

Luminescent Compounds for Targeting Formyl Peptide Receptors

Tamara Boltersdorf

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Imperial College London
Department of Chemistry

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of Doctor of Philosophy in Chemistry*

Declaration of Originality

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Abstract

Unregulated inflammation is implicated in a variety of disease states such as stroke, cancer and neurodegenerative conditions and often the extent of inflammatory response can determine the prognosis for a particular disease state. Human neutrophils express formyl peptide receptors one (FPR1) and two (FPR2/ALX, also known as the lipoxin A₄ receptor) and are known to both drive vascular inflammation and at the same time be involved in its resolution. Therefore, these receptors represent an attractive target for non-invasive visualisation of inflammation in disease states.

The work within this thesis focusses on designing luminescent imaging probes that selectively tag neutrophils in an inflammatory context. To achieve this, a small-molecule FPR2/ALX ligand was chosen based on ease of synthesis, ability for further functionalisation and a dose-dependent neutrophil response observed *in vitro*. The targeting system was initially combined with a rhodamine-based dye and binding via the neutrophil FPR family was confirmed via pharmacological blocking. *In vitro*, the probe did not affect neutrophil responses and *in vivo* preferential uptake by neutrophils in a model of acute inflammation compared to control groups was demonstrated. Via minor synthetic alterations, a pH-responsive version based on the rhodamine spirocyclisation equilibrium was prepared ($pK_{cycl} = 3.97$) and the compound was shown to be fluorescent in stimulated neutrophils *in vitro* but remained non-fluorescent under normal physiological conditions.

By appending the same targeting group to metal chelates, compounds with long-lived luminescence signals that can be separated from cellular autofluorescence via lifetimes, were prepared. Time-resolved microscopy on activated and unactivated human neutrophils incubated with FPR2/ALX-targeted lanthanide-based compounds was performed, but high concentrations were required. A second iteration of probe design, using a Re(I) tricarbonyl motif, resulted in time-resolved images of neutrophils at lower probe concentration.

Finally, a construct containing a lanthanide unit, a pH-responsive fluorophore and the targeting group was formed and the energy transfer processes in the Tb(III) analogue were investigated. The observed energy transfer provides a unique opportunity to enhance emission properties of the compound for time-resolved imaging via donor-sensitised, long-lived emission, providing an alternative radiative pathway that circumvents the formally forbidden, characteristically weak lanthanide emission.

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Abbreviations

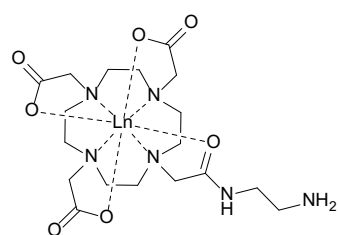
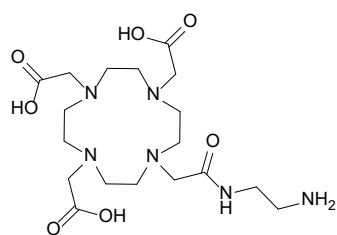
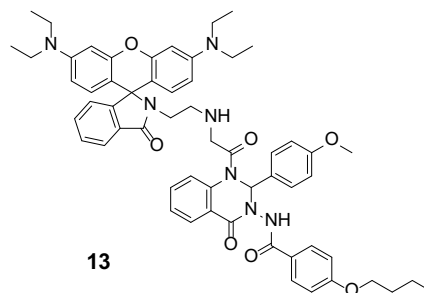
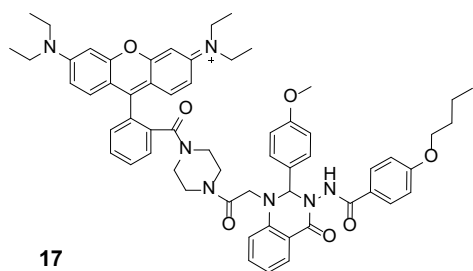
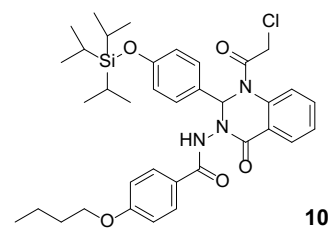
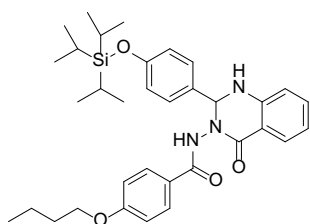
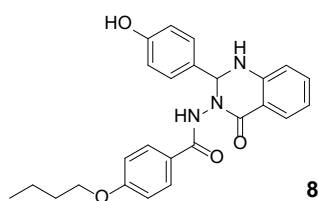
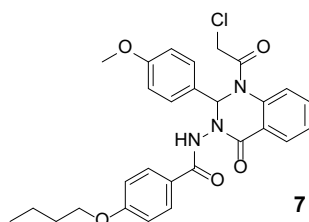
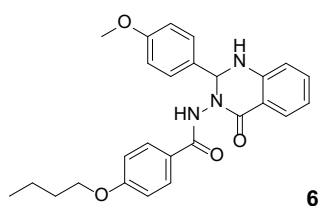
5-ALA	5-aminolevulinic acid
a.m.u.	atomic mass units
AnxA1	annexin A1
ANOVA	analysis of variance
a.u.	arbitrary units
BOC	<i>tert</i> -Butyloxycarbonyl
Boc2	<i>N</i> - <i>tert</i> -butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe
BODIPY	boron-dipyrromethene
COSY	correlation spectroscopy
cFLFLF	cinnamoyl-Phe-D-Leu-Phe-D-Leu-Phe-Lys-NH ₂
CsH	cyclosporin H
CT	computed tomography
Cyclen	1, 4, 7, 10- tetraazacyclododecane
Cy5	pentamethine cyanine
DAPI	4',6-diamidino-2-phenylindole
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMEM	Dulbecco's modified eagle medium
DO3A	1, 4, 7, 10- tetraazacyclododecane-1, 4, 7-trisacetic acid
DOTA	1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid
DOTAGA	2-(4, 7, 10-tris(carboxymethyl)-1, 4, 7, 10-tetraazacyclododecan-1-yl)pentanedioic acid
DTPA	diethylenetriaminepentaacetic acid
EC ₅₀	half maximal effective concentration
ESI	electrospray ionisation
fMLF	<i>N</i> -formyl-methionyl leucyl phenylalanine
FITC	fluorescein-5-isothiocyanate

FLIM	fluorescence lifetime imaging
FPR1	formyl peptide receptor 1
FPR2/ALX	formyl peptide receptor 2/lipoxin A4 receptor
FPR3	formyl peptide receptor 3
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOCl	hypochlorous acid
HOMO	highest occupied molecular orbital
HRMS	high-resolution mass spectrometry
HUVECs	human umbilical vein endothelial cells
HYNIC	hydrazinonicotinic acid
IC	internal conversion
IC ₅₀	the concentration of an inhibitor required to inhibit activity by 50%
ILCT	intraligand charge transfer
IR	infrared
IRF	instrument response function
ISC	intersystem crossing
K	Kelvin
K _x	stability constant
LCMS	liquid chromatography mass spectrometry
LMCT	ligand to metal charge transfer
Ln(III)	lanthanide +3 oxidation state
LUMO	lowest unoccupied molecular orbital
LRET	luminescence resonance energy transfer
LTB ₄	leukotriene B4
LXA ₄	lipoxin A4

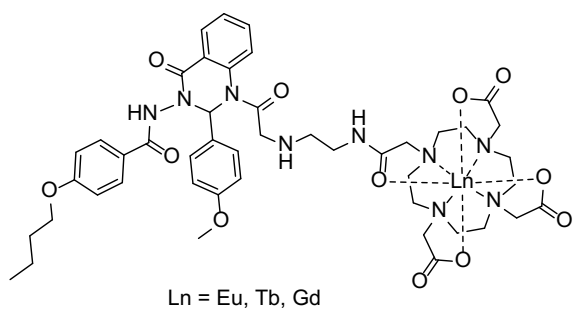
MALDI	matrix assisted laser desorption ionisation
MLCT	metal to ligand charge transfer
MPO	myeloperoxidase
MRI	magnetic resonance imaging
MS	mass spectrometry
NIR	near-infrared
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PET	positron emission tomography
PeT	photoinduced electron transfer
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
ppm	parts per million
q	hydration number
Quin C1	4-butoxy- <i>N</i> -(2-(4-methoxyphenyl)-4-oxo-1,4-dihydroquinazolin-3(2H)-yl)benzamide
Quin C7	4-butoxy- <i>N</i> -(2-(4-hydroxyphenyl)-4-oxo-1,4-dihydroquinazolin-3(2H)-yl)benzamide
R	distance
<i>r</i>	relaxivity
RBL	rat basophilic leukemia cells
ROS	reactive oxygen species
S _x	singlet state
SAAC	single amino acid chelate
SAP	square antiprism
SEM	standard error of mean
SPECT	single photon emission computed tomography

T_1	longitudinal relaxation
T_2	transverse relaxation
T_x	triplet state
TCSPC	time-correlated single photon counting
TFA	trifluoroacetic acid
TICT	twisted intramolecular charge transfer
TIPS	4-triisopropylsilyloxy
TLC	thin layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
TNF α	tumour necrosis factor alpha
Triflate	trifluoromethanesulfonate
TSAP	twisted square antiprism
UV-vis	ultraviolet-visible
WKYMVm	Trp-Lys-Tyr-Met-Val-D-Met-NH ₂
WRW4	Trp-Arg-Trp-Trp-Trp-Trp

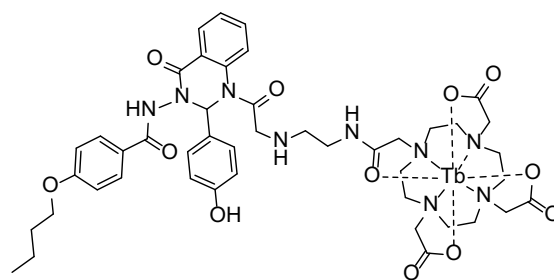
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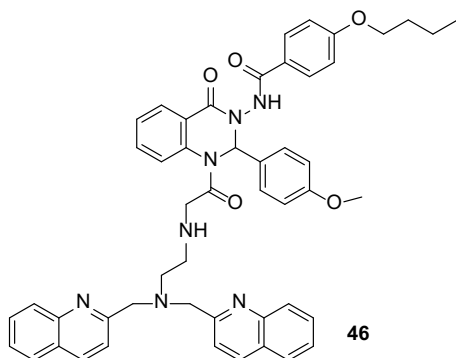
Ln = Eu, Tb, Gd



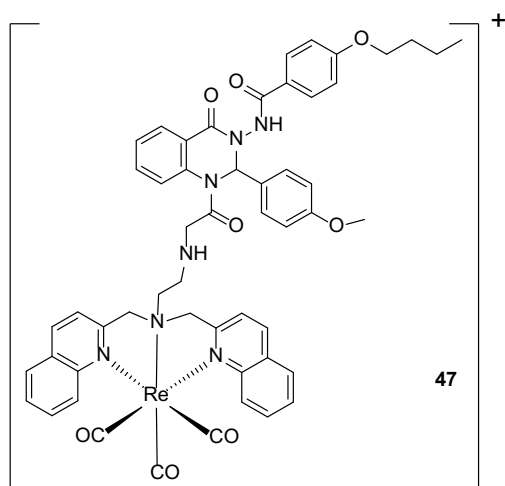
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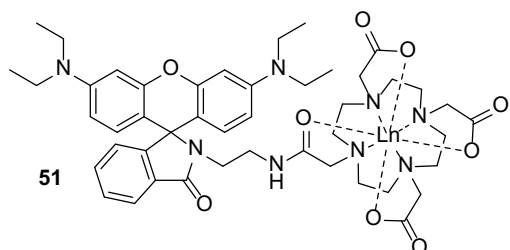
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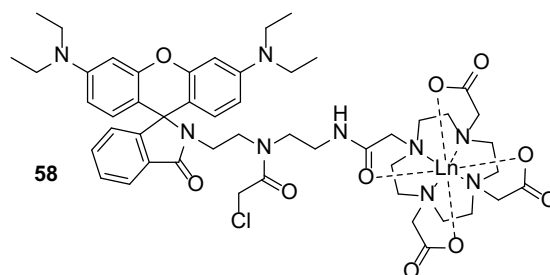
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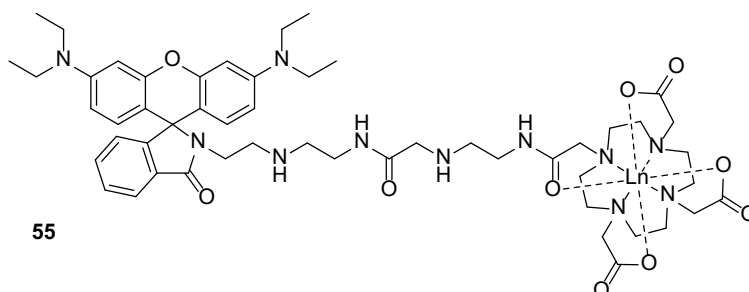
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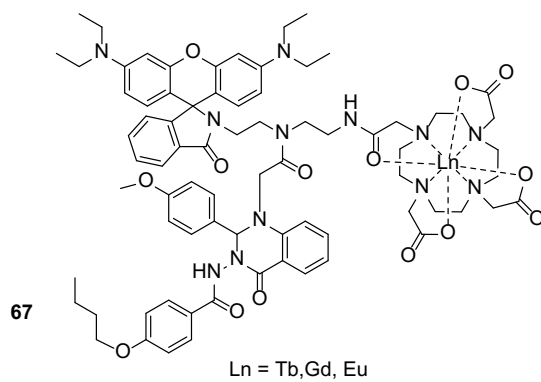
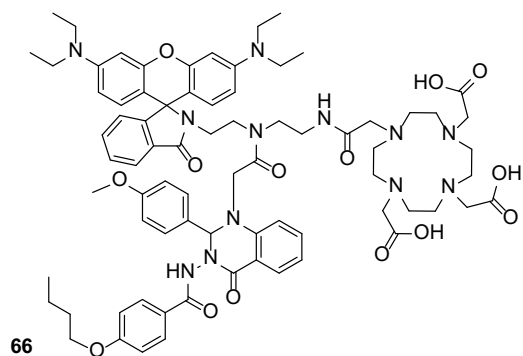
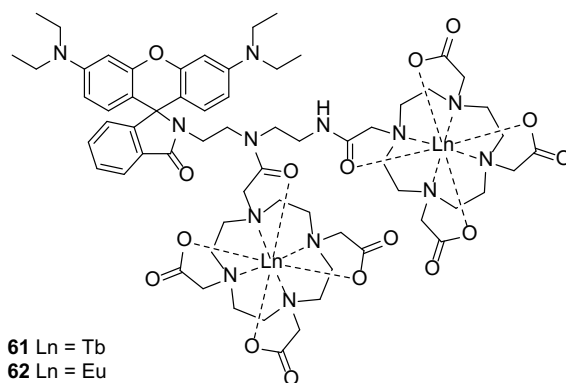
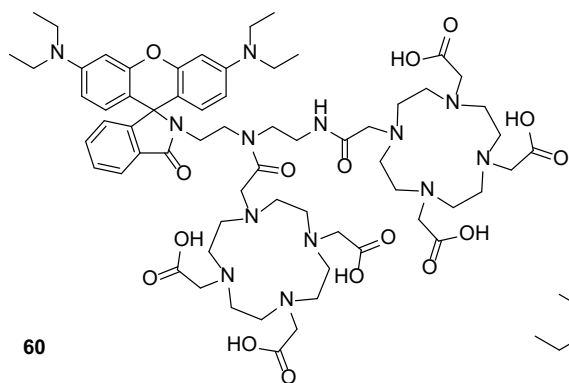
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Chapter 1

Introduction

This chapter aims to introduce the fundamental concepts involved in the themes discussed later in the thesis. Specifically, a broad survey of optical imaging, the use of organic fluorophores and lanthanide complexes in bioimaging applications as well as the basis of inflammation and an overview of formyl peptide receptors (FPRs) and their roles is provided. Each subsequent chapter will contain a brief, more subject-specific introduction focussing on the relevant area.

Briefly, Chapter 2 describes preparation and testing of two related, small-molecule, FPR2/ALX-targeting units. Their combination with an organic dye and a pH-sensitive organic fluorophore and subsequent imaging is outlined in Chapter 3. This is followed by discussion of a lanthanide-based, targeted probe in Chapter 4 and a switch to an emissive rhenium complex that also exhibits long-lived emission with potentially favourable properties in Chapter 5. Finally, Chapter 6 details combination of a switchable organic fluorophore with a lanthanide chelator and a targeting group, to form a complex with interesting photophysical properties as well as a synthetic strategy towards dual-modal, pH-sensitive FPR probes. The thesis aims and objectives are detailed in full at the end of this chapter.

1.1 Molecular imaging

Molecular imaging allows biological processes to be visualised and thus studied *in vivo* and non-invasively at a cellular and molecular level without perturbing the living system that is being investigated.^{1,2} As such, imaging techniques can be used in clinical diagnosis or to monitor disease states and guide therapies, as well as for biomedical research purposes. For instance, certain forms of cancer can be selectively identified at an early stage, and treatment plans can be modulated according to progress, thus allowing patients to be treated more efficiently.³

Historically, the field of medical imaging commenced with an X-ray image of Wilhelm Röntgen's wife's hand in 1895 and has developed ever since, with optical imaging, ultrasound, magnetic resonance imaging (MRI), computed tomography (CT) and nuclear imaging emerging as relevant techniques for *in vivo* imaging.¹ Many of these options are routinely used in a clinical context and the exact choice of imaging mode can be highly significant in identifying a specific issue.

Each modality is characterised by an inherent trade-off between factors such as sensitivity, spatial and temporal resolution, tissue penetration as well as cost and associated patient discomfort. Some of these factors associated with different modalities are summarised in Table 1.1.⁴ Scans can be combined with administration of relevant contrast agents, which are compounds designed to improve image quality, create signals for detection of anatomical information and/or provide a read-out of specific molecular-level events.⁵

	Radiation type	Spatial resolution	Scan time	Depth	Sensitivity	Contrast agent (e.g.)
MRI	Radio waves < 10 ⁻⁵ eV	4 - 100 µm	Minutes to hours	No limit	low	Gd-based, iron oxide nanoparticles
optical	Visible/NIR 2 - 3 eV	15 nm - 1 µm	seconds	< 1 cm	high	Photoproteins (e.g. GFP), fluorophores
PET	High energy γ rays 511 keV	1 - 2 mm	minutes	No limit	high	¹⁸ F, ¹¹ C, ¹⁵ O labels
SPECT	Lower energy γ rays 70 - 370 keV	1 - 2 mm	minutes	No limit	high	^{99m} Tc, ¹¹¹ In chelates
X-ray/CT	X-rays 10 ³ - 10 ⁵ eV	15 - 50 µm 50 - 200 µm	minutes	No limit	low	Iodinated agents, barium sulphate based
Ultrasound	High frequency sound < 10 ⁻⁷ eV	50-500 µm	seconds	mm to cm	high	Microbubbles, micelles, liposomes

Table 1.1: Overview of imaging modalities and their associated properties.^{1,2}

1.2 Designing molecular imaging probes

To create imaging agents which are designed to exploit specific molecular properties to non-invasively enhance the contrast or generate a signal for medical imaging and thus improve the physiological information obtained, several biological factors need to be considered. Probes need to be water-soluble, non-toxic and stable in the presence of endogenous proteins and metals within the host system i.e. kinetically inert as well as thermodynamically stable. This is particularly relevant in metal-based complexes to prevent release of heavy metal ions into a biological system and the associated toxicity. Further, administration of the imaging agent should not perturb the system it is imaging or elicit a pharmacological effect. In general, molecular design should be aimed at achieving high sensitivities in order to minimise the amount of compound that is administered to a patient to reduce toxicity and adverse effects which have been shown to increase with dose.⁵

More specifically, to improve existing clinical technologies by preparing more tailored imaging agents, there are several strategies, illustrated in Figure 1.1.^{6,7,8,9}

- 1) Design probes that optimise molecular parameters to provide enhanced imaging properties.
- 2) Target specific pathways and biochemical components to visualise markers of disease states and decrease non-specific uptake (lower doses).
- 3) Preparation of multi-modal probes to overcome inherent issues encountered with a single imaging technique.

- 4) Construction of responsive or “smart” probes to relay functional information upon sensing a stimulus.

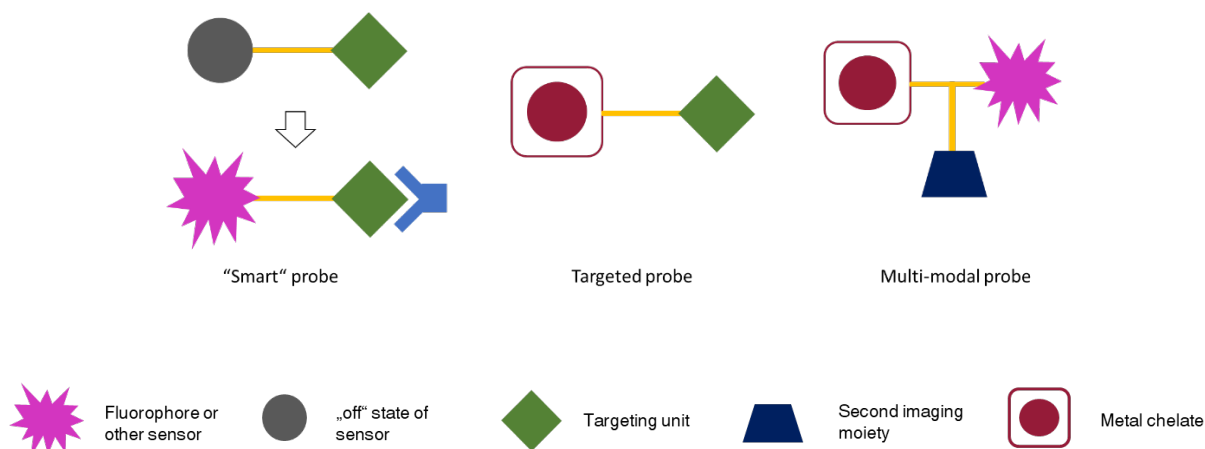


Figure 1.1: Schematic representation of strategies to improve imaging probes.

