-Ar), 128.5 (d, $^3J_{\text{CP}} = 8.7$ Hz, *m*-Ar), 117.8 (d, $^1J_{\text{CP}} = 84.5$ Hz, *i*-Ar), 33.1 (CH_2Br), 30.9 (d, $^1J_{\text{CP}} = 46.6$ Hz, CH_2P), 21.5 (CH_3). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 22.7. HRMS (ES-TOF+): m/z calcd for $\text{C}_{32}\text{H}_{35}\text{BrP}$ ($[\text{M} - \text{Br}]^+$) 529.1660, found 529.1643. Anal. calcd for $\text{C}_{32}\text{H}_{35}\text{PBr}_2$ (found): C, 62.97 (62.98); H, 5.78 (5.73).

General procedure 2 – coupling of triarylphosphane-substituted bromoxylene to DO3Atriesters hydrobromide (3)

The methodology was a modification of the procedure described by Kardashinsky *et al.*³⁷ To a solution of DO3Atriesters-HBr (1 eq.) and triarylphosphane-substituted bromoxylene (1 eq.) in MeCN (15 mL), Na_2CO_3 (5 eq.) was added, and the resulting suspension was heated to reflux and stirred under an N_2 atmosphere overnight. The inorganic base was removed by filtration and the solvent removed under reduced pressure. The resulting residue was recrystallized from acetone/ Et_2O to yield the desired product.

Triphenyl(4-((4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium bromide (3a). Following general procedure 2, the title compound was prepared from DO3Atriesters-HBr (0.30 g, 0.5 mmol), **2a** (0.26 g, 0.5 mmol) and Na_2CO_3 (0.26 g, 2.5 mmol) as a white solid (0.37 g, 76%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.78 (3H, tt, $^3J_{\text{HH}} = 6.9$, $^4J_{\text{HH}} = 1.7$ Hz, *p*-Ar), 7.74–7.63 (12H, m, *o*/*m*-Ar), 7.05–7.04 (4H, m, C_6H_4), 5.33 (2H, broad s, CH_2P), 3.64 (2H, s, $\text{C}_6\text{H}_4\text{CH}_2$), 3.41–2.11 (22H, m, macrocycle $\text{H}/\text{CH}_3\text{CO}_2$), 1.47 (18H, s, *cis*- $\text{C}(\text{CH}_3)_3$), 1.43 (9H, s, *trans*- $\text{C}(\text{CH}_3)_3$). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 173.7 ($\text{C}=\text{O}$), 172.7 ($\text{C}=\text{O}$), 135.6 (d, $^5J_{\text{CP}} = 4.0$ Hz), 135.3 (d, $^4J_{\text{CP}} = 2.7$ Hz), 134.4 (d, $^3J_{\text{CP}} = 9.8$ Hz), 131.6 (d, $^3J_{\text{CP}} = 5.3$ Hz), 130.9 (d, $^4J_{\text{CP}} = 1.8$ Hz), 130.4 (d, $^2J_{\text{CP}} = 12.8$ Hz), 129.9 (d, $^2J_{\text{CP}} = 8.7$ Hz), 117.1 (d, $^1J_{\text{CP}} = 86.0$ Hz), 83.1 (*cis*- $\text{C}(\text{CH}_3)_3$), 82.6 (*trans*- $\text{C}(\text{CH}_3)_3$), 57.9, 57.0, 50.9, 30.8 (d, $^1J_{\text{CP}} = 46.5$ Hz, CH_2P), 28.2 ($\text{C}(\text{CH}_3)_3$), 28.0 ($\text{C}(\text{CH}_3)_3$). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 23.1. HRMS (ES-TOF+): m/z calcd for $\text{C}_{52}\text{H}_{72}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{Br}]^+$) 879.5190, $\text{C}_{52}\text{H}_{73}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{Br} + \text{H}]^{2+}$) 440.2628, $\text{C}_{52}\text{H}_{74}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{Br} + 2\text{H}]^{3+}$) 293.8443. Found: 879.5258, 440.2600, 293.8420.