1.3 Optical imaging

Fluorescence microscopy is a widely used technique for visualisation of biological processes and its excellent spatial resolution (typically around 1 μm , but newer techniques enable even better resolution) allows observation of fluorescently tagged systems on a sub-cellular level.¹⁰ Additionally, it is inexpensive compared to other modalities, portable and can provide environment sensitive real-time data that can rapidly be acquired. These factors, in combination with the ionising-radiation-free imaging enables optical imaging to be used in image-guided surgical and endoscopic procedures as a valuable clinical technique.¹¹ The main drawback of fluorescence microscopy with respect to medical imaging, is the low tissue penetration arising from tissues absorbing, scattering, reflecting and refracting the illumination source which limits the depth at which traditional fluorophores can be detected.¹² This also means that although optical imaging is routinely used for examining cellular level processes and in pre-clinical investigations, the uses in a clinical context are currently limited to near-surface applications.

To address some of these issues, the depth penetration of the excitation and emission sources can be manipulated to improve optical properties within biological media. For instance, areas of low tissue absorption (dominated by water and haemoglobin) are found between 650 nm and 900 nm, and as such this area is more suited for fluorophore absorption and emission and is often referred to as the “near infrared (NIR) window”.^{13,12}

Nevertheless, applications of luminescent probes in biomedical research have dramatically increased over the last 20 - 30 years, which is likely due to more recent advances in the area such as single molecule imaging super-resolution microscopy which have enabled direct investigations on a molecular level into complex physiological processes.¹⁴

1.3.1 Principles of luminescence

Luminescence, a term encompassing both fluorescence and phosphorescence, occurs when a molecule absorbs a photon of the correct energy to excite an electron from the highest occupied molecular orbital (HOMO, also referred to as electronic ground state S_0) to an electronically excited state, followed by spontaneous emission of radiation upon relaxation back to the electronic ground state.¹⁵ When emission occurs from a singlet excited state, such as S_1 , the luminescence process is allowed by selection rules ($\Delta S = 0$) and termed fluorescence. Emission from triplet excited states, which involves a spin change compared to the S_0 electronic ground state ($\Delta S \neq 0$) and is therefore formally forbidden, is less probable and characterised by low intensity emission bands. However, population of, for instance, the T_1 state via intersystem crossing (ISC) from S_1 does occur and emission from this state is termed phosphorescence. A summary of these events is given in the Jablonski diagram below (Figure 1.2). The different timescales during which optical processes occur range from rapid excitation (10^{-15} s) and vibrational relaxation times (10^{-12} s) to phosphorescence, which can occur on up to a millisecond timescale. These timescales can therefore be used to differentiate processes as is the case when comparing phosphorescence ($\geq 10^{-6}$ s) and fluorescence ($\sim 10^{-9}$ s) emission.

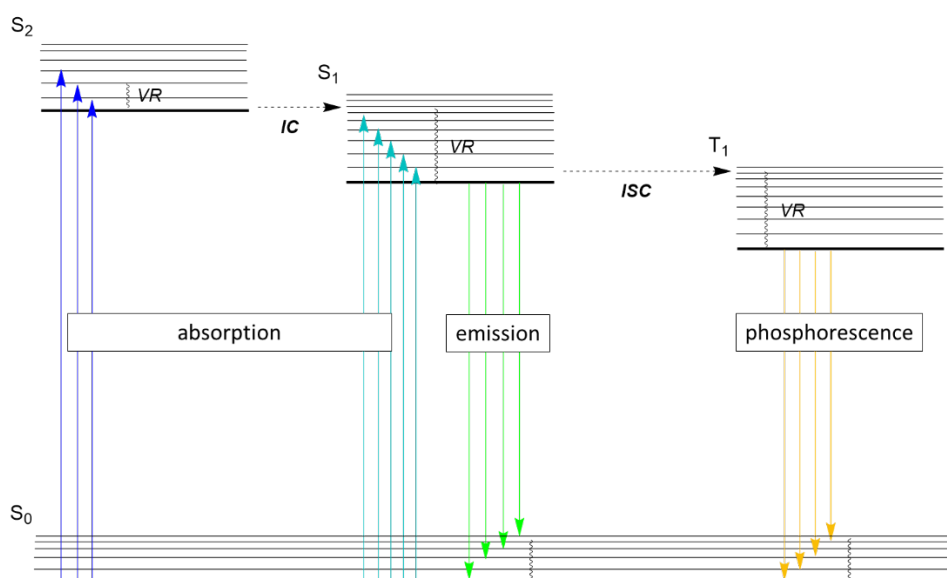


Figure 1.2: Jablonski diagram, S = singlet state, T = triplet state, VR = vibrational relaxation, ISC = intersystem crossing, IC = internal conversion.¹⁵

During the excitation process electronic, vibrational and rotational states within the molecule are altered and the molecule can undergo conformational changes and/or interactions with its environment in the excited state. As a consequence, the emitted photon will be of longer wavelength (lower energy) than the excitation source. The difference between wavelengths of excitation and emission is referred to as Stokes shift. In a biological context, endogenous fluorophore emission (autofluorescence) is typically characterised by small Stokes shifts making compounds with large Stokes shifts desirable for optical agents.¹⁶

A further point to consider is that the signal observed in medical diagnostics is proportional to the “brightness” of a complex, defined as the product of the molar extinction coefficient (for the absorption process) and luminescence quantum yield (for the emission process). It is therefore of great importance to prepare compounds with strong signals by maximising molar extinction coefficients and quantum yields, as this implies less of the probe is required to visualise the same process and as a result toxicity (which generally scales with dosage) is lower.¹⁷

1.3.2 Principles of resonance energy transfer

If two luminescent molecules come within proximity of each other, intra- or inter-molecular fluorescence resonance energy transfers (FRET) between a donor and an acceptor molecule can occur. This process is well-known and often proceeds via nonradiative dipole-dipole interactions (Förster mechanism).¹⁸ In order for this to occur, a spectral overlap between donor emission and acceptor absorption is required. On account of the well-defined dependence on distance between donor and acceptor unit for this interaction, such pairs can provide a useful means for measuring distances between the two within the 10 - 100 Å range and have been described as “molecular rulers”.^{19,20,21} Additionally, FRET (pioneered for instance by Ullman *et al*).²² or lanthanide-based luminescence resonance energy transfers (LRET) can be used in immunoassays to circumvent the need to separate bound and free tracer and thus allow for direct measurements in living cells.²³

The energy transfer process leads to a reduction in both donor lifetime and quantum yields as well as a simultaneous increase in the acceptor’s fluorescence intensity, as illustrated by Figure 1.3.

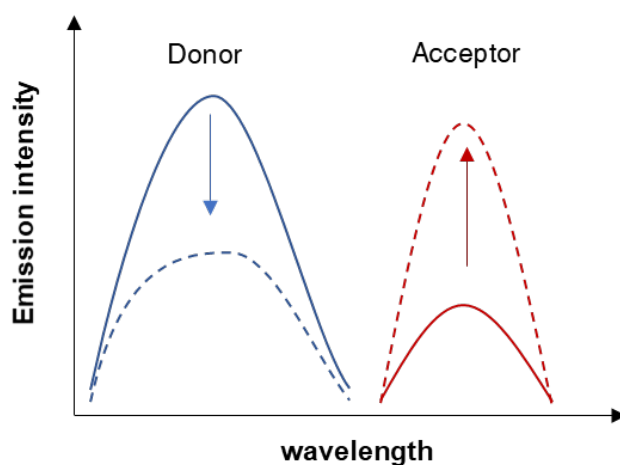


Figure 1.3: Schematic representation of changes in emission intensity of donor (blue) and acceptor (red) fluorophore when an energy transfer process occurs (dashed).

The distance between donor and the acceptor molecule (R) dependence is described by equation 1.1:

$$R = R_0 \left(\frac{1}{E} - 1 \right)^{-6} \quad (\text{eq 1.1})$$

Where E is the efficiency of the energy transfer and R_0 is the Förster radius given by equation 1.2. The Förster radius (R_0) is defined as the distance at which efficiency of the energy transfer is half maximal and can be used to estimate the theoretical intensity resulting from energy transfer processes between a fluorophore pair (eq 1.2)

$$R_0 = \left(\frac{8.785 \times 10^5 \kappa^2 \Phi_D J}{\eta^4} \right)^{\frac{1}{6}} \quad (\text{eq 1.2})$$

Where κ^2 expresses the orientation factor between the lumiphores (and is often approximated to be 2/3 in organic-based systems), Φ_D is the donor quantum yield, J defines the spectral overlap between donor emission and acceptor absorbance and η is the refractive index of the medium.^{24,19} Apart from a close donor-acceptor proximity, further requirements for an efficient FRET to occur include a good overlap between the donor emission spectrum and the acceptor excitation spectrum as well as non-perpendicular dipole-dipole angles.²⁵

In order to separate the FRET signals from direct acceptor fluorescence and donor bleed-through, images are usually acquired for 1) donor emission at donor excitation, 2) acceptor emission at donor excitation and 3) acceptor emission at acceptor excitation and can be processed after acquisition.²⁶ For short-lived donor-acceptor pairs such as two organic dyes, measurements are limited in their accuracy by the very short, multiexponential lifetimes of the fluorophores and the poor signal-to-noise ratio resulting from background fluorescence. A further limitation of FRET is the dependence of the energy transfer efficiency on the relative orientation of donor and acceptor (expressed by κ^2 , eq 1.2), which is often poorly known for organic components and introduces unknown error factors.²⁰

1.3.3 Luminescent probes for optical imaging

1.3.3.1 Organic Fluorophores

The most commonly applied fluorophores in microscopy are organic dyes that are typically composed of fused aromatic ring structures. Amongst these, many compound families that are notable for their exceptional brightness and biocompatibility have been reported, for instance, coumarins, acridines, rhodamines, cyanines and BODIPY (Figure 1.4).²⁷ Fluorescence properties can often be fine-tuned by synthetic modifications, and derivatives have been engineered for bioimaging applications as peptide or protein tags, for analyte or pH sensing, as molecular rotors and for cell or organelle staining in immunofluorescence applications.¹² More recently, genetically encoded fluorophores such as green fluorescent protein (GFP) have further revolutionised the ability to observe biological processes and protein interactions in live cells.¹⁷

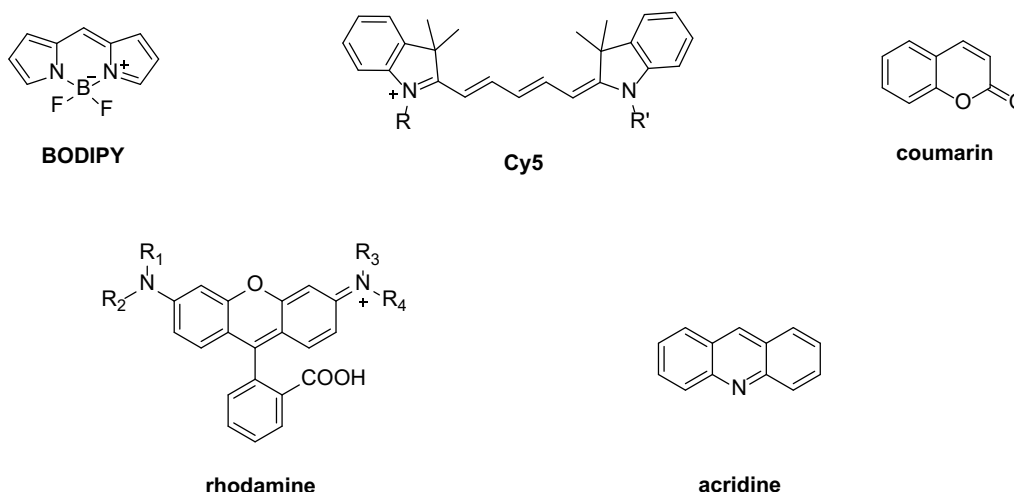


Figure 1.4: Core molecular structures of well-known organic dye families.

For imaging of cancers in fluorescence guided surgery, the fluorophores indocyanine green, 5-aminolevulinic acid (5-ALA) and methylene blue have been clinically approved (Figure 1.5).²⁸ Indocyanine green typically binds to plasma proteins due to its high hydrophobicity and can localise at cancerous sites via passive extravasation into tumour tissues, which are characterised by leaky vasculature (a phenomenon termed “enhanced permeability and retention” effect).^{29,30} 5-ALA has been approved for uses in glioblastoma imaging. The compound is metabolised via the haem synthesis route and converted into the protoporphyrin IX (Figure 1.5), with both fluorescent (excitation and emission in the visible range) and photodynamic properties.³¹ Similarly, methylene blue (Figure 1.5) is known for both photodynamic therapeutic availability and far-red fluorescence at low concentrations. The dye is unusual in the fact that it exists as various cations in solution and has been used for a variety of clinical applications such as imaging of different cancer types and treatment of infections.^{32,28}

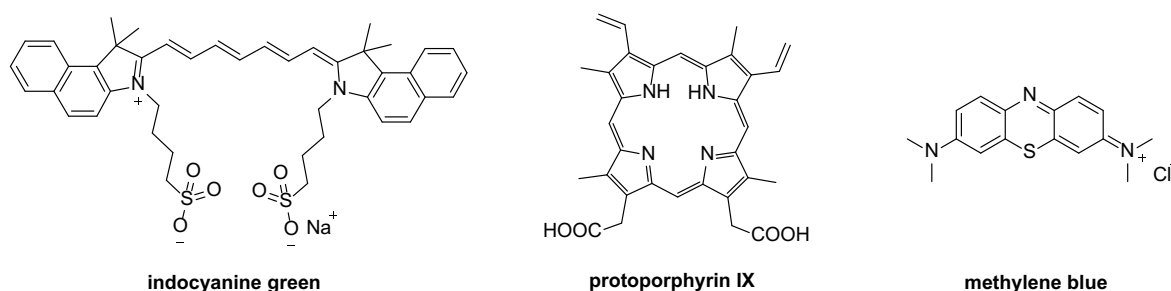


Figure 1.5: Molecular structures of clinically approved optical dyes for cancer detection.

None of these approved dyes however are selective for cancers and can accumulate non-specifically, reducing the signal to noise ratios as well as resulting in false positives.³⁰ To improve sensitivity and specificity of detection and thus their utility, an ongoing surge in efforts to develop dyes that are targeted

to cancer markers, or become activatable in cancerous environments, has occurred. Amongst numerous successful preclinical examples a selection of targeted probes have been put forward to the clinical trials stage.^{33,34,35}

For cellular imaging, many approaches to probe design have been reported that enable functional information about the cellular status to be relayed. A recent example by Umezawa *et al* reports preparation of a compound to detect glutathione (GSH) dynamics, which are essential for cellular homeostasis and can aid understanding of pathophysiological processes in disease states, in live-cell imaging and real-time.³⁶ Their rhodamine-based platform was constructed to produce a rapid and reversible reaction to cellular glutathione and allowed ratiometric concentration quantification (Figure 1.6).

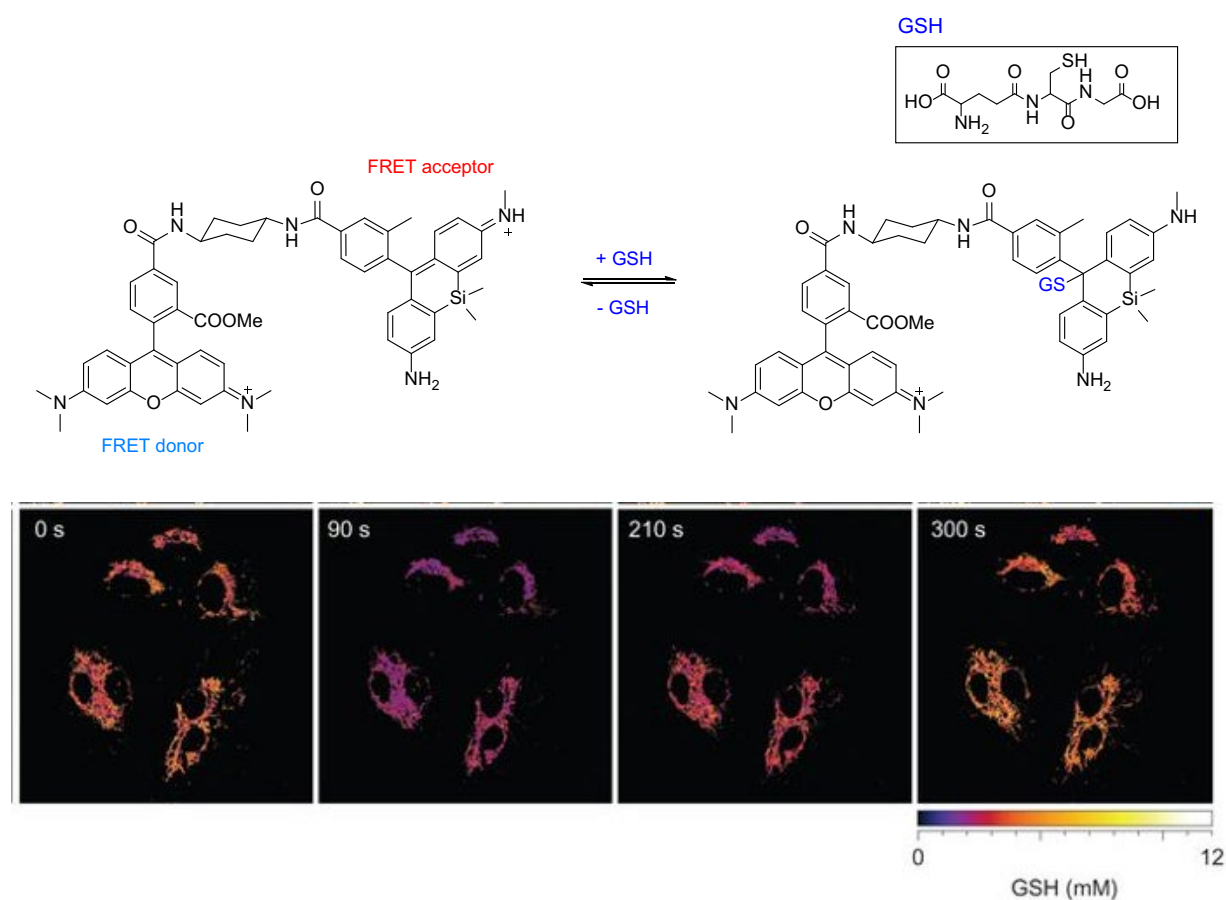


Figure 1.6: Molecular structure of glutathione sensitive probe (above) and glutathione (inset) and ratio images indicating GSH concentration taken at different time frames in HUVEC cells (below). Hydrogen peroxide was administered at 30 s – 90 s, resulting in a reduction of cellular GSH concentration, which was restored upon reperfusion with H_2O_2 -free medium. Excerpt of images reproduced with permission from reference.³⁶

Despite the functionality of organic probes in many bioimaging applications their uses are often compromised by low photostability (particularly in the NIR range), high hydrophobicity and small Stokes shifts. Some of these issues can be addressed by using metal-based luminescence.

1.3.3.2 Inorganic coordination compounds for optical imaging

When manipulated correctly, metal-based luminescent compounds can overcome many of the issues associated with currently more common organic fluorophores. In particular, d^6 transition metal species as well as Ln(III) complexes are often characterised by suitable photophysical properties including large Stokes shifts, long luminescence lifetimes in the micro- to millisecond range based on metal-centred emission and improved photostability.^{16,37} In addition, ligand systems can be easily functionalised to incorporate bioconjugation vectors, control cellular localisation properties and tune emission features.³⁸

(a) Transition metal-based systems:

For cellular applications, the most commonly applied d^6 metals are iridium(III), rhenium(I) and ruthenium(II). Kinetically inert, biocompatible complexes based on all three metals have been abundantly reported within the literature for cellular imaging purposes.^{16,37}

For instance, Vellaisany *et al* recently reported a typical, iridium-binding ligand framework that was modified to include a dopamine receptor agonist (Figure 1.7).³⁹ They demonstrated good photostability and low cytotoxicity of the complex in cells, and were further able to monitor binding to dopamine receptors and agonist-mediated cellular internalisation as well as visualise the complex by two-photon excited, time-resolved microscopy (Figure 1.7).

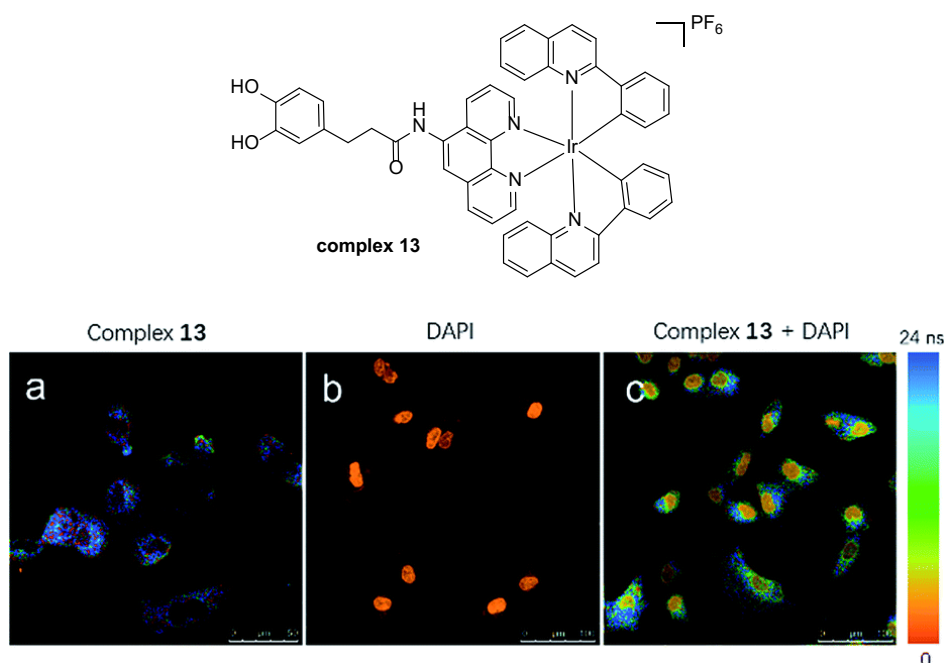


Figure 1.7: Molecular structure of compound **13** (above) published by Vellaisany *et al* and lifetime maps of A549 cells incubated with compound **13** (a), DAPI (b) and **13** and DAPI (c). Reproduced from reference with Creative Commons license (CC BY 3.0).³⁹

Ruthenium-based systems have been used as DNA stains in living cells and applied for therapeutic purposes.^{40,41} Further, studies conducted by Sun *et al* have indicated that in chiral ruthenium(II) polypyridyl complexes, one enantiomer acted as a more efficient luminescent tag and could preferentially bind to G-quadruplex DNA over duplex DNA in cells.⁴²

Stable, kinetically inert and luminescent rhenium(I) tricarbonyl complexes, which are typically combined with polypyridyl or bisquinoline ligands, have similarly been investigated for their cellular imaging abilities and as sensors of cellular products and are discussed in more detail in 5.⁴³

(b) Lanthanide-based systems

Similar to transition metal complexes, lanthanide complexes can provide photostable and inert architectures with large Stokes shifts and long luminescence lifetimes that greatly surpass those of both organic dyes and d-block metals. However, excitation pathways can be more complex and emission intensities are often low compared to organic dyes due to the formally Laporte forbidden nature of the f-f transition.

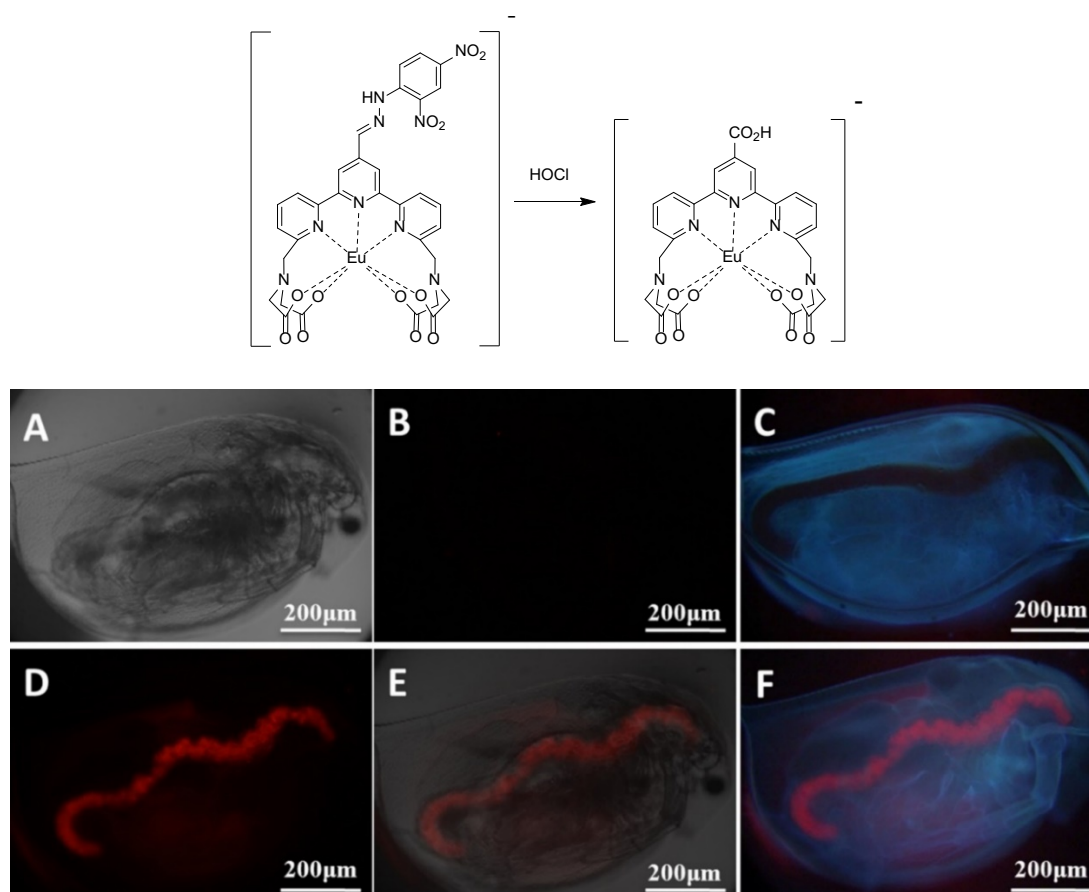


Figure 1.8: Above, molecular structure of a hypochlorous acid sensitive probe. Below, luminescence images of *Daphnia magna* treated with the compound. A) brightfield, B) time-gated acquisition before treatment with hypochlorous acid, C) steady-state acquisition before treatment with hypochlorous acid, D) time-gated luminescence after treatment with hypochlorous acid, E) the merge between image A and D and F) steady-state acquisition after treatment with hypochlorous acid. Reproduced from reference with permission.⁴⁴

Nevertheless, numerous cellular imaging examples have been reported (see Chapter 4 for more details) and lanthanides have further become relevant for therapeutic *in vivo* purposes and optical imaging.^{45,46}

One such example is a recently reported hypochlorous acid sensor using a luminescent europium-complex. The Eu(III) chelating motif was combined with luminescence-quenching group that was cleaved upon reaction with HOCl, resulting in an “on” response (Figure 1.8). This “on” response was observed by time-gated methods in stimulated macrophages as well as in living organisms (*Daphnia magna*) treated with HOCl.^{44,47} Using a similar strategy, the same group recently prepared mitochondria targeted, biothiol sensing combined europium and terbium complexes.⁴⁸

1.4 Lanthanide coordination compounds for bioimaging applications

The characteristic $[Xe]f^n$ electronic configuration of lanthanides with contracted, core-like 4f orbitals allows them to essentially retain their free ion spectroscopic and magnetic properties in their predominantly trivalent coordination complexes.⁴⁹ Bonding to lanthanide ions with large ionic radii occurs typically via octa- or nona-dentate complexation and has an ionic nature (with only small contributions from covalent interactions). The lack of f-orbital participation in bonding implies that coordination geometries are routinely controlled by steric constraints of the ligand rather than ligand field effects and careful consideration is required to select donor sets leading to kinetically inert complexes.

1.4.1 Optical properties of lanthanide complexes

From a photophysical point of view, lanthanide-based probes for cellular imaging can offer several advantages over the more common organic fluorophores. Firstly, their luminescent lifetimes are several orders of magnitude longer (in the micro/millisecond range) and secondly, their emission profiles are characterised by large Stokes shifts.⁵⁰ Both factors contribute to the possibility of excluding cellular autofluorescent background signals associated with π -conjugated systems such as flavins, hemes and other metabolites, either by time-gating techniques or the use of emission filters.^{46,51} Additionally, their emission profiles are often sharp, distinct peaks that are only minimally affected by their environment due to the core-like character of the $4f^n$ electronic configuration and their luminescence is not quenched by the oxygen triplet ground state that is present in aerated biological media.

However, the absorption cross-section of lanthanide ions is low, as a result of small dipole strengths of formally Laporte forbidden 4f-4f transitions, making strong luminescence from direct excitation difficult.^{52,53} The selection rules are somewhat relaxed by spin-orbit coupling, but nevertheless the bands tend to be weak. To circumvent this issue, so-called “antenna” chromophores can be used to absorb radiation and then transfer it to the emissive lanthanide ion excited state by a process termed sensitisation, formally defined as “light conversion via an absorption-energy transfer-emission sequence involving an absorbing ligand and an emitting metal” (Figure 1.9).⁵⁴

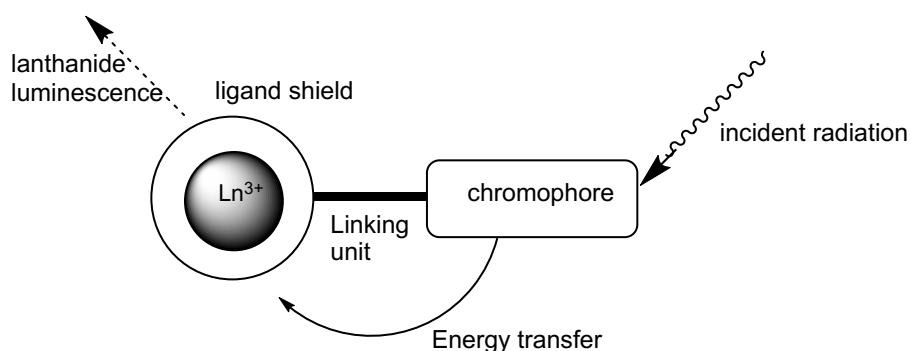


Figure 1.9: Illustration of lanthanide sensitisation process via an organic antenna unit. Redrawn from reference.⁵³

Lanthanide complex excited, emissive states are determined by the microstates arising from f-electron configurations (Russell Saunders spin-orbit coupling) and their luminescence spans a wide area of the electromagnetic spectrum from UV for Gd(III) through visible for Eu(III), Tb(III), Sm(III) and Dy(III) to near IR regions of Nd(III), Yb(III), Er(III) and Pr(III).⁵⁵ Emission intensities vary and often, sensitised Eu(III) and Tb(III) chelates prove most useful for imaging purposes, as a result of the large energy gap between ground and excited states and associated largest lanthanide emission intensities (energy gap law).⁵⁶

To optimise the sensitisation process, there are two main factors to account for: Firstly, photophysical efficiency of the chromophore donor to lanthanide acceptor state energy transfer process and secondly the competing non-radiative deactivation pathways. The energy transfer typically occurs via a Laporte allowed ligand absorption, intersystem crossing to a long-lived, ligand-based T_1 state followed by energy transfer to the lanthanide excited state and subsequent metal emission,⁵⁷ although competing processes can occur via for instance CT participation, particularly if the Ln(II) oxidation state is accessible (Figure 1.10).^{58,59,60}

An ideal energetic match between the antenna triplet state and lanthanide excited state is a balance between energy levels close enough to enable fast sensitisation kinetics, while at the same time maintaining a large enough gap to avoid thermally activated back energy transfer to the sensitiser (BT, Figure 1.10).⁶¹ The typical Eu(III) emissive state $^5D_0$ is found at $\sim 17,200\text{ cm}^{-1}$ and the Tb(III) counterpart, $^5D_4$ lies around $\sim 20,400\text{ cm}^{-1}$ and antennae are usually considered suitable if they have a triplet states $> 2000\text{ cm}^{-1}$ above the lanthanide acceptor state.^{62,60,63}

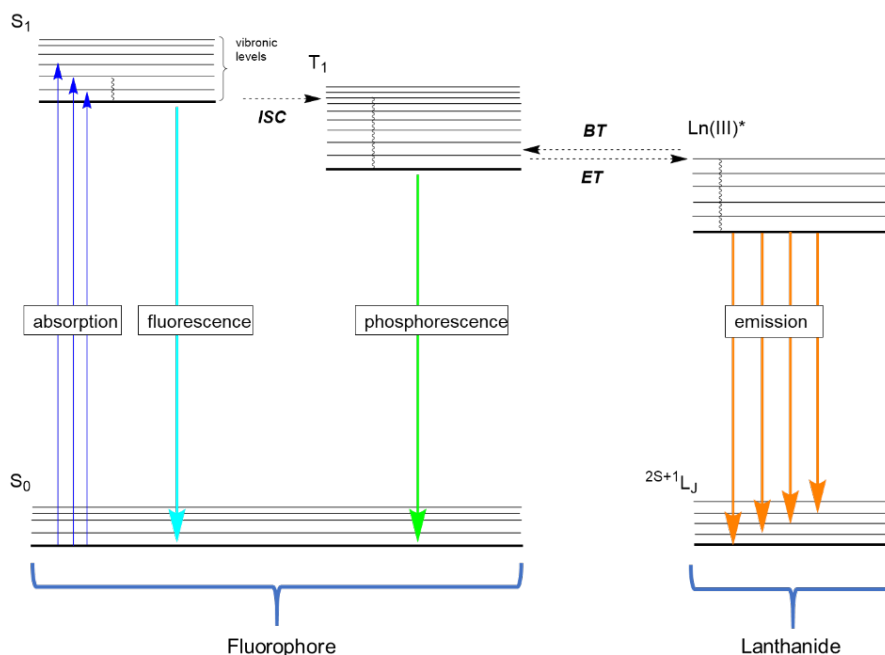


Figure 1.10: Jablonski diagram illustrating a typical energy transfer pathway to the lanthanide excited state. S = singlet state, T = triplet state, ISC = intersystem crossing, ET = energy transfer, BT = back transfer.

Furthermore, spatial proximity between the two groups is essential as the transfer process usually requires one of two points: either physical orbital overlap (for an electron exchange as routinely observed for Dexter exchange mechanism energy transfer processes⁶⁴) or a Coulombic resonance interaction (as described by a Förster mechanism⁶⁵). The energy transfer rate via the Dexter mechanism decays exponentially with distance between the donor and acceptor, and significant orbital overlap is usually described up to 30 - 50 pm.⁵⁵ The Förster mechanism, where non-radiative resonance is achieved between energy release from an excited donor group and a ground state acceptor group leading to excitation of the acceptor, also has a distance dependent term ($\propto R^{-6}$). In this case, excitation of the acceptor can occur over longer distances compared to the Dexter mechanism. Often, the energy transfer is likely to occur via a combination of both mechanisms, although some examples have been reported, where a specific sensitisation mechanism prevails.⁶⁶ For example, Sykes *et al* reported a Dexter-based energy transfer mechanism for lanthanide sensitisation in europium/iridium dyads.⁶⁷

For luminescent lanthanide compounds, non-radiative quenching processes by solvent O-H oscillator vibrations are particularly effective, due to the large Franck Condon overlap with the metal-excited states (Figure 1.11). To a lesser degree, vibrational overtones of N-H, C-H and C=O are also known to deactivate excited state lanthanide luminescence. To minimise these interactions, encapsulating, hydrophobic, organic ligands are often constructed that can shield the metal from solvents.^{68,69} The effect of luminescence quenching by O-H vibrational modes can be employed to gain insights into the amount of first coordination sphere bound water molecules (q-value), by comparing the effect of O-D and O-H oscillator quenching on luminescence lifetimes, using a well-established linear relationship between the two.⁷⁰

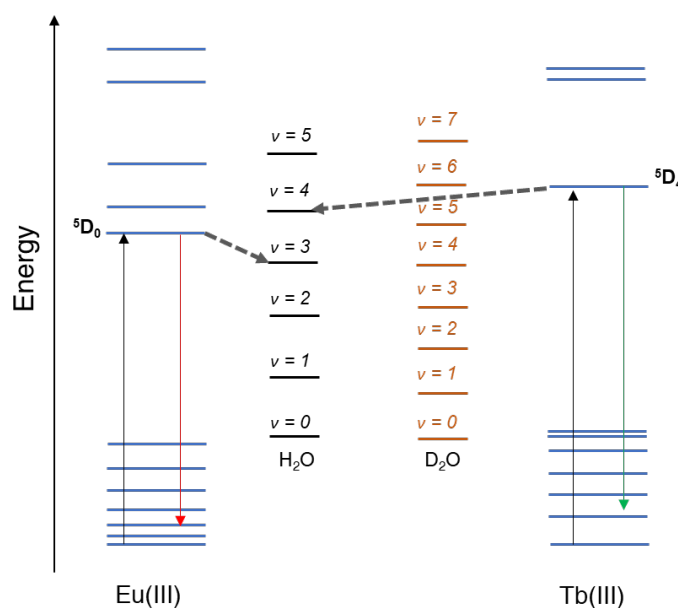


Figure 1.11: Representation of Eu(III) and Tb(III) excited states and associated vibrational quenching by H_2O and D_2O . Redrawn from reference.⁷⁰

1.4.2 DOTA macrocycles as ligand systems

DOTA or DO3A derived macrocycles, first reported in 1976 have generated sustained interest as metal-binding ligands in biomedical applications as a result of the kinetically inert and thermodynamically stable, octadentate lanthanide coordination complexes they form.⁷¹ Thermodynamic stability constants ($\log K_{Gd}$) for Gd(III) DOTA for instance have been reported as 24.7, compared to 22.46 for DTPA, an acyclic analogue.⁷² In its uncomplexed form, the macrocycle is flexible and adopts a variety of conformations. When binding to a metal, the system is characterised by a preorganised donor set of eight “hard” oxygen/nitrogen Lewis bases that bind via the four cyclen-based amines and the four acetate arms. Together with the excellent size match for lanthanide binding, this has proven useful in a variety of biomedical applications and clinically approved contrast agents such as ProHance, Dotarem and Gadovist are based on this structure (Figure 1.13).^{73,74,75}

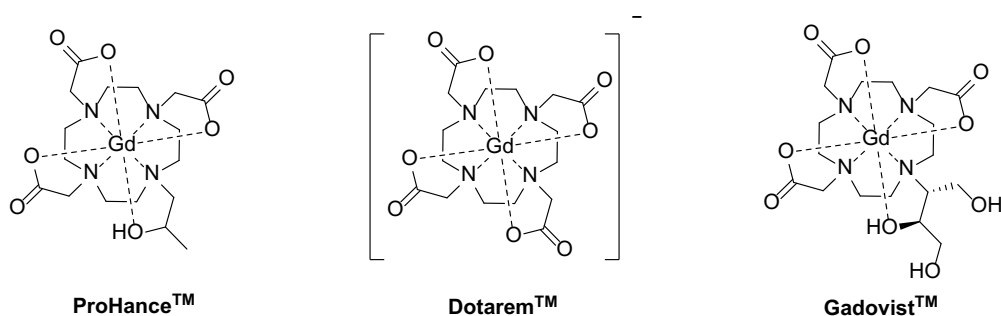


Figure 1.13: Molecular structures of clinically approved DOTA-derived contrast agents.

In the context of luminescent or MRI applications, these chelators have three main functions: Firstly, to encapsulate the bound lanthanide and avoid decomplexation under physiological conditions. This is critical as even low concentrations of free gadolinium can severely disrupt endogenous signalling, in particular Gd(III) has a comparable ionic radius (108 pm) to Ca(II) (114 pm), but the higher charge density of the lanthanide ion means it can outcompete calcium.⁷⁶ Secondly, they enable site-specific attachment of the metal with a well-defined coordination environment and thirdly, the ligand acts as a shield to minimise excited state quenching of lanthanide luminescence from nonradiative deactivation by surrounding solvent molecules.