Tri-*p*-tolyl(4-((4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium bromide (3b). Following general procedure 2, the title compound was prepared from DO3Atriesters-HBr (0.30 g, 0.5 mmol), **2b** (0.28 g, 0.5 mmol) and Na_2CO_3 (0.26 g, 2.5 mmol) as a white solid (0.23 g, 47%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.57–7.52 (6H, m, *o*-Ar), 7.43 (6H, dd, $^3J_{\text{HH}} = 8.3$, $^4J_{\text{HH}} = 3.3$ Hz, *m*-Ar), 7.07 (2H, dd, $^3J_{\text{HH}} = 8.3$, $^4J_{\text{HH}} = 2.3$ Hz, C_6H_4), 7.03 (2H, d, $^3J_{\text{HH}} = 8.1$ Hz, C_6H_4), 5.18 (2H, d, $^2J_{\text{HP}} = 14.4$ Hz, CH_2P), 3.68 (2H, s, $\text{C}_6\text{H}_4\text{CH}_2$), 3.43–2.18 (16H, m, macrocycle H) 3.04–3.00 (6H, m, CH_2CO_2), 2.47 (9H, s, ArCH_3) 1.48 (18H, s, *cis*- $\text{C}(\text{CH}_3)_3$), 1.43 (9H, s, *trans*- $\text{C}(\text{CH}_3)_3$). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 173.7 ($\text{C}=\text{O}$), 172.7 ($\text{C}=\text{O}$), 146.4 (C_6H_4), 135.6 (C_6H_4), 134.3 (d, $^2J_{\text{CP}} = 10.3$ Hz, *o*-Ar), 131.5 (d, $^4J_{\text{CP}} = 6.4$ Hz, C_6H_4), 131.0 (d, $^3J_{\text{CP}} = 12.5$ Hz, C_6H_4), 130.8 (*m*-Ar), 127.2 (d, $^4J_{\text{CP}} = 8.5$ Hz, *p*-Ar), 114.6 (d, $^1J_{\text{CP}} = 88.7$ Hz, *i*-Ar), 83.0 ($\text{C}(\text{CH}_3)_3$), 82.6 ($\text{C}(\text{CH}_3)_3$), 58.0 ($\text{C}_6\text{H}_4\text{CH}_2$), 56.9 (CH_2CO_2), 56.0 (CH_2CO_2), 51.0 (broad, macrocycle C), 49.4 (broad, macrocycle C), 31.1 (d, $^1J_{\text{CP}}$, CH_2P), 28.2 (*cis*- $\text{C}(\text{CH}_3)_3$), 28.0 (*trans*- $\text{C}(\text{CH}_3)_3$), 22.0 (Ar-CH_3). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 22.1. HRMS (ES-TOF+): m/z calcd for $\text{C}_{55}\text{H}_{78}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{Br}]^+$) 921.5659, $\text{C}_{55}\text{H}_{79}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{Br} + \text{H}]^{2+}$) 461.2863. Found: 921.5684, 461.2828.

Tris(3,5-dimethylphenyl)(4-((4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium bromide (3c). Following general procedure 2, the title compound was prepared from DO3Atriesters-HBr (0.30 g, 0.5 mmol), **2c** (0.31 g, 0.5 mmol) and Na_2CO_3 (0.26 g, 2.5 mmol) as a white solid (0.36 g, 70%). ^1H NMR (400 MHz, CDCl_3) δ_{H} (ppm): 7.35 (3H, s, *p*-Ar), 7.21 (6H, d, $^3J_{\text{HP}} = 13.1$ Hz, *o*-Ar), 7.11 (2H, d, $^3J_{\text{HH}} = 8.0$ Hz), 7.05 (2H, dd, $^3J_{\text{HH}} = 8.2$, $^4J_{\text{HP}} = 2.4$ Hz), 5.15 (2H, d, $^2J_{\text{HP}} = 14.2$ Hz, CH_2P), 3.68 (2H, s), 3.05 (2H, s), 3.01 (4H, s), 2.97–2.16 (16H, m, macrocycle H), 2.36 (18H, s, Ar-CH_3), 1.48 (18H, s, *cis*- $\text{C}(\text{CH}_3)_3$), 1.44 (9H, s, *trans*- $\text{C}(\text{CH}_3)_3$). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, CDCl_3) δ_{C} (ppm): 173.6 ($\text{C}=\text{O}$), 172.7 ($\text{C}=\text{O}$), 140.2 (d, $^2J_{\text{CP}} = 12.6$ Hz, C_6H_4),