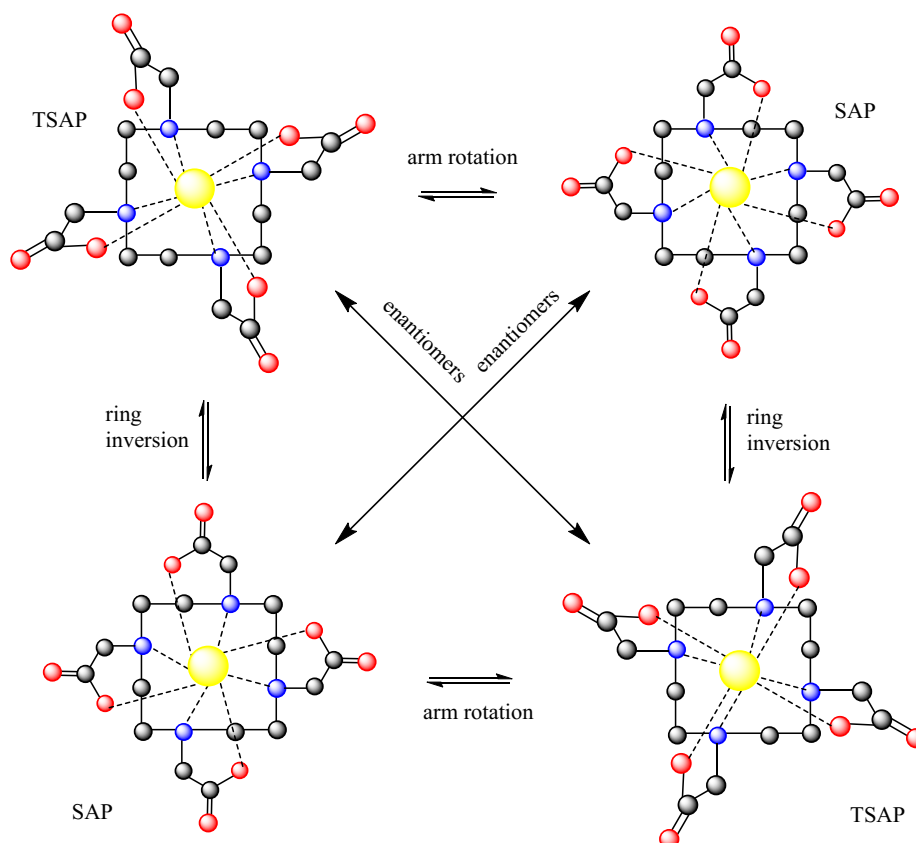


Figure 1.14: Stereoisomerism in lanthanide DOTA complexes. Adapted from reference.⁷⁷

Ln(III) DOTA structures are known to form two different diastereomers (of which one is usually more abundant than the other) in solution that can interconvert on an NMR timescale, of which each is part of an enantiomeric pair, that is however indistinguishable by NMR (Figure 1.14).⁷⁸ The first stereoisomer can be visualised as a square antiprism (SAP) and is also termed the major isomer, with a twist angle between the oxygen and nitrogen binding planes of $\sim 40^\circ$. The second, minor isomer is referred to in the literature as a twisted square antiprism (TSAP) and the twist angle between the two planes is closer to $\sim 25\text{--}30^\circ$ (illustrated in Figure 1.15).⁷⁷

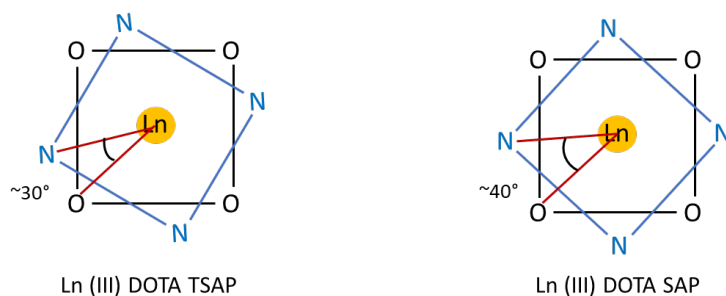


Figure 1.15: Representation of Ln(III) DOTA complex twist angles in the SAP and TSAP isomers.⁷⁷

The exact geometry of the Ln(III) DOTA complexes is dependent on the metal ion size, with smaller lanthanides including Eu(III) reported as square antiprismatic (SAP) solid state structures^{79,80} whereas the larger lanthanides adopt capped twisted square antiprismatic (TSAP) structures.⁸¹ In solution interconversion between the two geometries occurs either by ring inversion, or rotation of the acetate arm and can be controlled by various factors such as temperature and pressure as well as ionic strength of the solution.^{82,83} For example, although the SAP conformation is the dominant form for most Ln DOTA complexes, increasing the steric demand on the metal centre is known to favour the TSAP conformation and raising the amount of salt present will similarly shift the equilibrium towards the more polarisable TSAP conformation (which is better stabilised by outer sphere salt binding).^{84,78} This is particularly relevant with respect to utilising the Gd(III) analogues as contrast agents, as water exchange rates and the associated relaxivity values increase with reduction of bite angles, as the bound water molecule lies further away from the lanthanide ion and consequently the TSAP geometry is more favourable. In fact, water exchange is up to 50 times faster in the TSAP isomer compared to the SAP isomer.⁸⁵ Recently it was reported that introducing chiral centres into the DOTA macrocyclic ring afforded complexes that were more kinetically inert than traditional Ln(III) DOTA complexes, displayed favourable water exchange kinetics (and preference for the TSAP isomer depending on substituent) and, remarkably, no gadolinium deposition within mice brains in *in vivo* studies, which has become a serious safety concern with clinically used agents (Figure 1.16).⁸⁶

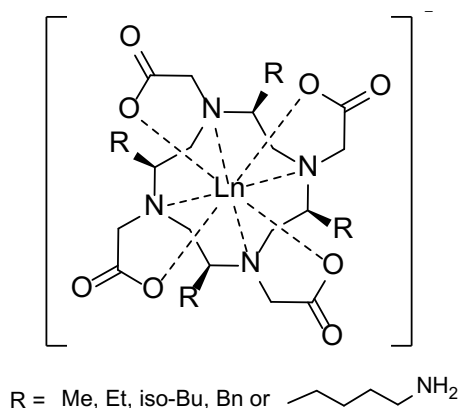


Figure 1.16: Molecular structure of recently reported, chiral DOTA-derived structures for molecular imaging.⁸⁶

1.5 Inflammation

Inflammation plays a significant role in the progression of numerous disease states including neurodegenerative and cardiovascular disorders such as Alzheimer's disease,⁸⁷ stroke,⁸⁸ sickle cell disease⁸⁹ and cancer.⁹⁰ Consequently, a sustained interest exists in gaining a better understanding of the underlying physiological mechanisms using tools to non-invasively detect the immune response and hence develop improved therapeutic measures.

1.5.1 The inflammatory response mechanism

The inflammatory response is an essential mechanism designed to fight infection or combat tissue injury, with the central goal being to restore and maintain homeostasis within a host system. On the flip side however, unregulated immune activation is involved in pathogenic effects of a large variety of diseases.⁹¹

Inflammation can be viewed as a series of complex biological signalling cascades that are classed into three main categories: initiation, propagation and resolution. In a simplified view of the process, initial activation can be triggered by recognition of a pathogen or other invading agents (for instance a bacterial infection) by endogenous receptors such as toll-like receptors (TLRs) or nucleotide oligomerization domain (NOD)-like receptors (NLRs).^{91,92} Activation causes local macrophages and mast cells to excrete inflammatory mediators such as cytokines (for example, interleukins and tumour necrosis factor- α (TNF α)) and histamines which propagate the downstream response and ultimately lead to increased vascular permeability (vasodilation).⁹³ The effect of this is to recruit blood components from inside the vessels to the extravascular site of infection or injury via a process termed diapedesis, transmigration or extravasation (Figure 1.17). The main cell type to migrate towards injury sites are neutrophils. Selectivity in the extravasation process is provided by activated blood vessel endothelial surface selectins that bind leukocyte integrins to prevent loss of erythrocytes from the vessel.⁹⁴ Originally, leukocyte (neutrophil, lymphocyte and monocyte) transmigration through the blood vessels was described by the three central steps of rolling, activation and firm adhesion. More recently, a more complex picture of the leukocyte adhesion cascade has emerged, including various functional steps from cellular capture (or tethering) to slow rolling, adhesion strengthening and spreading, intravascular crawling and finally transmigration (Figure 1.17).⁹⁵

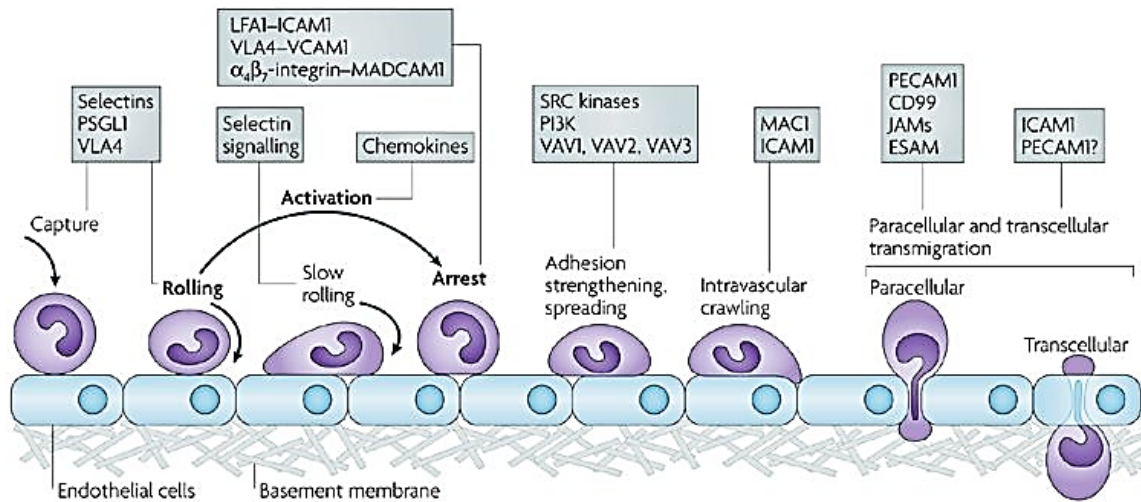


Figure 1.17: Representation of the updated leukocyte adhesion cascade. Reproduced from reference with permission.⁹⁵

Once at the target site, neutrophils can become activated either via direct contact with, for example, a microbial pathogen, or presence of endogenous signalling molecules excreted by local cells. This leads to three possible killing mechanisms. Firstly, neutrophils can release toxic compounds (such as reactive oxygen species (ROS), reactive nitrogen species, proteinase 3, cathepsin G, elastase and myeloperoxidase (MPO)), which are usually contained within their granules and this generates unselective damage to both host tissue and exogenous targets in an attempt to eradicate the invading pathogen. Secondly, when neutrophils become highly activated, they are able to release extracellular traps (NETs) containing reactive species and thus immobilise pathogens and thirdly, when directly encountering a microorganism, they can encapsulate them and eliminate the pathogen via phagocytosis (Figure 1.18).⁹⁴

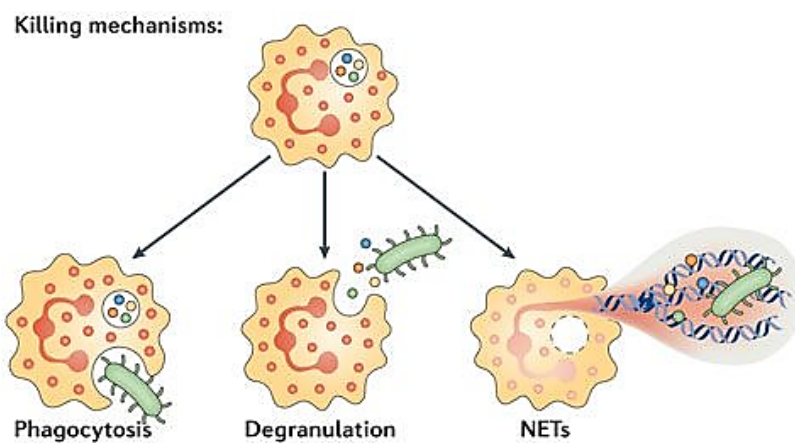


Figure 1.18: Neutrophils and three common mechanisms to kill pathogens. Reproduced from reference with permission.⁹⁴

Successful elimination of the pathogen leads to termination of the acute response and a switch to a resolution and repair phase, mediated by a macrophage-induced change from the pro-inflammatory prostaglandin lipid mediators to anti-inflammatory lipoxins. Monocytes are mobilised in order to remove dead cells, while at the same time recruitment of neutrophils to the site of injury is terminated.⁹⁶ If on the other hand the pathogen persists, neutrophils are replaced with macrophages, and in some cases T cells, and eventually formation of granulomas and tertiary lymphoid tissues occurs, which are both characteristic of a pathological, chronic inflammatory state.⁹⁷

1.5.2 Inflammation imaging

Visualisation of inflammation on a molecular level is central to 1) gaining mechanistic insights into the physiological processes occurring 2) facilitating the development of a screening tool for drug discovery and 3) monitoring and modulating clinical treatment plans for patients.

In the past, suitable probes have been localised at inflammatory sites of interest via both active and passive accumulation methods.⁹⁸ Passive localisation strategies exploit the characteristically increased blood supply, metabolic increase and vascular permeability of the affected regions to enhance increased non-specific uptake of an imaging agent compared to uptake in healthy tissues. Methods include administration of ⁶⁷Ga citrate (Figure 1.19), ^{99m}Tc-labelled albumin and radiolabelled immunoglobulin. However, often high doses of radiation are administered which limits the clinical applicability.^{99,100} Another option is to monitor increased glucose uptake of immune activated granulocytes and macrophages via PET scans using labelled fluorodeoxyglucose (FDG, Figure 1.19), although the main drawback about this is that FDG also accumulates at sites of other pathologies such as tumours, or could flag up benign reasons for metabolic increases.^{101,99}

Encompassed within the concept of active targeting strategies are compounds designed to target inflammatory markers such as leukocyte receptors or protein mediators. Historically, diagnostic successes have been reported for *ex vivo* labelling of white blood cells with ⁶⁷Ga and ¹¹¹In chelates that were subsequently re-injected into the patient, despite the complicated procedure and risky handling of potentially contaminated blood.¹⁰² More recently, direct administration of targeted tracers has replaced these *ex vivo* methods. A variety of markers for inflammation that relate to different aspects of the signalling cascade have been identified including metabolic functions (such as increased choline metabolism), expression or overexpression of membrane receptors on cells or vessels, endogenous CO production and inflammatory cytokines.^{103,104} Examples of this include targeted-visualisation of upregulated TSPO receptor expression on immune cells in disease states (using for instance [¹¹C]-PK11195, Figure 1.19) or imaging of increased CBR levels on activated microglial cells in neuroinflammatory conditions.^{105,106,107,108,109} Both strategies have been extensively investigated in *in vivo* PET studies. Similarly, COX-2, an endogenous cytokine associated with inflammatory activity and cancer has been targeted using radiolabelled analogues of the anti-inflammatory drug CelecoxibTM (Figure 1.19).¹¹⁰

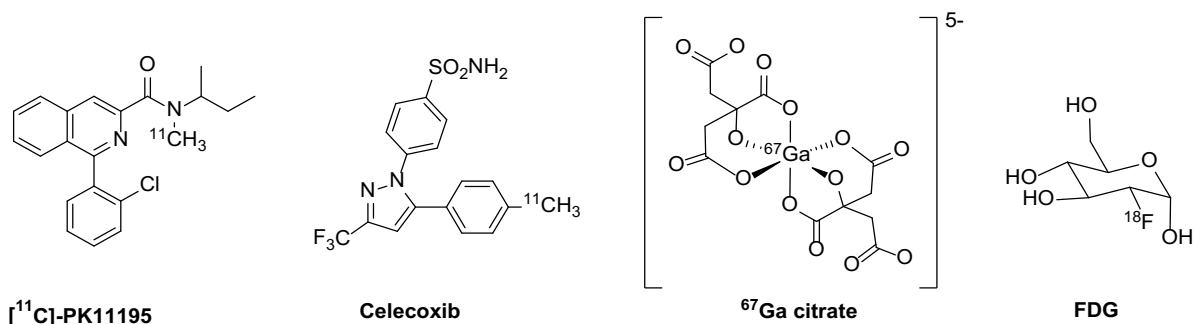


Figure 1.19: Examples of radiopharmaceutical-based inflammation imaging probes.

Neutrophils are abundant immune cells and often the first to arrive at sites of inflammation. They are further well-known to control both pro- and anti-inflammatory aspects of the host response. For these reasons targeted tracers that can be injected and can track neutrophils or neutrophil activity provide valuable insights on their diverse physiological roles.

One method to achieve this, is to visualise neutrophil-associated enzymes. For instance, the presence of MPO at inflammatory foci, an enzyme usually contained within neutrophil granulocytes which exerts essential antimicrobial activity,¹¹¹ has been used in activatable, gadolinium-based MRI contrast agents. By using a DTPA-Gd derivative, bis-5-hydroxytryptamide-diethylenetriamine-pentatacetate gadolinium, or a similar Gd-DOTAGA derivative (Figure 1.20), selective visualisation of inflamed tissues in cerebrovascular inflammation and an *in vivo* stroke model was achieved.^{112,113,114} This class of probes either oligomerised or bound to proteins upon encountering MPO activity and thus enhanced MRI contrast by increasing the rotational correlation times.^{112,113,114} Along the same lines, luminol (Figure 1.20) has also been shown to selectively reveal MPO activity via optical imaging methods in preclinical inflammatory models, although the low tissue penetration of blue-light emission of the probe somewhat limits current applications.¹¹⁵ Matrix metalloproteinases (MMPs) are a family of immune-cell associated enzymes that degrade proteins in inflammatory diseases states. Amongst others, probes to monitor neutrophil elastase MMP9 activity in acute lung injury and MMP12 activity in macrophages have been developed.^{116,117} Using one of the fluorescent probes, cleavage by MMP12 was shown on activated macrophages by demonstrating loss of the previously observed FRET emission and “unquenching” of the donor fluorophore (Figure 1.20).¹¹⁷

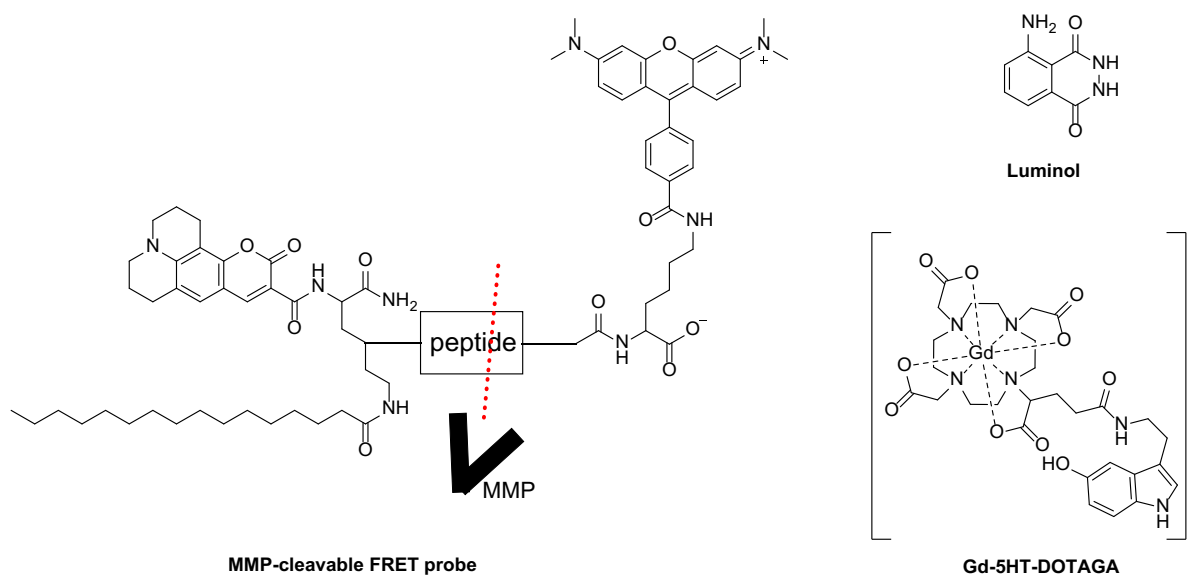


Figure 1.20: Probes to detect MPO activity (Gd-5HT-DOTAGA¹¹⁴ and luminol¹¹⁵) and structure of an activatable probe that shows a fluorescence response upon MMP12 cleavage (peptide cleavage by the enzyme is represented by black shape and dashed line).¹¹⁷

This project focusses on targeting FPRs in inflammation, which are a family of receptors that are expressed on the neutrophil cell surface and thus enables direct tracking of these cells. Imaging units targeted to FPRs have been previously reported and are discussed in more detail in section 2.1.1. These probes however, to the best of our knowledge, have been based solely on highly potent FPR agonist or antagonist peptide motifs and are thus restricted by the sensitive nature of peptide-based synthetic modifications, high production costs and the possibility of activation or inhibition of the receptor by the probe even at low concentrations (*vide infra*).

1.6 Formyl peptide receptors as targets for inflammation

G-protein coupled receptors (GPCRs) constitute the largest group of membrane proteins and are structurally characterised by their 7-transmembrane configuration. Each of the seven α -helical segments is connected by alternating intra- and extra-cellular loops.¹¹⁸

Amongst these, FPRs belong to the classical chemotactic GPCR subfamily and are expressed by a variety of immune cells such as human neutrophils.¹¹⁹ This receptor family is involved in all phases of the inflammatory response, from initiation and identification of chemotactic signals, through propagation and resolution pathways to tissue repair functions and are therefore attractive therapeutic targets of immune activation. In addition, a lot of their specific physiological functions and signalling pathways, in particular for FPR2/ALX and FPR3 (which is not expressed on neutrophils) still remain unclear, creating a need for methods to selectively visualise these receptors in order to elucidate their mechanisms of action and improve therapies.¹²⁰

1.6.1 FPR family

Historically, the presence of a functional receptor on neutrophils, which was later defined as the FPR, was first discovered when Schiffmann *et al* showed that synthetic *N*-formyl peptides stimulated neutrophil chemotaxis and lysosomal enzyme release.¹²¹ Later, GPCR-mediated internalization of ligands and FPRs in neutrophils was observed using a fluorescent ligand by time-lapse video microscopy and time-resolved flow cytometry, which could confirm internalisation of the ligand-receptor complex.¹¹⁹ Final identification of the FPR as a GPCR was achieved in the 1980s and it was shown that the Gi family of heterotrimeric G-proteins couple to the receptor, by inhibiting agonist (fMLF) stimulated neutrophil functions upon exposure to pertussis toxin.¹²² In 1990, Boulay *et al* managed to clone the human *FPR* gene, which ultimately enabled a description of the receptor in the defined environment of transfected cells.¹²³ In addition, a further receptor with 351 amino acids, FPR2/ALX, with significant sequence homology to human FPR1 (69% of shared amino acids) was identified, followed by another gene of 352 amino acids with 56% shared sequence identity with FPR1, named FPR3.¹¹⁹

The genes for all three receptor subtypes within the human system, FPR1, FPR2/ALX and FPR3 are clustered on chromosome 19q13.3 and, although a high level of sequence homology between the FPRs is observed, they differ in ligand recognition and binding.¹²⁴ Within different mammalian species, the FPR family shows significant evolutionary divergence.¹²⁵ In mice, which are often used to model the human system, the differential gene expansion is particularly apparent as eight different FPR gene members have been identified: Fpr1, Fpr2, Fpr3, and Fpr-related sequences Fpr-rs3 to Fpr-rs7, amongst which one is a pseudogene (not coding for a functional receptor). Fpr1 is viewed as the murine orthologue of FPR1 and Fpr2 and Fpr3 are most similar to FPR2/ALX (interestingly, there is no murine equivalent to FPR3).¹²⁶

FPRs are expressed by a variety of cells in the human system and their distribution on different tissues has been previously reviewed and is summarised in Table 1.2.^{127,128,129} Notably, human neutrophils express both FPR1 and FPR2/ALX on the cell surface, but do not express FPR3, whereas macrophages and monocytes are known to express all three receptor types.

	FPR1	FPR2/ALX	FPR3
Cell types	Neutrophils Monocytes/macrophages Immature dendritic cells Endothelial cells Platelets Hepatocytes Astrocytes Microglial cells Fibroblasts Vascular smooth muscle cells	Neutrophils Monocytes/macrophages Immature dendritic cells T cells/B cells Endothelial cells Epithelial cells Microglial cells Astrocytes Fibroblasts Hepatocytes Carcinoma cells	Monocytes/macrophages Dendritic cells Eosinophils differentiated HL-60 cells
Tissues	Thyroid Adrenal Central nervous system Liver Lung Spleen Heart Uterus Ovary Placenta Kidney Eye Stomach Bone marrow Colon Neuroblastoma	Placenta Brain Bone marrow Spleen Lung Testis Neuroblastoma	Spleen Lymph nodes Lung Trachea Liver Adrenal Small intestine placenta

Table 1.2: Summary of cells and tissues that express FPRs in the human system.^{127,128,129}

1.6.2 Physiological roles and receptor subtypes

The role of FPR receptors is often considered to be regulatory in host defence and inflammation.¹²² Under unstimulated conditions, mice lacking Fpr1 or Fpr2 behave normally, but in disease models Fpr1 and/or Fpr2 deficient mice (Fpr1^{-/-} and Fpr2^{-/-} respectively) respond distinctly from the wild type.¹³⁰ At a functional level, the receptor family has been shown to be involved in many pro-inflammatory signalling processes: they can induce chemotaxis, mobilise adhesion molecules and receptors from granules and secrete proteolytic enzymes and ROS.¹³¹ FPR signalling is also reported to regulate resolution of inflammation via neutrophil apoptosis or FPR2/ALX-mediated inhibition of neutrophil migration.¹²⁰ This

dual role suggests that careful modulation of FPR activity is important for controlling inflammation-associated pathologies.

FPR1

The neutrophil FPR1 exerts important roles associated with the inflammatory response and has been shown to be implicated in chemotaxis, adhesion, degranulation, production of ROS, cytokine production as well as other functions.^{127,128} Studies have further shown, that although the receptor is expressed in inactive neutrophils, inflammatory stimuli lead to a rapid upregulation of the receptor expression as well as an increased protein synthesis. Activation of the receptor results in ligand-specific responses, for example activation with distinct agonists have been shown to result in isolated, specific inflammatory responses rather than stimulation of all known pathways. One example is CaG, an FPR1 agonist that can cause weak mitogen-activated protein kinase phosphorylation, chemotaxis and recruitment of dendritic cells but does not induce a Ca^{2+} flux, which is typically seen using fMLP as an agonist.¹³² This functional variety was postulated to be related to low affinity binding to the receptor.¹²⁹

FPR2/ALX

The FPR2/ALX has been shown to be strongly involved in the resolution of inflammation, with an absence of the receptor being coupled to heightened and progressive disease states, while at the same time pro-inflammatory roles have also been reported.^{120,96,133} For these reasons, FPR2/ALX is considered an unusual GPCR as the receptor is activated by a diverse set of ligand motifs and, depending on ligand binding, can elicit both pro- and anti-inflammatory pathways.¹³⁴ For example, binding to leucine 37 has been reported to induce pro-inflammatory effects such as cytokine release and NF- κ B activation, whereas annexin A1 (AnxA1) exerts a dual anti-inflammatory effect of inhibiting neutrophil migration as well as promoting neutrophil apoptosis. Lipoxin A₄, also known for its pro-resolving activity, on the other hand only inhibits neutrophil migration, thus causing a subtly different effect to AnxA1 (which acts as both an anti-inflammatory and a pro-resolving mediator).^{96,135}

Although the mechanistic basis for how opposite cues can be triggered by FPR2/ALX agonists is yet to be fully understood, Cooray *et al* attributed such effects to distinct receptor dimerisation states after receptor binding.¹³⁶ They found that this caused alterations in the receptor configuration, which in turn resulted in changes to intracellular signalling. Finally, it is likely that multiple receptor cues are represented within an inflamed lesion and hence the receptor role is largely determined by the abundance of each.

FPR3

Only a poor grasp of the actions of human FPR3 has been achieved and most of its functions *in vivo* are still unclear. Devosse *et al* established presence of the receptor type on eosinophils, monocytes, macrophages and dendritic cells and suggested a role in allergic diseases.¹³⁷ Although FPR2/ALX and FPR3 share a high degree of sequence homology, reports suggest that FPR3 is fairly insensitive to FPR2/ALX ligands and, in a resting state, FPR3 was highly phosphorylated and localised in intracellular vesicles.¹³⁸

1.6.3 FPR ligands

As stated above, FPRs bind to structurally diverse ligand motifs, that can elicit varied responses upon binding. For instance, it is known that receptor interactions are specific to ligand type. This behaviour is suggestive of so-called 'biased agonism', which describes the GPCRs ability to adopt distinct conformational states linked to different signal transduction pathways.^{139,140} Recently, FPR biased agonism in the context of post-myocardial infarction injury was demonstrated *in vivo* and *in vitro*, for two small-molecule FPR agonists: Amgen compound 43 and compound 17b (Table 1.3).^{141,142} Compound 17b was found to stimulate a cardioprotective effect, while signalling was biased away from the intracellular Ca²⁺ signalling pathway, whereas compound 43 did not exhibit these properties.¹⁴³ Such discoveries can have profound effects on clinical outcomes and emphasise the importance of understanding the role receptor ligands can play in disease states. A non-exhaustive list of select FPR ligands is found in Table 1.3.

Ligand	Action	Receptor target
Host derived		
Annexin A1	agonist	FPR2/ALX > FPR1, FPR3
Ac2-26	agonist	FPR1, FPR2/ALX
F2L	agonist	FPR2/ALX, FPR3
Humanin	agonist	FPR2/ALX, FPR3
Lipoxin A4	agonist	FPR2/ALX
fMLF	agonist	FPR1 > FPR2/ALX
SAA	agonist	FPR2/ALX
Boc-1	antagonist	FPR1, FPR2/ALX, FPR3
Boc-2	antagonist	FPR1, FPR2/ALX, FPR3
Cyclosporin H	antagonist	FPR1
Peptide library		
WKYMVm/WKYMVM	agonist	FPR2/ALX > FPR1 > FPR3
cFLFLF	agonist	FPR1
MMK-1	agonist	FPR2/ALX
WRW4	antagonist	FPR2/ALX, FPR3
Isopropylureido-FLFLF	antagonist	FPR1
Small molecule		
Quin C1	agonist	FPR2/ALX
Compound 17b	agonist	FPR1, FPR2/ALX
Compound 43	agonist	FPR1, FPR2/ALX
AG-14	agonist	FPR1
BVT173187	antagonist	FPR1
Quin C7	antagonist	FPR2/ALX

Table 1.3: Select formyl peptide receptor ligands and modes of action, collated from references.^{130,144}

1.7 Aims and objectives

Despite the abundance of both agonist and antagonist ligands reported for the FPR family and the established role of these receptor types in a series of disease states, no clinically relevant therapies based on these receptors are available. This is partly attributed to the fact that the complicated and often contrasting physiological roles of GPCRs are still poorly understood and off-target effects therefore could cause more damage than benefits in an *in vivo* context. As a result, there is a significant need for tools to aid understanding of the pathways and mechanisms of FPRs. Furthermore, although examples of FPR1-specific imaging agents have been reported, the functions of the FPR2/ALX receptor are underexplored.

In line with the issues described, this project aims at preparing luminescent compounds that can selectively bind to FPRs and thus allow neutrophils to be tracked *in vivo*. In inflammation-associated disease states these receptors are known to firstly be overexpressed on the cell surface and secondly exist in an activated state.¹²⁸ Both factors contribute to the fact that neutrophils in an inflammatory context preferentially take up such compounds.

In this work, ligand systems to target FPR2/ALX are prepared based on findings from a reported compound library hit, characterised and tested for their *in vitro* effect on neutrophil function, which is discussed in Chapter 2.

Based on these results, a FPR2/ALX agonist is further functionalised to yield a targeted fluorophore system in Chapter 3 (Figure 1.21, **A**). The aim of this conjugate is to establish that the system binds via the FPR family and does not cause activation of the receptor system *in vitro*. Once verified, the construct will be carried forward into a disease model to determine whether it can be used as an inflammation-specific imaging agent and to further explore the role of FPR2/ALX in a pre-clinical setting. Finally, successful validation of the agent prompted preparation of a related probe via facile synthetic modifications, which displayed pH sensitivity (Figure 1.21, **B**). The pK_{cycl} of the pH-sensitive fluorescent probe was found to be within biologically relevant values, that are further lowered in an inflammatory context as a result of tissue acidosis. A selective “on” response could be observed in neutrophils that had been activated prior to exposure to the compound thus validating its potential use as an inflammation-associated pH sensor.

Next, in order to incorporate long-lived luminescent signals that allow for separation from endogenous fluorescence, three structurally analogous lanthanide complexes (Ln = Tb, Eu, Gd, Figure 1.21, **C**) with distinct spectroscopic properties are described. Cellular confocal imaging using the Tb version permitted time-resolved acquisition in neutrophils, but required high concentrations of the probe for visualisation.

To mitigate the weak emission observed for the terbium compound in cells, a Re(I) tricarbonyl species was introduced in Chapter 5 instead, that still offers long-lived emission, but is expected to be brighter.

Finally, in Chapter 6, FPR-targeted compounds that contain two imaging moieties and a switchable unit- for potential pH-sensing, dual-emission, luminescence resonance energy transfer or dual-modality, are

described. These molecules (Figure 1.21, **D**) incorporate the previously described building blocks and exhibit interesting photophysical properties.

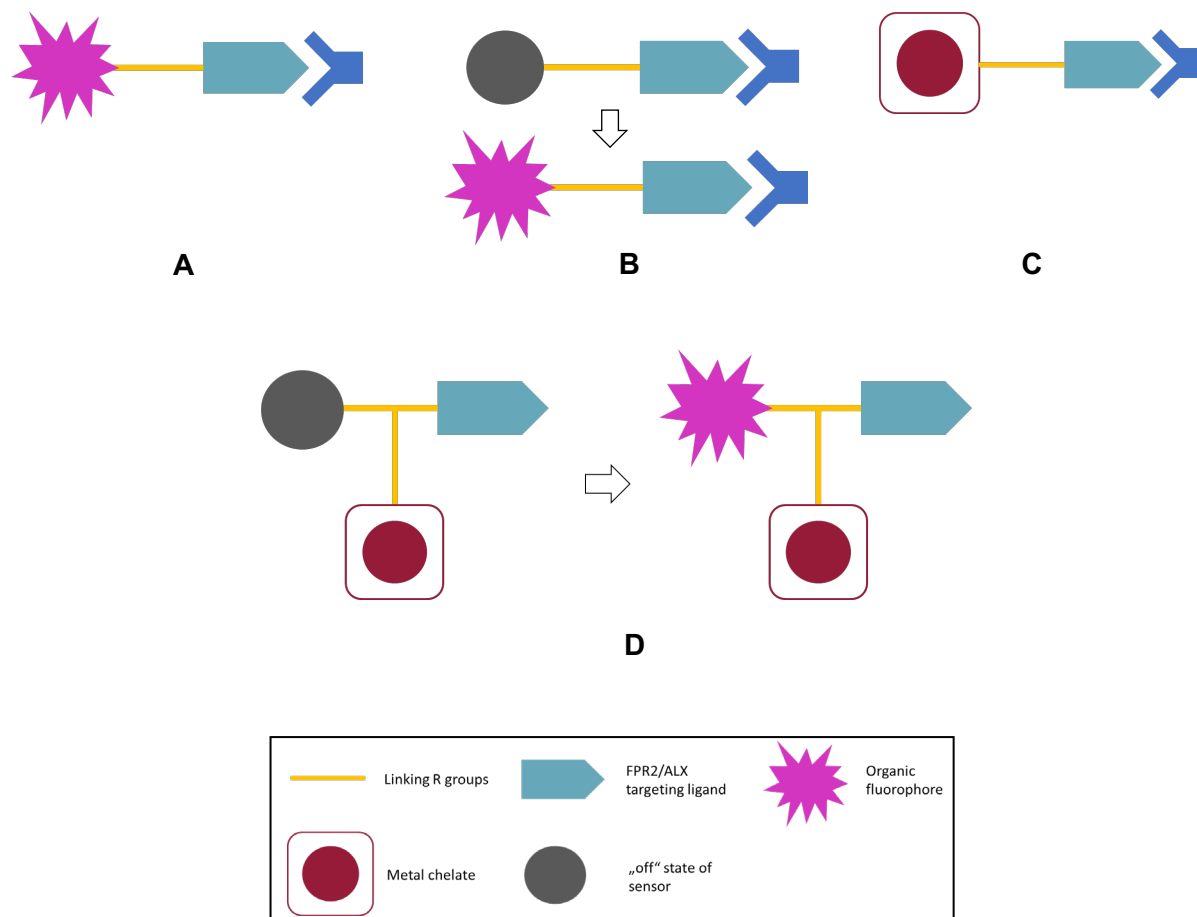


Figure 1.21: Strategies for formation of FPR-targeted fluorescent compounds (**A**), incorporating a sensor (**B**), a metal chelate for long-lived emission signals (**C**) or all three of the above (**D**).

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Chapter 2

Ligands for targeting FPR2/ALX

2.1 Introduction

The neutrophil surface FPR receptors, FPR1 and FPR2/ALX, can act as markers of inflammation and, as such, are convenient targets for visualising the inflammatory response. In addition, the complex roles these receptors play in the progression of inflammation-associated pathologies are only partly understood. Methods to non-invasively visualise them in a cellular context can aid discovery of new treatment options. The FPR family is known to bind to a variety of ligands of both endogenous and exogenous origin. Most of the reported ligand structures are peptide-derived, but a progressive interest exists in finding small-molecule structures, which are typically synthetically and metabolically more robust, cheaper and easier to prepare and produce fewer off-target responses.^{1,2,3}

2.1.1 Peptide-based motifs for binding to FPRs

N-formyl-methionyl leucyl phenylalanine (fMLF, Figure 2.1), one of the first synthetic chemotactic peptides and a potent FPR agonist was reported to selectively bind to FPR motifs ($EC_{50} = 0.1\text{--}1\text{ nM}$).^{4,5} For this reason, fMLF and related structures were modified by solid-phase peptide synthesis to produce radiotracers and optical probes for localisation in inflammation or cellular visualisation of FPRs.^{6,7,8,9} However, the utility of these motifs for imaging purposes is compromised by the ability of fMLF-derived peptides to cause a pro-inflammatory response even at low doses in a host system.¹⁰

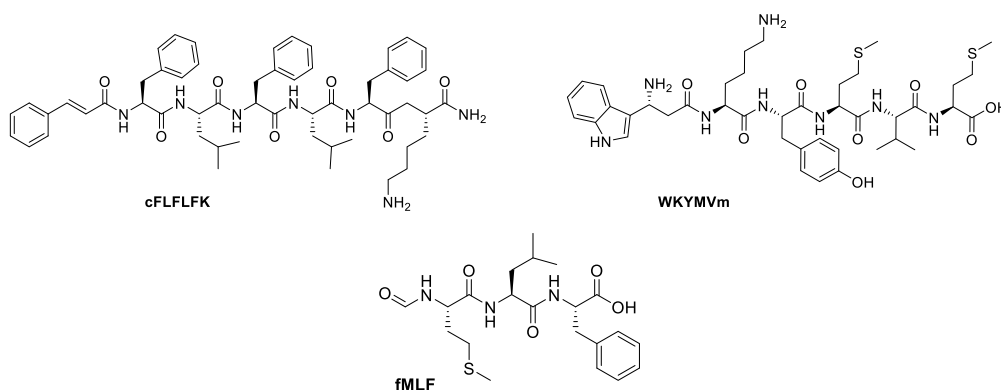


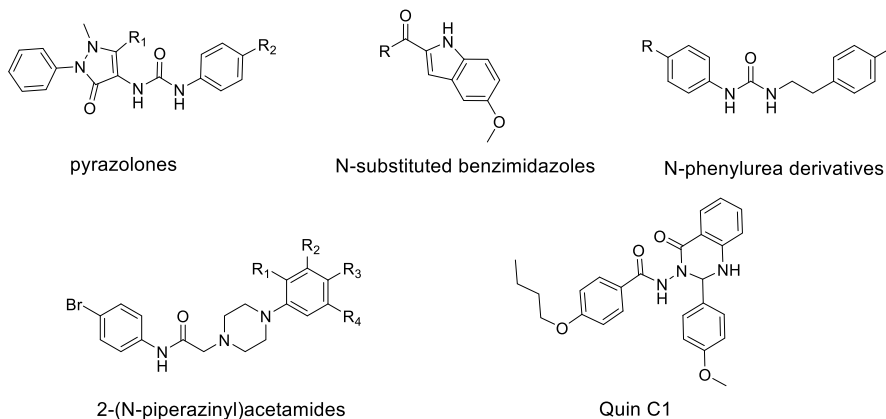
Figure 2.1: Molecular structures of peptide-derived FPR binding motifs cFLFLFK (FPR1 antagonist), fMLF (FPR agonist) and WKYMVm (FPR2/ALX agonist).

To avoid this effect, one peptide-derived motif in particular, the FPR1-antagonist synthetic peptide cinnamoyl-F-(D)L-F-(D)L-F (cFLFLF, Figure 2.1),¹¹ has been extensively derivatised to prepare a range of FPR1-targeted imaging agents. Amongst these, FPR1-targeted contrast agents for magnetic resonance imaging (MRI),¹² positron emission tomography (PET),¹³ optical and single-photon emission computed tomography (SPECT),^{14,15} as well as a dual (NIR/MRI) probe¹⁶ and a ^{68}Ga -doped iron oxide nanoplatfrom surface-coated with cFLFLF¹⁷ have been reported. Chen *et al* demonstrated effective *in vivo* imaging in a preclinical context using a HYNIC- $^{99\text{m}}\text{Tc}$ -cFLFLF conjugate, which allowed evaluation

of the therapeutic response to antibiotics in an acute osteomyelitis inflammation model.¹⁸ A follow-up study using FPR1-specific imaging probes cFLFLF-PEG-Cy7 and ^{99m}Tc-HYNIC-PEG-cFLFLF to track leukocyte infiltration at disk herniation sites by simultaneous NIR and SPECT imaging was reported.¹⁹ However, one of the drawbacks with these constructs is that they suffer from poor target to background ratios as a result of the high hydrophobicity of cFLFLF, which often necessitates incorporation of a further large substituent to increase solubility. Labelling efficiencies using this peptide are reported to be low.¹⁷

Less common in the literature are reports of FPR2/ALX-specific ligands. A synthetic peptide FPR2/ALX agonist, Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm, EC₅₀ = 75 nM, Figure 2.1), was identified and has been used as the “gold standard” FPR2/ALX agonist to investigate the role of this receptor.^{20,21,22,23} However, similar to fMLF, the potent agonistic activity of this ligand in addition to the sensitivity in handling harsher reaction conditions, make it a less than ideal candidate for development of imaging agents.

Agonists



Antagonists

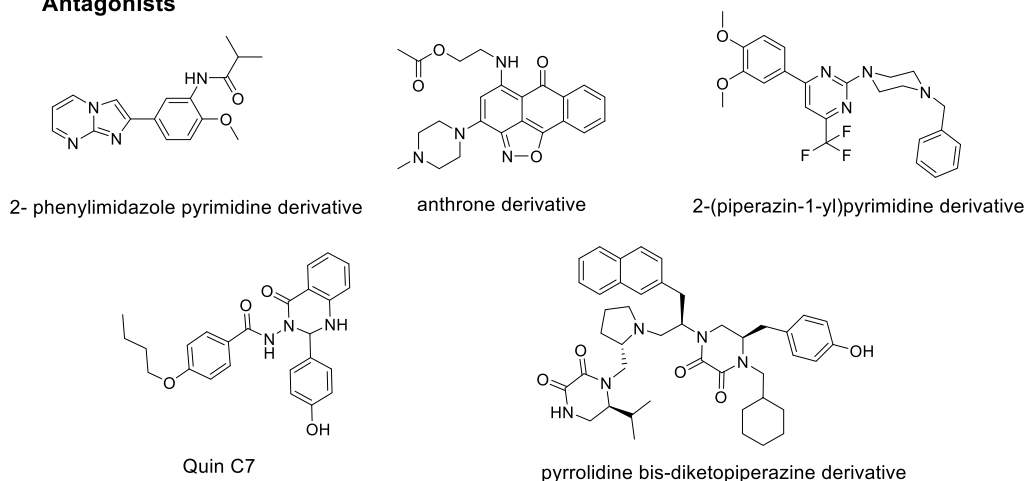


Figure 2.2: Selection of molecular structures of small molecule FPR2/ALX ligands reviewed in reference.²⁴

More recently, efforts to identify small-molecule FPR2/ALX binding candidates using large scale screening for binding motifs have resulted in a series of ligands which were recently reviewed by Schepetkin *et al* and selected examples are depicted in Figure 2.2.²⁴ Derivatisation of these structures could provide a convenient way of overcoming synthetic and biological limitations associated with the previously reported peptide-based probes, and represent a more promising approach as targeting units for imaging probes.