136.8 (*p*-Ar), 135.9 (C_6H_4), 131.6 (*o*-Ar), 131.5 (C_6H_4), 130.7 (C_6H_4), 127.5 (d, $^3J_{\text{CP}} = 8.3$ Hz, *m*-Ar), 117.7 (d, $^1J_{\text{CP}} = 84.9$ Hz, *i*-Ar), 82.9 (CH_2CO_2), 82.5 (CH_2CO_2), 57.9 ($\text{C}_6\text{H}_4\text{CH}_2$), 56.7 (CH_2CO_2), 55.9 (CH_2CO_2), 50.8 (broad, macrocycle $\underline{\text{C}}$), 49.2 (broad, macrocycle $\underline{\text{C}}$), 30.9 (d, $^1J_{\text{CP}} = 48.5$ Hz, CH_2P), 28.1 (*cis*- $\text{C}(\text{CH}_3)_3$), 27.9 (*trans*- $\text{C}(\text{CH}_3)_3$), 21.5 (Ar-CH_3). $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3) δ_{P} (ppm): 22.4. HRMS (ES-TOF⁺): m/z calcd for $\text{C}_{58}\text{H}_{84}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{Br}]^+$) 963.6129, $\text{C}_{58}\text{H}_{85}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{Br} + \text{H}]^{2+}$) 482.3098. Found: 963.6139, 482.2990.

General procedure 3 – deprotection of *tert*-butyl protected DO3A-xy-TAP compounds using trifluoroacetic acid (4)

The methodology was a modification of the procedure described by Kardashinsky *et al.*³⁷ *tert*-butyl protected compounds were dissolved in trifluoroacetic acid (reaction mixture concentration 0.1 g mL⁻¹) and stirred at rt under an N₂ atmosphere for 24 h. The acid was removed *in vacuo*, before the residue was taken up in H₂O (50 mL) and washed with CHCl_3 (3 $\times$ 10 mL). The aqueous solvent was removed under reduced pressure, before the residue was purified *via* semi-preparative HPLC methods.

DO3A-xy-TTP trifluoroacetate. Following general procedure 3, the title compound was prepared from **3a** (0.25 g, 0.26 mmol) and trifluoroacetic acid (2.5 mL) as a white solid (0.16 g, 73%). ^1H NMR (400 MHz, MeOD) δ_{H} (ppm): 7.91 (3H, td, $^3J_{\text{HH}} = 7.3$, $^4J_{\text{HH}} = 1.8$ Hz, *p*-Ar), 7.78–7.62 (12H, m, *o,m*-Ar), 7.51 (2H, d, $^3J_{\text{HH}} = 7.8$ Hz, C_6H_4), 7.08 (2H, dd, $^3J_{\text{HH}} = 8.0$, $^4J_{\text{HP}} = 2.7$ Hz, C_6H_4), 4.95 (2H, d, $^2J_{\text{HP}} = 15.1$ Hz, CH_2P), 4.34 (2H, s), 3.97 (2H, s), 3.64 (2H, s), 3.39 (4H, s), 3.29–3.02 (16H, m). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, MeOD) δ_{C} (ppm): 162.8 ($\text{C}=\text{O}$), 162.5 ($\text{C}=\text{O}$), 136.6 (*p*-Ar), 135.3 ($^3J_{\text{CP}} = 9.1$ Hz, *m*-Ar), 133.2 ($^3J_{\text{CP}} = 5.2$ Hz, C_6H_4), 131.5 ($^2J_{\text{CP}} = 13.6$ Hz, *o*-Ar), 119.6 (C_6H_4), 119.0 ($^1J_{\text{CP}} = 86.9$ Hz, *i*-Ar), 116.7 (C_6H_4), 58.5, 55.6, 54.1, 50.5 (broad s), 50.4 (broad s), 30.3 ($^1J_{\text{CP}} = 48.6$ Hz, CH_2P). $^{19}\text{F}\{^1\text{H}\}$ -NMR (377 MHz, MeOD) δ_{F} (ppm): –73.0. $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, MeOD) δ_{P} (ppm): 26.8. HRMS (ES-TOF⁺): m/z calcd for $\text{C}_{40}\text{H}_{48}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{CF}_3\text{CO}_2]^+$) 711.3311, $\text{C}_{40}\text{H}_{49}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{CF}_3\text{CO}_2 + \text{H}]^{2+}$) 356.1689. Found: 711.3320, 356.1727.

DO3A-xy-TTP trifluoroacetate. Following general procedure 3, the title compound was prepared from **3b** (0.21 g, 0.23 mmol) and trifluoroacetic acid (2.1 mL) as a white solid (0.10 g, 57%). ^1H -NMR (400 MHz, MeOD) δ_{H} (ppm): 7.58–7.52 (12H, m, *o,m*-Ar), 7.47 (2H, d, $^3J_{\text{HH}} = 8.2$ Hz, C_6H_4), 7.09 (2H, broad s, C_6H_4), 4.83 (2H, d, $^2J_{\text{HP}} = 15.3$ Hz, CH_2P), 4.48 (2H, broad s), 4.15 (2H, broad s), 3.78–3.43 (4H, m, $\text{CH}_2\text{CO}_2\text{H}$), 3.29–2.90 (16H, m, macrocycle $\underline{\text{H}}$). 2.50 (9H, s, CH_3). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, MeOD) δ_{C} (ppm): 174.2 ($\text{C}=\text{O}$), 171.7 ($\text{C}=\text{O}$), 148.2 (*p*-Ar), 135.2 (d, $^3J_{\text{CP}} = 10.3$ Hz, C_6H_4), 133.2 (d, $^4J_{\text{CP}} = 5.4$ Hz, C_6H_4), 132.0 (d, $^2J_{\text{CP}} = 12.4$ Hz, *o*-Ar), 121.9 (C_6H_4), 119.0 (*m*-Ar), 115.7 (d, $^1J_{\text{CP}} = 90.2$ Hz, *i*-Ar), 113.2 (C_6H_4), 58.6, 55.4, 53.6 ($\text{CH}_2\text{CO}_2\text{H}$), 50.8 (broad, macrocycle $\underline{\text{C}}$), 30.7 (d, $^1J_{\text{CP}} = 48.7$ Hz, CH_2P), 21.8 (CH_3). $^{19}\text{F}\{^1\text{H}\}$ -NMR (377 MHz, MeOD) δ_{F} (ppm): –77.3. $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, MeOD) δ_{P} (ppm): 22.0. HRMS (ES-TOF⁺): m/z calcd for $\text{C}_{43}\text{H}_{54}\text{N}_4\text{O}_6\text{P}$

($[\text{M} - \text{CF}_3\text{CO}_2]^+$) 753.3781, $\text{C}_{43}\text{H}_{55}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{CF}_3\text{CO}_2 + \text{H}]^{2+}$) 377.1924. Found: 753.3786, 377.2102.

DO3A-xy-TXP trifluoroacetate. Following general procedure 3, the title compound was prepared from **3c** (0.33 g, 0.32 mmol) and trifluoroacetic acid (3.3 mL) as a white solid (0.17 g, 57%). ^1H NMR (400 MHz, MeOD) δ_{H} (ppm): 7.52 (3H, s, *p*-Ar), 7.49 (2H, d, $^3J_{\text{HH}} = 7.7$ Hz, C_6H_4), 7.18 (6H, d, $^3J_{\text{HP}} = 13.1$ Hz, *o*-Ar), 7.00 (2H, d, $^3J_{\text{HH}} = 6.7$ Hz, C_6H_4), 4.68 (2H, d, $^2J_{\text{HP}} = 15.0$ Hz, CH_2P), 4.24 (2H, s), 3.83 (2H, s), 3.64 (2H, s), 3.42–3.01 (18H, m, macrocycle $\underline{\text{H}}$), 2.36 (18H, s, CH_3). $^{13}\text{C}\{^1\text{H}\}$ -NMR (100 MHz, MeOD) δ_{C} (ppm): 161.6 ($\text{C}=\text{O}$), 161.3 ($\text{C}=\text{O}$), 140.4 (d, $^2J_{\text{CP}} = 13.7$ Hz, C_6H_4), 136.6 (d, $^4J_{\text{CP}} = 3.7$ Hz, *p*-Ar), 132.0 (d, $^4J_{\text{CP}} = 5.5$ Hz, C_6H_4), 131.5 (C_6H_4), 131.2 (d, $^2J_{\text{CP}} = 10.4$ Hz, *o*-Ar), 118.3 (C_6H_4), 117.5 (d, $^1J_{\text{CP}} = 84.7$ Hz, *i*-Ar), 115.4 (*m*-Ar), 56.8, 54.4, 49.8 (broad s), 49.1 (broad s), 48.7 (broad s), 29.2 (d, $^1J_{\text{CP}} = 49.7$ Hz, CH_2P), 19.9 (CH_3). $^{19}\text{F}\{^1\text{H}\}$ -NMR (377 MHz, MeOD) δ_{F} (ppm): –72.6. $^{31}\text{P}\{^1\text{H}\}$ -NMR (162 MHz, MeOD) δ_{P} (ppm): 22.3. HRMS (ES-TOF⁺): m/z calcd for $\text{C}_{46}\text{H}_{60}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{CF}_3\text{CO}_2]^+$) 795.4250, $\text{C}_{46}\text{H}_{61}\text{N}_4\text{O}_6\text{P}$ ($[\text{M} - \text{CF}_3\text{CO}_2 + \text{H}]^{2+}$) 398.2159. Found: 795.4326, 398.2164.