2.1.2 Quinazolinone-derived motifs for binding to FPR2/ALX

2,3-Dihydroquinazolinones are well-known in the literature and encompass a wide range of heterocycles that can exhibit a variety of pharmacological and biological activities. Examples are as diverse as reports of anticancer activity,²⁵ antiparasitic properties, for instance in malaria,²⁶ anti-inflammatory properties,²⁷ or activity as transient receptor potential melastatin 2 (TRPM2) inhibitor.²⁸

Quin C1, a substituted 2,3-dihydroquinazolin-4(1*H*)-one was first reported as a hit from a FPR2/ALX binding compound library screen and tested *in vitro* using human neutrophils.²⁹ In the original paper, Nanamori *et al* found that the compound could stimulate neutrophil chemotaxis and β -glucuronidase release in a dose-dependent manner. On the other hand, the typical neutrophil superoxide production response was not observed, indicating the compound may act as a partial agonist. The potency was estimated at 1/1000 of the known peptide FPR2/ALX agonist WKYMVm.²⁹ Using cells stably transfected with either FPR1 or FPR2/ALX, they further demonstrated that Quin C1 selectively stimulates calcium mobilisation via FPR2/ALX and did not stimulate the same response in FPR1 transfected cells. Interestingly, the results obtained using human neutrophils contrast subsequent validation in mouse cells, where Quin C1 could induce calcium mobilisation in both rat basophilic leukemia (RBL) mFpr1 and mFpr2 cells, with better EC₅₀ values determined for mFpr1 cells.³⁰ The report by Nanamori *et al* further showed that FPR2/ALX internalisation (a typical GPCR response to ligand binding) occurs above 1 μ M, but they did not investigate binding to FPR1. An incomplete competitive binding study suggests that Quin C1 interacts with FPR2/ALX via a binding site that is distinct, but possibly adjacent to the site of WKYMVm binding, resulting in partial competition.

In 2005, the compound structure was extended and a patent was published encompassing compounds of the general structure **A** (Figure 2.3) to modulate FPR2/ALX functionality.³¹ The synthetic compound library high-throughput screening (HTS) campaign that initially identified the FPR2/ALX agonist Quin C1, further identified that substitutions at the *para* position of the 2-phenyl group were found to be key to the resulting compounds' bioactivity profiles. Bulky substituents or no substituent on the 2-phenyl *para* position both lead to complete loss of activity. Other agonists within the C-series (Figure 2.3) were also identified and, perhaps most intriguingly, substitution of the methoxy group with a hydroxyl functionality yielded a reversal of activity with a full antagonist, Quin C7.³²

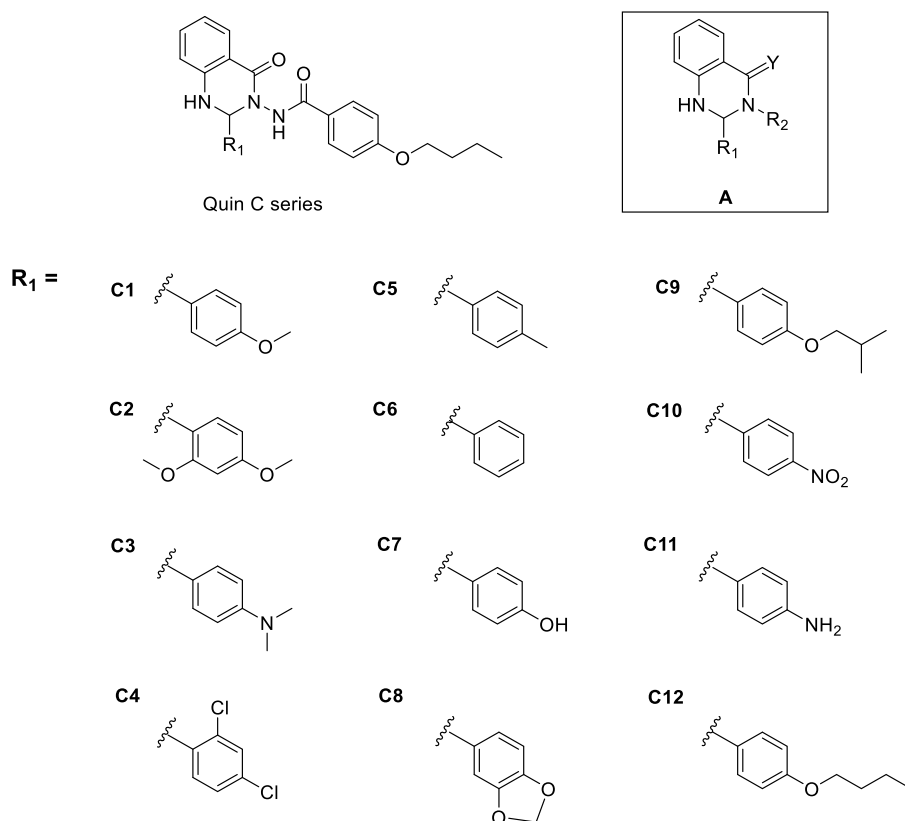


Figure 2.3: General structure for group of compounds **A** and Quin C-series with substituents.³²

Quin C7 was shown to antagonise WKYMVm-stimulated calcium mobilisation in a dose dependent manner in RBL-FPR2/ALX cell lines, as well as to suppress chemotaxis induced by both WKYMVm and Quin C1. Competitive binding studies carried out on several members of the Quin C-series using FPR2/ALX-RBL cells indicated mediocre IC₅₀ values (92 ± 1 nM for Quin C1) compared to known peptide agonists and antagonists. The limited solubility of Quin C7 prevented exact determination of values, as at 100 µM, WKYMVm was not completely displaced. In cells transfected with FPR1, Quin C7 could not compete with [³H]fMLf, indicating selectivity of binding for FPR2/ALX.

In vivo studies demonstrated that administration of Quin C7 in a model of ear edema inhibited associated inflammation, which was suggested to be related to an *in vivo* inhibitory or anti-inflammatory effect.³² In a mouse model of bleomycin-induced lung injury, administration of Quin C1 was found to reduce neutrophil and lymphocyte counts in bronchoalveolar lavage fluid as well as decreasing expression of TNFα, IL-1β, keratinocyte-derived chemokine and TGF-β1.³⁰

Intriguingly, it appears that both the antagonist Quin C7 and agonist Quin C1 exhibit anti-inflammatory properties within defined concentration ranges. In the context of this PhD work, it is further notable that the *in vivo* anti-inflammatory effect of Quin C1 was observed at a narrow range of doses (namely between 0.04 and 0.2 mg)³⁰ and the effect was not observed at higher doses or lower doses tested. This is in accord with a similar effect observed using lipoxin A4 (LXA₄), a pro-resolving inflammatory

mediator group that acts specifically via FPR2/ALX (also termed the LXA₄ receptor) and produces anti-inflammatory responses at low doses, whereas other ligand properties become apparent at higher doses.^{33,34,35}

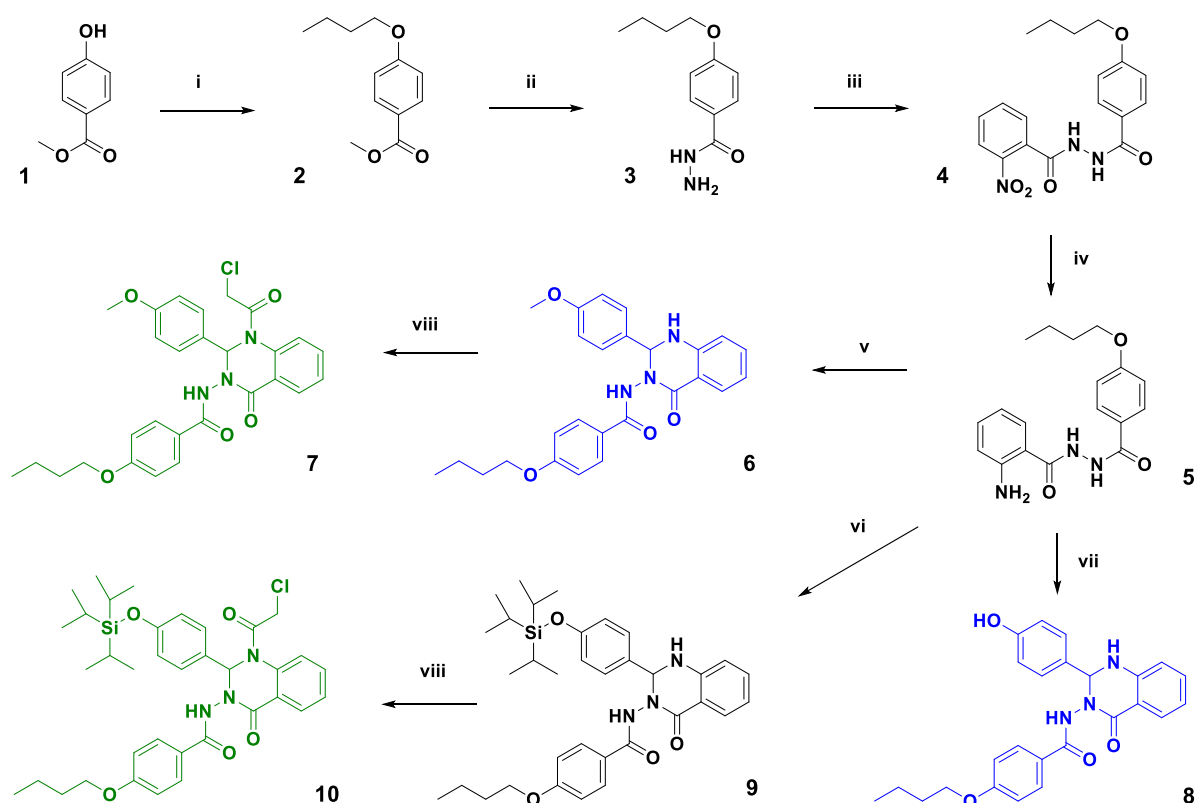
FPR2/ALX ligands have been observed to activate only selected neutrophil functions and their potencies in specific assays do not necessarily correlate with their receptor binding affinities,^{34,36,37} which led to the notion that ligands may have different intrinsic, concentration-dependent abilities of inducing receptor conformational changes.³³

2.2 Aims

In this chapter, preparation of two FPR2/ALX targeting groups, Quin C7 and Quin C1, their spectroscopic characterisation and *in vitro* validation is described. Both groups are based on the previously reported quinazolinone-derived scaffold from a high throughput screening hit. Of the two, Quin C1 exhibits agonist activities whereas Quin C7 is a FPR2/ALX antagonist. Both compounds are reported to be less potent ligands than peptide-based groups that have previously been investigated for their potential in imaging constructs and thus are more suited as targeting units that do not elicit a pharmacological response at relevant doses. The aim of this chapter is therefore to prepare and characterise these two motifs as well as to find and define a derivatisation site and strategy that enables them to be utilised in the design of FPR2/ALX-targeted imaging probes. These building blocks will then be combined with reporter moieties in the following chapters, while hopefully still retaining their FPR2/ALX binding properties.

2.3 Results and discussion

The synthetic pathway towards preparation of the FPR2/ALX agonist Quin C1 (**6**) and antagonist Quin C7 (**8**) is summarised in Scheme 2.1. To make the compounds available for combination with an imaging unit, they were further functionalised using chloroacetyl chloride on the secondary amine-terminus. As changes to the *para* phenyl position have been shown to drastically impact biological activity, this position was protected for compound **8** using a triisopropylsilyloxy (TIPS) group prior to formation of the functionalised analogues **7** and **10**, that can be directly appended to imaging moieties.

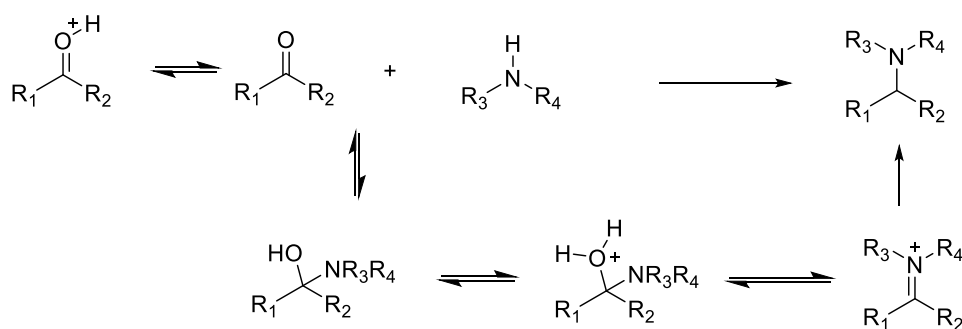


Scheme 2.1: Strategy for preparation of FPR2/ALX agonist Quin C1 (**6**, blue) and antagonist Quin C7 (**8**, blue) and respective functionalised derivatives, **7** and **10** (green). Reagents: i) 1-bromobutane, potassium carbonate, methanol, 60 % yield, ii) hydrazine monohydrate, ethanol, 70 % yield, iii) 2-nitro-benzoyl chloride, potassium carbonate, dichloromethane, 43 % yield, iv) zinc dust, acetic acid, dichloromethane, 70 % yield, v) 4-methoxy benzaldehyde, citric acid, ethanol, 83 % yield, vi) 4-(triisopropylsilyloxy) benzaldehyde, citric acid, ethanol, 14 % yield vii) 4-hydroxy benzaldehyde, citric acid, ethanol, 47 % yield viii) chloroacetyl chloride, triethylamine, dichloromethane, 60 % yield (**7**) and 13 % yield (**10**).

2.3.1 Preparation of FPR2/ALX ligands Quin C1 and Quin C7

Preparation of the target molecules **6** and **7** was carried out following modified literature protocols.^{38,32,29} Initially, 1-bromobutane was reacted with commercially available 4-hydroxymethyl benzoate under basic conditions. The product was isolated by column chromatography and the resulting methyl 4-butoxybenzoate (**2**) was further reacted with hydrazine monohydrate to yield **3**. A switch of solvent system from methanol (as described within the literature)³² to ethanol and addition of excess hydrazine reagent afforded **3** and substantially improved reaction yields (70 %) compared to previous reports. Within the ¹H-NMR spectrum, this resulted in a shift change for the proximal aromatic protons compared to **2**. In addition, loss of the 3H singlet resonance at 3.89 ppm that had been assigned to the methyl ester protons was apparent by ¹H-NMR spectroscopy. Acylation of **3** using nitrobenzoyl chloride was attempted under several conditions and the choice of accompanying base proved imperative for reducing side products, with potassium carbonate affording the best results. Next, the nitro group in compound **4** was reduced using zinc dust in dichloromethane/acetic acid to yield **5**, a reactive, primary, aromatic amine. Reductive amination of **5** using the corresponding 4-methoxy benzaldehyde, 4-hydroxy benzaldehyde or 4-TIPS benzaldehyde to form **6**, **8** and **9** respectively was carried out. Prior to this final step, 4-TIPS benzaldehyde was prepared according to literature protocols from 4-hydroxy benzaldehyde and 4-triisopropylsilyl chloride.³⁹

Reductive amination in the presence of an acid catalyst usually proceeds via a protonation step, followed by nucleophilic addition of the amino group and formation of the imine (Schiff base) intermediate and subsequent Schiff base reduction (Scheme 2.2). Here, the reduction of the Schiff base or the imine formation step is substituted by intramolecular addition of a further amino group to form the cyclic motif seen for compounds **6**, **8** and **9**.



Scheme 2.2: Generalised reaction mechanism for acid catalysed reductive amination.

Previous protocols reported for formation of **6** and **8** using acetic acid were found to be very low-yielding.^{29,32} In search of a better method, formation of dihydroquinazolinones by grinding of the reagents on a solid support system was investigated, as exemplified within the literature by Ding *et al.*³⁸ Although some of the desired product was formed using this technique, the yields obtained were

similarly low (~5 – 10 %). Evangelista *et al* indicated that the rate determining step in similar reductive amination reactions is acid catalysed and as such is likely to follow the Bronsted equation⁴⁰

$$\log k_{\text{cat}} = -\alpha \text{pK}_{\text{a}} + \text{constant} \quad (\text{eq 1.1})$$

Where k_{cat} is the catalytic rate constant, pK_{a} is the added acid strength and α (between 0 and 1) is the Bronsted slope. This relationship implies that yields correlate with the pK_{a} of the acid in question and a literature precedent looking at acid-catalysed reductive amination of N-acetylamino sugars demonstrated that a switch to citric acid ($\text{pK}_{\text{a}} = 3.1$) compared to acetic acid ($\text{pK}_{\text{a}} = 4.75$) improved yields. When the acid pK_{a} was further decreased, for instance, using malonic or maleic acid (pK_{a} of 2.8 and 1.91 respectively), they found a small reduction in yield (contrary to the expected trend) and suggested this is a result of protonation of the amino group at low pH values.⁴¹ Encouraged by these results, the reaction was found to produce moderate but improved yields using citric acid for all three compounds. Compound **6** in particular could be prepared in up to 83 % yields. Isolation of the pure products was achieved after purification by column chromatography.

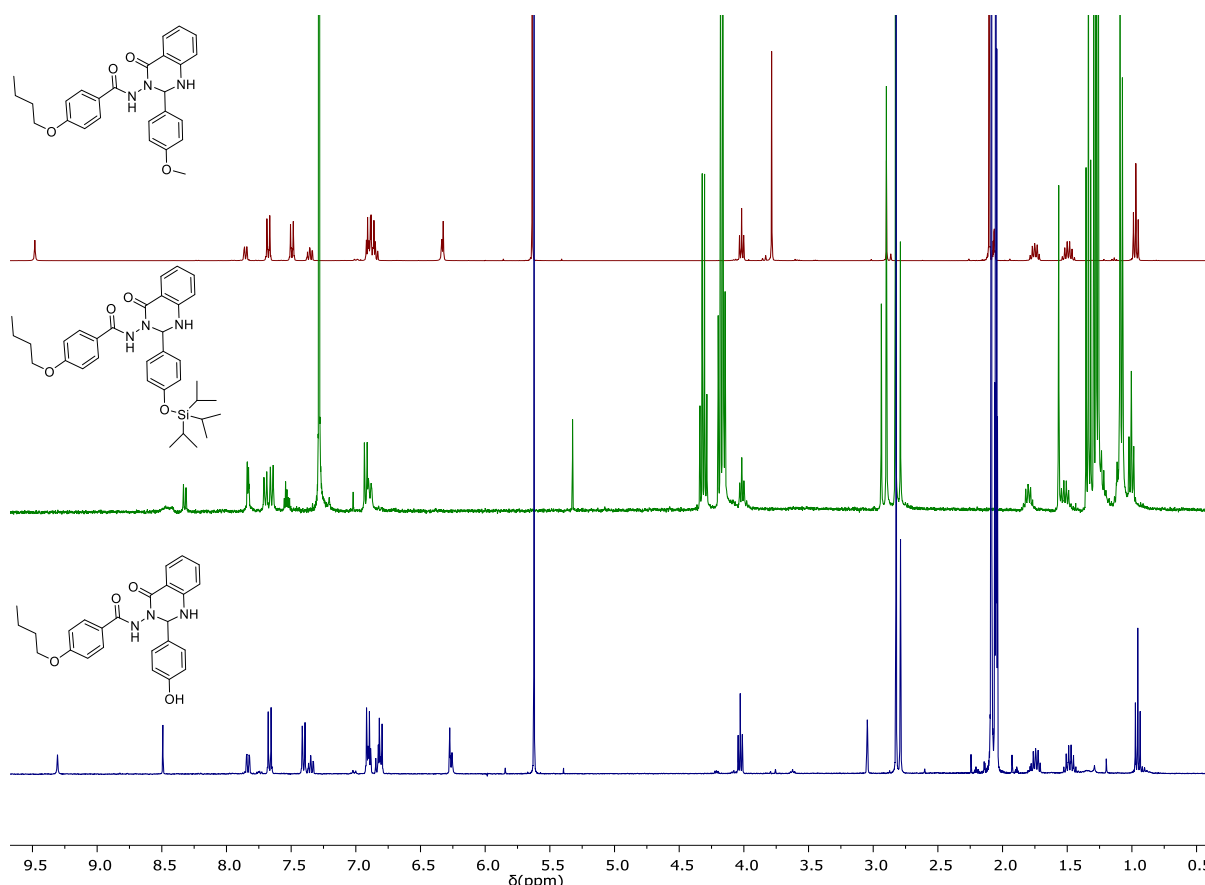


Figure 2.4: Stacked ¹H-NMR spectra run at 400 MHz and 298 K of **6** (top in d₆-acetone), **9** (middle in CDCl₃) and **8** (below in d₆-acetone). Citric acid impurities are present in the spectrum of **9**.