Radiochemistry procedures

General synthesis of [^{68}Ga]Ga-DO3A-xy-TAP. The methodology was a modification of the procedure described by Wang *et al.*²⁸ To a solution of DO3A-xy-TAP trifluoroacetate (approx. 100 μg) in NH_4OAc buffer (0.1 M, 0.5 mL), an aliquot of [^{68}Ga]GaCl₃ in 0.1 M HCl (approx. 150 MBq, 0.5 mL) was added, and the resulting mixture was heated at 100 $^\circ\text{C}$ for 30 min to yield the desired product. Initial QC was performed using iTLC and a mobile phase of 0.1 M disodium EDTA and by the analytical HPLC method described previously.

Determination of Log *D* values. As an index for lipophilicity, octanol/phosphate buffered saline (PBS) partition coefficient (log *D*) values of the [^{68}Ga]Ga-DO3A-xy-TAP compounds were determined. A general methodology is as follows: aliquots of the radiolabelled compound (10 μL , ~4 kBq) were added to vials containing a layered mixture of *n*-octanol (500 μL) and PBS solution (pH 7.4, 500 μL). After vortex mixing for 1 min, the mixture was centrifuged to separate the octanol–PBS phases (10⁴ rpm, 2 min), before aliquots of the two phases (100 μL) were taken, and transferred into vials for counting. Relative amounts of test compound in each phase were determined by an automatic gamma counter. The log *D* value was reported as the average of data obtained in 6 independent experiments.

In vitro tumour uptake study

For the tumour uptake study, MDA-MB 231 breast cancer cells were used. [^{68}Ga]Ga-DO3A-xy-TXP and [^{68}Ga]Ga(m) were dissolved in serum-free cell medium to give a concentration of 1 MBq mL⁻¹. 100 kBq aliquots of radiotracer were added to cell samples (2 mL, 5 $\times$ 10⁵ cells per mL) and incubated for 30 min. The cell supernatant was transferred to tubes for gamma counting, before the cells were washed with PBS which was subsequently added to the cell supernatant. The cells were harvested by addition of trypsin (300 μL) and washing with a

serum-free cell medium (700 μ L). The supernatant and the harvested cells were assayed in an automatic gamma counter to determine percentage tracer uptake. The experiments were performed in triplicate.

Langendorff isolated heart perfusion

Animals. Male Wistar rats (240–275 g; Charles River Laboratories) were used for all experiments. Animal procedures were in accordance with the Animals (Scientific Procedures) Act, UK, 1986.

The triple- γ -detector system. We developed a system for characterizing the pharmacokinetics of radionuclide passage through an isolated perfused heart comprising 3 orthogonally orientated lead-collimated Na/I γ detectors positioned: (i) 3 cm downstream of a radiotracer injection port on the arterial line, 15 cm upstream of the heart cannula (to provide a radiotracer input function); (ii) directly opposite the heart itself; and (iii) over the venous outflow line (to provide an output function). Each was connected to a modified GinaSTAR instant thin-layer chromatography system running Gina software (Raytest Ltd).

Experimental protocol. Rats were anaesthetized with sodium pentobarbital and heparin sodium (200 mg kg⁻¹ and 200 IU kg⁻¹ respectively, i.p.) and their hearts were excised and cannulated in the Langendorff mode as previously described.⁵¹ The hearts were perfused at 14 mL min⁻¹ constant flow with modified Krebs–Henseleit buffer (mKHB) containing NaCl (118 mM), KCl (5.9 mM), MgSO₄ (2.3 mM), NaHCO₃ (25 mM), EDTA (0.65 mM), glucose (11.1 mM) and CaCl₂ (2.5 mM). The mKHB was freshly prepared and filtered before use, and was gassed with 95% O₂ and 5% CO₂ and used at 37 °C. A left ventricular balloon was used to measure contractile function.

After a 10 min stabilization period, a bolus of radiotracer (1 MBq) was injected into the arterial line, and the activity at the arterial, heart and venous detectors was recorded (Fig. 4A). After 15 minutes, a 6 μ M solution of CCCP in mKHB heated to 37 °C was infused *via* the arterial line at 1.4 mL min⁻¹, whilst the flow rate at the peristaltic pump was reduced by 10%. A further 1 MBq radiotracer bolus was administered 5 minutes after the commencement of CCCP infusion. The radiotracer retained after 15 min in both the healthy and CCCP-infused hearts was reported as the average of the normalized activity between 14–15 minutes after the radiotracer injection.

Author contributions

N.L. and R.S. jointly supervised the work. A.S. carried out the synthetic chemistry, radiochemistry and isolated heart perfusion. P.G. carried out the cell work. A.S., N.L., R.S., P.B. and M.M. co-authored the manuscript.

Conflicts of interest

There are no conflicts to declare.

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