Compounds were analysed by ^1H -NMR, ^{13}C -NMR and HRMS spectroscopy. Molecular ion peaks corresponding to $[\text{M}+\text{H}]^+$ were evident in the respective high resolution mass spectra for **6** at 446.2089 a.m.u. and **8** at 432.1921 a.m.u, whereas formation of the protected Quin C7-analogue **9** was evidenced as a $[\text{M}-\text{H}]^+$ molecular ion peak at 586.3124 a.m.u. A view of the stacked ^1H -NMR spectra of **6**, **8** and **9** is depicted in Figure 2.4. The butyl chain resonances were apparent for all compounds within the alkyl region and display the characteristic multiplets around ~ 1.5 and ~ 1.75 ppm depending on the compound. Further, a 2H triplet assigned to the oxygen-adjacent protons around 4 ppm is evident, as is a 3H triplet assigned to the proton environment on the terminal carbon of the alkyl chain. The triisopropyl protons for **9** appear within the alkyl region at 1.20 - 1.37 ppm, and a 3H singlet is observed around 3.78 ppm for the methoxy group in compound **6**. Exact assignment of the aromatic ^1H -NMR regions was aided by the nuclear Overhauser effect spectroscopy (NOESY) correlations detected and exemplified for compound **6** in Figure 2.5 and 2.6.

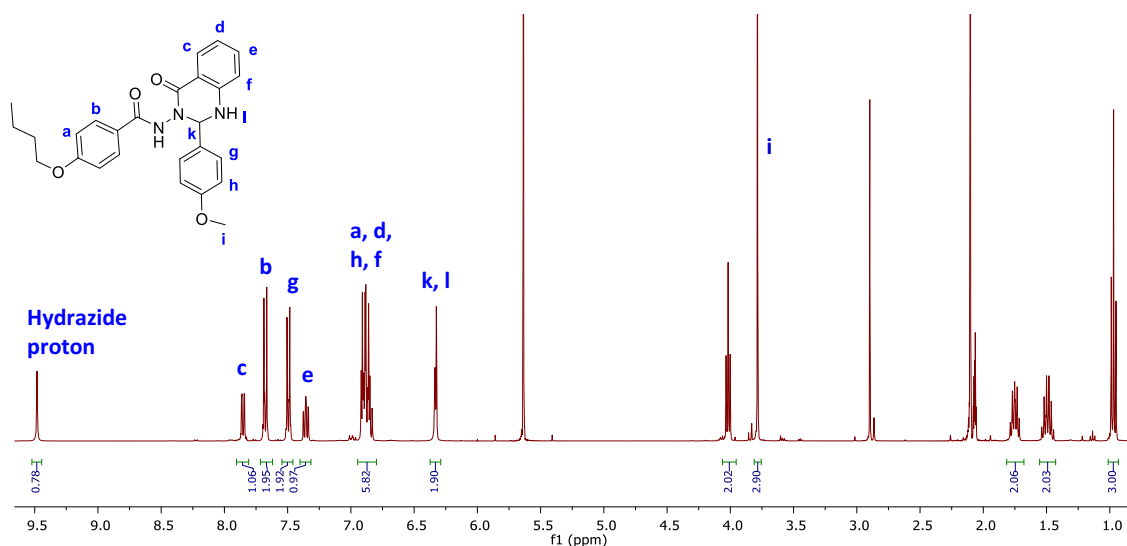


Figure 2.5: ^1H -NMR spectrum of **6** in d_6 -acetone at 400 MHz and 298 K with proton assignments enabled via NOESY spectroscopy.

Within the aromatic region of **6**, five sets of proton signal regions were observed: a 6H multiplet consisting of several overlapping resonances between 6.83 ppm and 6.90 ppm, a triplet of doublets ($^3J_{\text{ortho}} = 8.0$ Hz, $^4J_{\text{meta}} = 1.6$ Hz), integrating to 1H at 7.35 ppm, two consecutive 2H signals at 7.49 and 7.66 ppm and a doublet of doublet ($^3J_{\text{ortho}} = 7.8$ Hz, $^4J_{\text{meta}} = 1.5$ Hz) integrating to one proton at 7.84 ppm (Figure 2.5). In order to reliably assign the specific shifts to protons, through-space nuclear Overhauser effect (NOE) correlations were obtained. NOEs between the region at 6.30 ppm, containing both a resonance for the aminal proton (H_k) as well as the aromatic NH (H_l), were observed for shifts at 7.49 ppm and 6.83 - 6.90 ppm, allowing H_g and H_f to be identified (Figure 2.6, circled). Similarly, H_h and H_a could be assigned by correlations with the respectively close alkyl regions. The 2H signal at 7.66 ppm was assigned to H_b , upon observation of a NOE with the hydrazide proton signal at 9.38 ppm

(Figure 2.6, circled). As there is no correlation observed between the two ^1H signals at 7.35 and 7.84 ppm, these two protons cannot be adjacent. Further, both have a cross-peak with the region at 6.83 - 6.90 ppm and the splitting patterns indicate that the doublet of doublets must belong to H_c or H_f and the triplet of doublets to H_e or H_d , thus enabling the final assignment as seen in Figure 2.5.

Similarly, signals for compound **8** could be systematically assigned using 2D NMR spectroscopic methods.

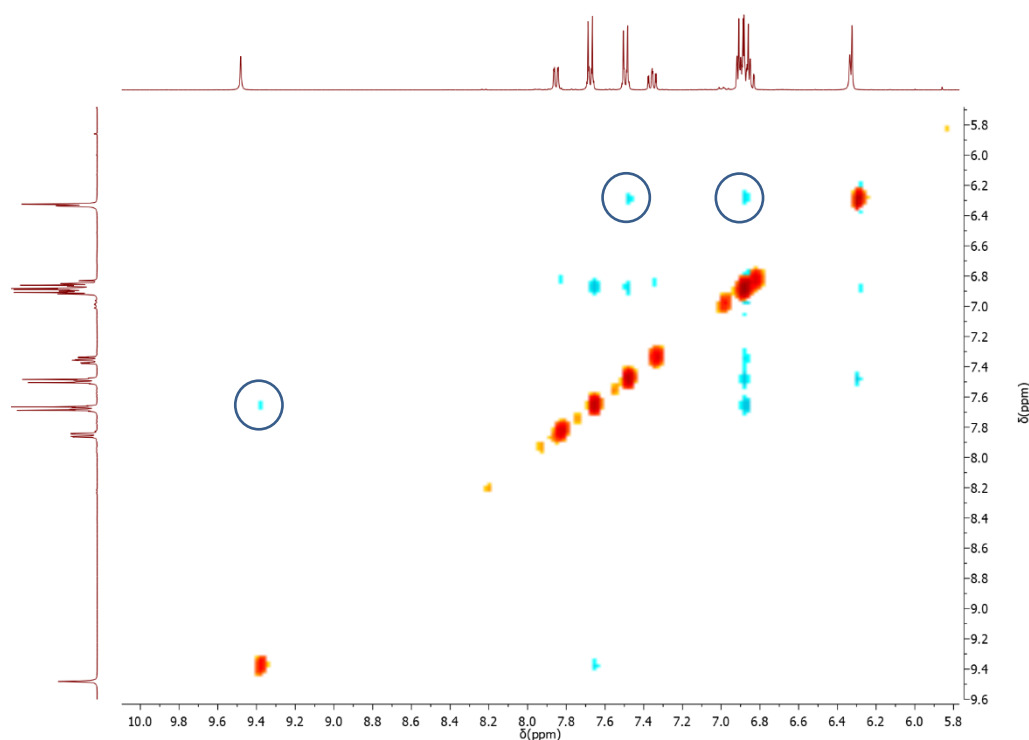


Figure 2.6: Excerpt of NOESY correlations found for compound **6** in d_6 -acetone at 400 MHz. The enlarged aromatic region is shown and key resonances that enabled relevant assignments are circled.

2.3.2 Solid state structure of compound **6**

Crystals were grown by slow diffusion and the solid-state structure of Quin C1 (**6**) was obtained by X-ray crystallographic analysis by Dr Andrew White at Imperial College London and data were collected on an Agilent Xcalibur diffractometer. In the solid state, there are two twists within the molecule along the C3-C24 bond and along the C12-C13 bond, creating three roughly perpendicular planes (Figure 2.7), as assigned using Mercury 4.1.0 software.

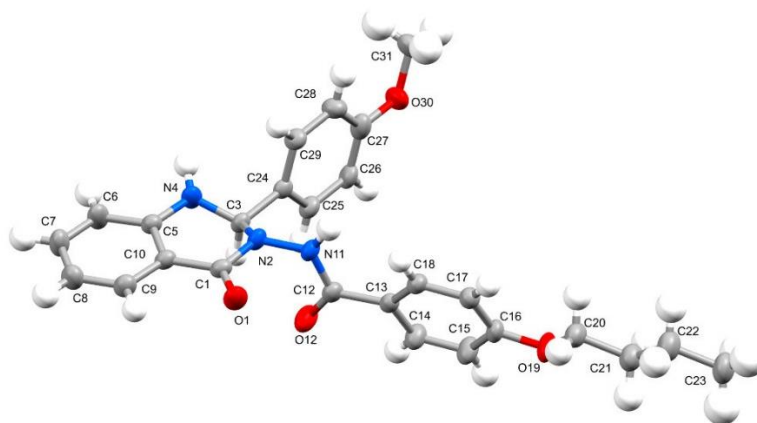


Figure 2.7: Solid state structure of **6** obtained by X-ray crystallography with 50% thermal ellipsoids, shown here for *R* enantiomer. The modelled disorder within the compound introduced by the second orientation is shown in the experimental Section 8.9, Figure 8.1.

The crystal structure further demonstrated the presence of both the *R* and the *S* enantiomer, which co-crystallised in an alternating pattern. The second orientation around the chiral carbon (C3) introduced the disorder observed within the adjacent C₆H₄OMe group unit (figure 2.8, **A** showing both enantiomers and Section 8.9, Figure 8.1 showing the modelled disorder). A complete list of averaged bond lengths and angles in the molecule is given in experimental section 8.9. H-bonding between O1 and H4A (on N4) was observed with a calculated bond distance of 2.272 Å (Figure 2.8, **B**) and the dihedral angle for the twist around C24-C3-N2 bond was determined as 36.2 degrees.

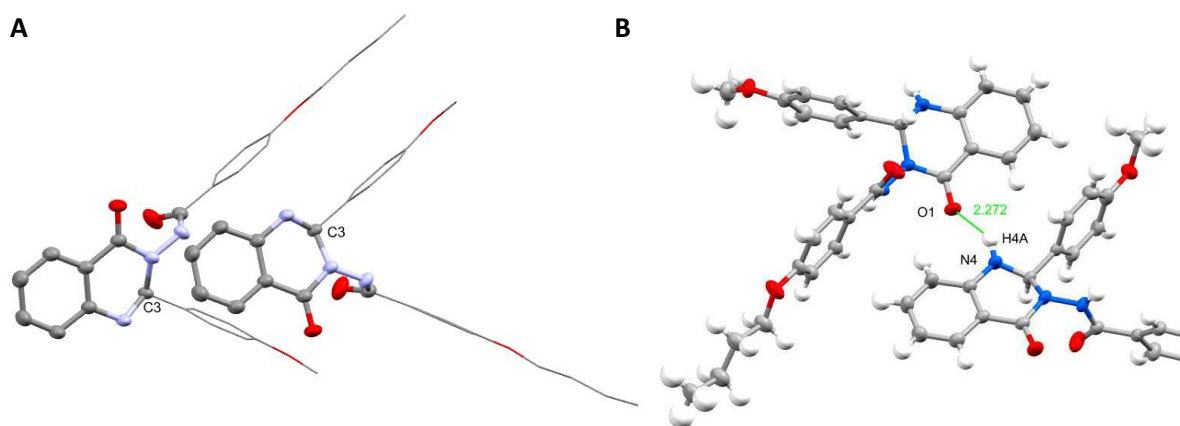


Figure 2.8: 3D view of **6** obtained by X-ray crystallography (**A**) showing packing of alternate *R* and *S* enantiomers with the chiral centre marked as C3 (hydrogen atoms omitted and wireframe plot of aromatic substituents for clarity). Excerpt of structure showing H-bonding interaction and bond distance (**B**).

2.3.3 Photophysical characterisation

Absorption, excitation and emission profiles for Quin C1 (**6**) and Quin C7 (**8**) were recorded in aerated methanol solutions. Absorption maxima were observed at 228 nm, 255 nm and 341 nm (for **6**) and 227 nm, 255 nm and 336 nm (for **8**) respectively and the profiles of the two compounds are extremely similar. Correspondingly, the excitation and emission spectra of the two compounds recorded were almost identical and are depicted below (Figure 2.9). Both compounds are characterised by large Stokes' shifts and broad, featureless emission maxima around 455 nm when excited at 350 nm.

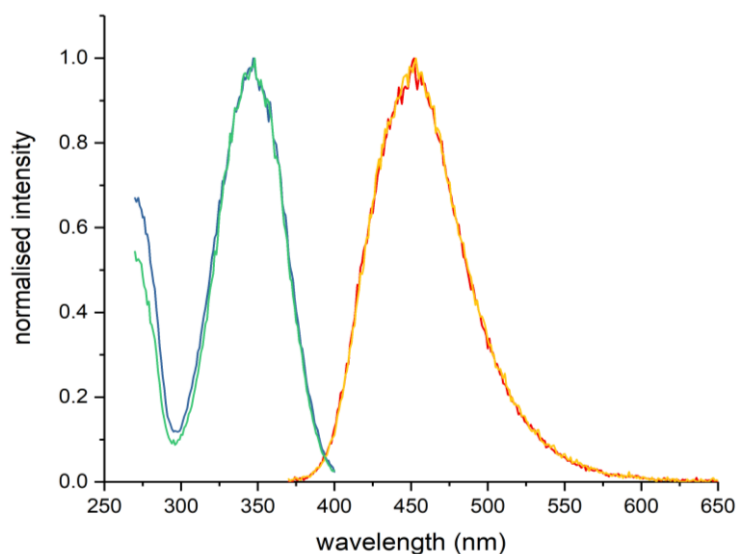


Figure 2.9: Normalised excitation and emission spectra ($\lambda_{em} = 450$ nm and $\lambda_{exc} = 350$ nm) of **6** (in green and yellow respectively) and **8** (in blue and red respectively) recorded in methanol solutions.

Dihydroquinazolinone-derivatives have previously been reported to exhibit solvatochromic behaviour and the bathochromic shifts often observed within the emission bands were attributed to a polar excited state.^{42,43} When varying the solvent system, the emission maxima of both Quin C1 and Quin C7 showed a strong dependence on solvent, likely related to the polarity and H-bonding ability of the solvent system in question (Figure 2.10).

Taken together, these results are suggestive of the presence of a charge transfer state, as is common in systems with an electron donating and an electron accepting unit connected via π conjugation.⁴⁴

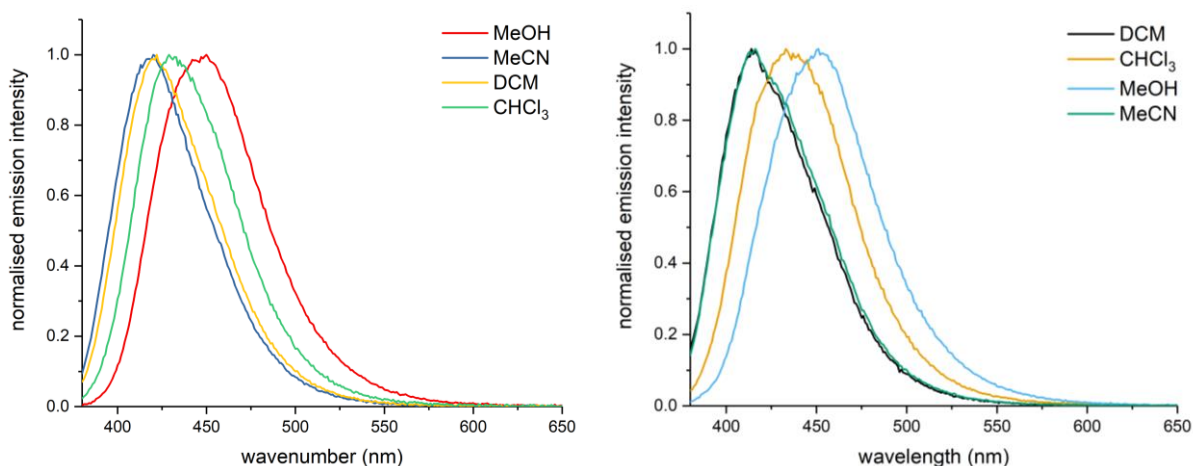


Figure 2.10: Normalised emission spectra ($\lambda_{exc} = 350$ nm) of **6** (left) and **8** (right) recorded in different solvent systems.

2.3.4 Functionalisation of ligands to enable combination with imaging moieties

In the absence of a crystal structure for the FPR family, molecular docking studies describing the FPR2/ALX binding site have been conducted using a rhodopsin model or multiple homology models (based on the opioid receptor and CXCR4 crystal structures).² One of these proposed models indicated that small-molecule compounds are likely to bind in three binding pocket areas, one of which contains a positively charged group (subpocket I) and all three of which are considered hydrophobic. Subpocket II is proposed to incorporate non-polar groups, while electronegative heteroatoms were placed in subpocket III.^{45,46} In another report, docking studies of four quinazolinone derivatives were investigated and most of them resulted in similar poses. Subpocket I was suggested to likely accommodate the quinazolinone group with possible aromatic interactions, whereas the *para*-methoxy phenyl group of Quin C1 is suggested to be located deeper within the protein forming a hydrogen bond (Figure 2.11).^{2,24} Models used and the resulting binding modes that have been previously reported vary, but the results overall imply that tight control of the precise attachment position of the bulky imaging unit could have a profound effect on receptor binding abilities of the compound.

To enable attachment of an imaging unit, the aromatic secondary amine groups were acylated using chloroacetyl chloride in an addition elimination reaction. This strategy has been abundantly reported in the literature for amide formation using both primary and secondary amines.⁴⁷ The reagent is generally considered highly reactive and was therefore chosen for peptide formation with the secondary amine, as these motifs can be problematic in peptide coupling reactions. In addition, chloroacetyl chloride provides an alkyl chloride site for further functionalisation. The reaction was carried out under a nitrogen atmosphere and yielded the expected adducts **7** and **10** respectively after purification by column chromatography.

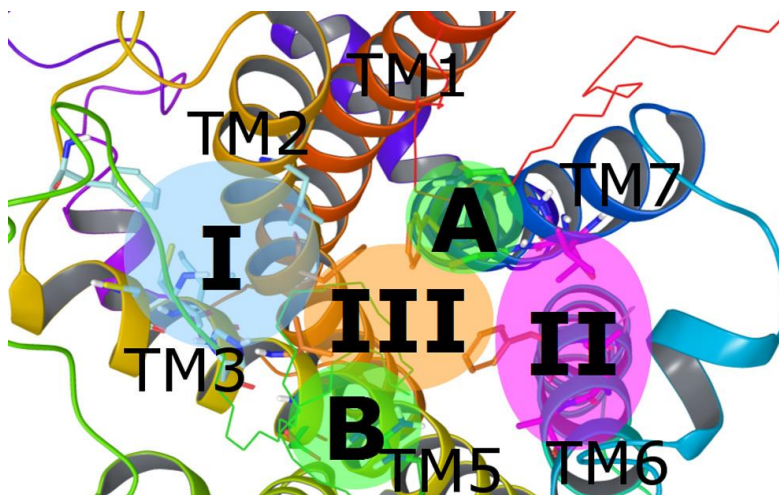


Figure 2.11: Representation of the three hydrophobic clusters (I, II, III) and two polar clusters (A, B) identified by Stepniewski et al to be considered important for binding using their FPR2/ALX homology model. Figure reproduced with permission from reference.²

Presence of **7** and **10** was verified by high resolution mass spectrometry and the structures were further assessed by $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and NOESY spectroscopy. The signals corresponding to alkyl and methoxy protons were comparable to those of the starting materials (**6** and **9**) and integrated correctly with respect to the aromatic signals observed. In addition, appearance of a broad signal at 4.84 ppm in the $^1\text{H-NMR}$ spectrum of **7** with a relative integration of 2H was assigned to the chloride linker protons (Figure 2.12). In compound **7**, the two signals expected around 6.3 ppm (as observed in $^1\text{H-NMR}$ spectra for **6** and similarly for **8**) assigned to the aminal proton and the adjacent NH were not apparent. Instead, the aminal proton signal was shifted to 7.13 ppm and appeared broadened. Attachment position of the chloride linker on the aromatic amine rather than the hydrazide amine as well as assignment of the aminal proton was confirmed by the NOESY correlations detected. Interactions through space for compound **7** were observed between H_f and H_m as well as the broad aminal proton resonance at 7.31 ppm and H_m (Figure 2.12). A NOESY cross-peak was evident for the resonance observed for H_g and the NH proton at 10.37 ppm as well as between H_b and the shift at 10.37 ppm, which establishes assignment positions for **7** as depicted below (Figure 2.12). Further, the marked shift of the aminal hydrogen, as well as the disappearance of the original aminal carbon signal within the $^{13}\text{C-NMR}$ could suggest a partial displacement of the hydrogen towards the intramolecular carbonyl (tautomerism). Attachment of the linker directly onto the aminal carbon rather than onto the nitrogen could be ruled out based on the correlations through space detected by NOESY.

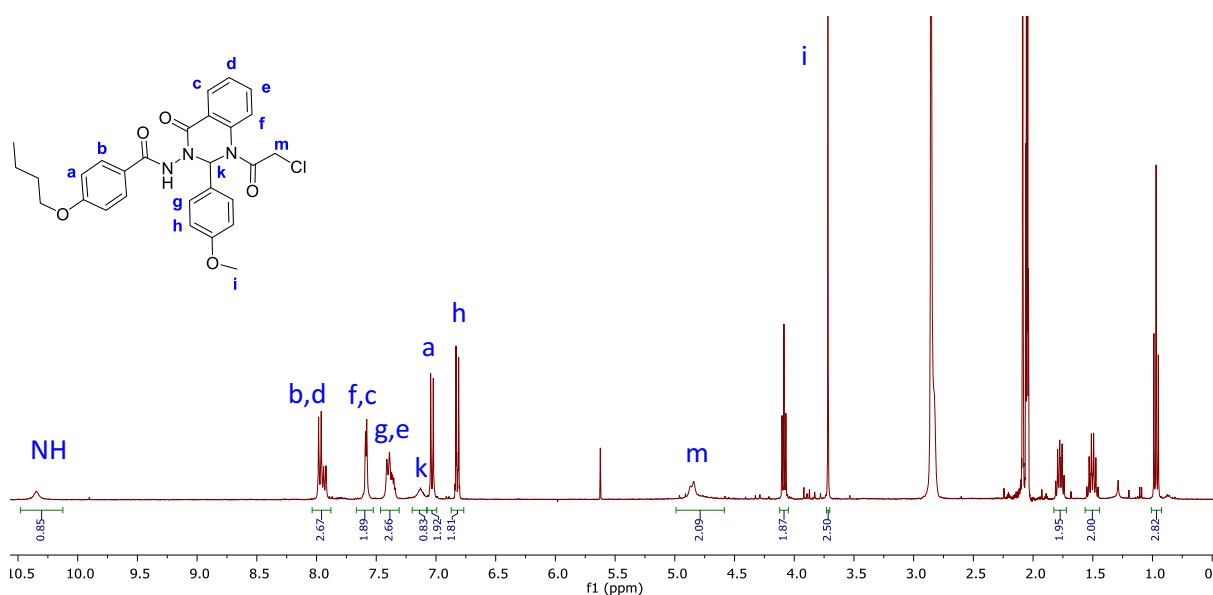


Figure 2.12: ^1H -NMR spectrum of **7** with proton assignments enabled by the NOESY correlations detected run at 400 MHz in d_6 -acetone.

Equivalent assignment was conducted for compound **10**. Within the ^1H -NMR spectrum of **10**, the chloro-linker protons appeared as a set of roofed doublets at 4.55 and 4.82 ppm, showing coupling between the two geminal protons (Figure 2.13).

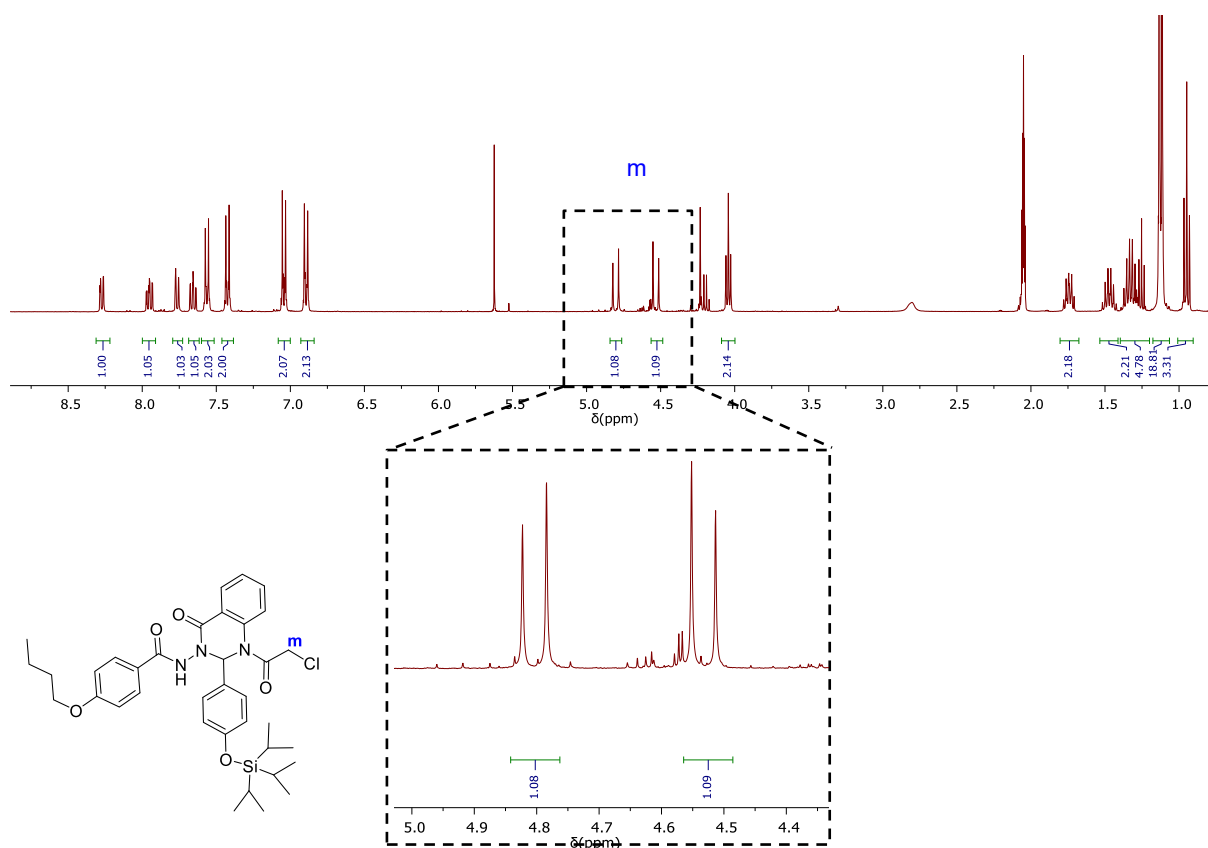


Figure 2.13: ^1H -NMR spectrum of **10** run at 400 MHz in d_6 -acetone. Below, enlarged region showing a set of roofed doublets at 4.55 ppm and 4.85 ppm.

2.3.5 *In vitro* validation of **6** and **8** using neutrophil functional assays

For the FPR2/ALX ligands to provide a useful platform towards formation of receptor targeted tracers, it is crucial to ensure that the ligand itself (with known agonist/antagonist properties) will not perturb the system it is imaging at doses relevant for detection. To assess whether presence of the compounds evoke a neutrophil response, the effects of varying concentrations (10^{-6} M, 10^{-7} M, 10^{-8} M and 10^{-9} M) of **6** and **8** were analysed using assays that simulate three central neutrophil functions: chemotaxis, transmigration and granule (e.g. myeloperoxidase (MPO)) release.

Firstly, the ability of a neutrophil to migrate in response to a chemical gradient was validated in a chemotaxis assay. Secondly, during the host inflammatory response, neutrophils can extravasate through the blood vessel endothelium upon stimulation by endogenous signalling pathways.⁴⁸ This process is simulated in the transmigration assay, where migration through an endothelial cell layer in response to a chemokine was tested. Finally, upon activation, neutrophils can release reactive species from their granules to eliminate invading pathogens. This functionality was validated in the presence of varying concentrations of compound by quantifying MPO release from stimulated neutrophils.

Each *in vitro* assay described here or in subsequent chapters was run using cells prepared from at least four independent healthy donor samples and experiments were run in duplicate or triplicate either under supervision of the Gavins lab or as repeat iterations by Gavins lab members. Analysis was performed using GraphPad Prism 8 software.

2.3.5.1 Neutrophil isolation

Neutrophils were obtained at LSUHSC Shreveport by healthcare professionals from healthy donors who gave informed consent. The study was approved by the institutional review board of the LSUHSC-S (STUDY00000261) and conducted in accordance with the Declaration of Helsinki. Isolation protocols were carried out by Dr Junaid Ansari and Miss Gaganpreet Kaur at LSUHSC Shreveport. The leukocyte layer was extracted from the blood samples using a dextran sedimentation technique and further separated over a histopaque 1077 gradient. Residual red blood cells were lysed and after several washing steps, neutrophils were resuspended in DMEM and counted using a Neubauer hemocytometer (diluted 1:1 with trypan blue). The resulting cell count was diluted to the number of cells required per assay performed.

2.3.5.2 Chemotaxis assay

The chemotactic response of neutrophils was assessed using a 96-well plate set-up, where each well contained a solution with either vehicle (phosphate buffered saline (PBS)) or a chemoattractant and was covered with a porous polycarbonate membrane surrounded by a hydrophobic area (illustrated in Figure 2.14). Human neutrophils were preincubated with vehicle or compound (at concentrations of 10^{-6}

M, 10^{-7} M, 10^{-8} M and 10^{-9} M) and a drop of cell suspension was added to each membrane surface. The neutrophils commence migration in response to the chemical gradient they experience (or random migration, when no stimulus is present) and the extent of migration was assessed either spectrophotometrically (when the cells have been pre-treated with calcein AM) or via manual cell counting.

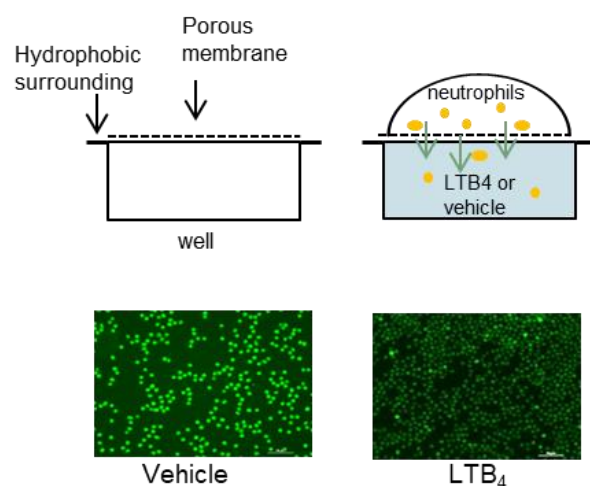


Figure 2.14: Schematic overview of chemotaxis assay set-up. Neuroprobe 96-well plates with polycarbonate membrane were loaded with vehicle or LTB₄ solution in the chamber below and cellular suspensions incubated with vehicle or relevant compound concentrations were added above. Neutrophil migratory response was quantified and could be visualised as depicted below, with few cells migrating in response to vehicle (bottom, left) and substantially more cells when stimulated by chemoattractant LTB₄ (bottom, right).

Results for the chemotaxis assay were reported as a migration index, which was calculated by dividing numbers of neutrophils migrated in response to leukotriene B₄ (LTB₄, 10^{-6} M) by the number of cells migrated in response to vehicle. The rationale behind choosing LTB₄ as a chemoattractant is that the compound is known to induce a potent chemoattractant response in neutrophils via engagement of BLT1 and BLT2 (high- and low- affinity respectively) receptors, and is independent of FPR signalling pathways.^{49,50}

The data obtained for both compounds shows that they do in fact significantly change neutrophil function in response to the chemoattractant compared to the control at certain concentrations. When the cells were pre-treated with higher concentrations of Quin C7 (10^{-6} M and 10^{-7} M), less directed neutrophil mobility in response to LTB₄ compared to the relevant controls was apparent. These results are consistent with the reported antagonist characteristics of compound **8** and indicate that inhibition of neutrophil function occurs at higher concentrations. Presence of Quin C1, a reported FPR2/ALX agonist, did not affect neutrophil migration at the highest and lowest concentrations tested (10^{-6} M and 10^{-9} M respectively). However, a dose-dependent response can be observed, where at intermediate concentrations the chemotactic response of the cells is slightly, but statistically significantly reduced at 10^{-7} M and 10^{-8} M concentrations (Figure 2.17). The fact that such a response occurs only at intermediate doses is in accord with similar observations made, on the anti-inflammatory effect of the

compound which is only observed within a narrow range of doses but not at higher or lower concentrations.³⁰

2.3.5.3 Transmigration assay

Migration in response to a chemotactic gradient was further evaluated in a transmigration assay, which assesses the ability of neutrophils to transmigrate through an endothelial cell layer in response to activation through a chemical stimulus. To achieve this, human umbilical vein endothelial cells (HUVECs) were grown on a transwell insert with a porous membrane by Miss Gaganpreet Kaur of LSUHSC Shreveport. The insert was suspended in a solution containing vehicle or LTB₄ (10^{-6} M) and cells were added to the top, as illustrated schematically in Figure 2.15.

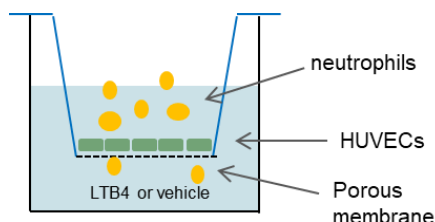


Figure 2.15: Schematic representation of transmigration assay setup with a layer of endothelial cells (HUVECs) through which neutrophils migrate.

The data obtained from this assay are consistent with what was observed during the chemotaxis assay. At high concentrations (10^{-6} M and 10^{-7} M), Quin C7 inhibits neutrophil transmigration, whereas for lower concentrations no significant change is apparent. Cells incubated with Quin C1 on the other hand behave like the control group except at an intermediate concentration of 10^{-7} M, where a suppression of trans migratory response compared to the control group becomes evident (Figure 2.17).

2.3.5.4 MPO release assay

Activated human neutrophils release MPO from their granules that can produce collateral oxidative damage to tissue via formation of, for example, hypochlorous acid (HOCl) in the presence of hydrogen peroxide and a chloride ion. This process typically occurs during the neutrophil respiratory burst.^{51,52} The produced HOCl is known to react with diverse endogenous targets and generate products such as aldehydes, diverse chloramines and tyrosyl radicals that contribute to antimicrobial activities.⁵³

As such, MPO can be used as a quantitative indicator of neutrophil activation. Addition of the chromogenic substance 3,3',5,5'-tetramethylbenzidine (TMB, Figure 2.16) to activated neutrophils (that have released MPO), forms a blue solution. The hydrogen donor abilities of TMB during the myeloperoxidase mediated reduction of hydrogen peroxide to water results in TMB diimine formation (Figure 2.16) and hence the colour change occurs.

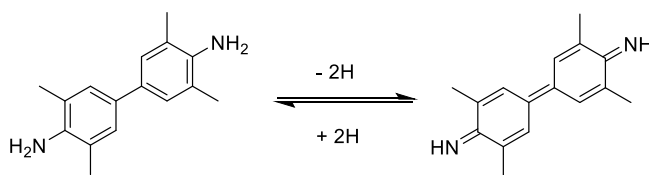


Figure 2.16: 3,3',5,5'-Tetramethylbenzidine structure and reversible reaction to form the diimine derivative causing a colour change (blue), and the corresponding diamine (yellow).

After 5 minutes, the reaction is terminated by addition of sulfuric acid, inducing a further colour change as the diamine is re-formed, which allows release of MPO to be quantified spectrophotometrically relative to a TMB standard, using absorption wavelengths of 450 nm to read the diamine product. Using this method, the level of MPO release was measured from stimulated neutrophils in the presence and absence of Quin C7 and Quin C1. Results indicate that at lower concentrations (10^{-7} to 10^{-9} M), Quin C7 shows no statistically significant effect on neutrophil function in the MPO release assay. However, the highest concentration of Quin C7 (10^{-6} M) caused a reduction in MPO release compared to controls, consistent with observations made in the transmigration and chemotaxis assays and indicative of reported antagonistic properties. Treatment with Quin C1 on the other hand had no effect on neutrophil MPO release compared to controls except at 10^{-8} M concentrations, where MPO release appeared to be significantly decreased, again illustrating the apparent narrow dose response window (Figure 2.17).

The data collected highlight that the unfunctionalised ligand systems **6** and **8** both influence neutrophil function across all three assays in a concentration dependent manner. For Quin C1, only a specific, narrow concentration range was associated with decreased neutrophil activity compared to the control, which indicates that the compound could be incorporated into imaging systems and used at relevant biological concentrations without affecting neutrophil function. Quin C7 on the other hand was found to inhibit neutrophil function with increasing concentration and as a result is less suitable for imaging applications.

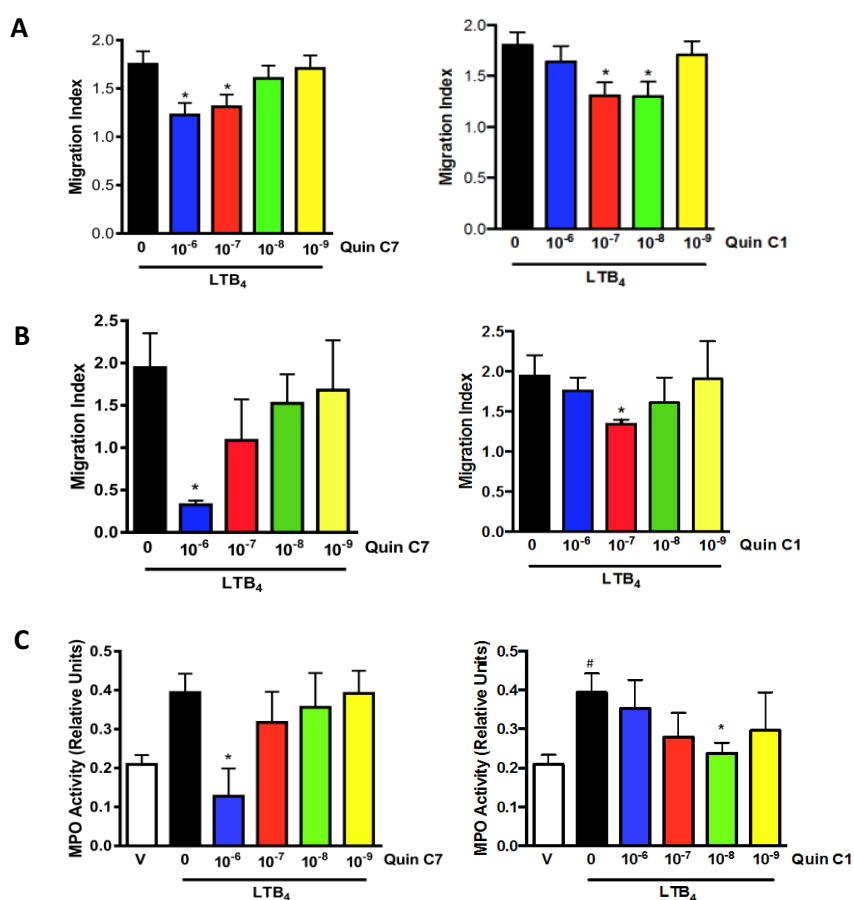


Figure 2.17: Assay data shows freshly isolated human neutrophils incubated with different concentrations of the compounds (Quin C7, left and Quin C1, right) added to the top wells of a Neuroprobe chemotaxis chamber (A), whilst LTB_4 (10^{-6} M) was added to the bottom wells to assess whether the compounds affect neutrophil chemotactic responses or transmigration responses upon migration through an endothelial cell layer on a transwell insert (B). Migration indices were calculated by dividing numbers of neutrophils migrated to LTB_4 by numbers of cells migrated to vehicle. Below, data shows stimulated MPO release in human neutrophils at varying molar concentrations of compounds as indicated above with a constant LTB_4 concentration of 10^{-6} M (C). Data are overall average $\pm$ SEM of 4 separate experiments, prepared with different neutrophil donors. Each experiment was run in duplicate. * $P < 0.05$ vs. corresponding vehicle control group. (ANOVA and Bonferroni).

2.4 Conclusions

Preparation and spectroscopic characterisation of two small-molecule FPR2/ALX ligands was described. Both moieties were further functionalised using chloroacetyl chloride as a bifunctional linking group. This provides a useful building block to prepare FPR2/ALX targeted probes relevant to a variety of imaging modalities such as MRI, PET and optical microscopy, depending on the choice of group appended or metal ion that is chelated. For Quin C7 (**8**), a prior protection strategy was devised to prevent unwanted substitution reactions on the hydroxyl group which has previously been shown to be crucial for the compounds' bioactivity profile. The two compounds were assayed *in vitro* for their effects on human neutrophil function, specifically their ability to influence chemotaxis, transmigration and MPO release. The obtained results show that a concentration dependent effect on neutrophil function in all three assays is observed for both compounds. Quin C7 (**8**) inhibits neutrophil function as the concentration is increased, which becomes apparent at the highest concentrations tested (10^{-6} M and 10^{-7} M). For Quin C1 (**6**) a more complex response was observed. Decreased neutrophil activity was seen across assays only at specific, intermediate concentrations (10^{-7} M and/or 10^{-8} M) whereas higher and lower amounts are not differentiated from the control group. The data collected suggest that the compounds do not influence the inflammatory functioning of neutrophils at relevant concentrations for visualisation and thus are suitable to act as a FPR2/ALX targeting unit incorporated into an effective imaging agent, which is encouraging for their future use and application later in this thesis.

2.5 References

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Chapter 3

Fluorescent compounds for targeting FPR2/ALX in inflammation

3.1 Introduction

Chemical probes designed for fluorescence applications have demonstrated exceptional abilities to identify structure, stimuli and analytes with low detection limits and high sensitivity in a cellular context and therefore are particularly desirable for biological applications. For example, organic dyes derived from the xanthene core (Figure 3.1) have been extensively applied for fluorescence cell imaging due to their relatively long excitation and emission wavelengths, high extinction coefficients and quantum yields in combination with good photostability. Amongst these, the rhodamine family, with the core structure depicted in Figure 3.1 was first prepared in 1905¹ and has since been widely used in a range of diverse applications.²

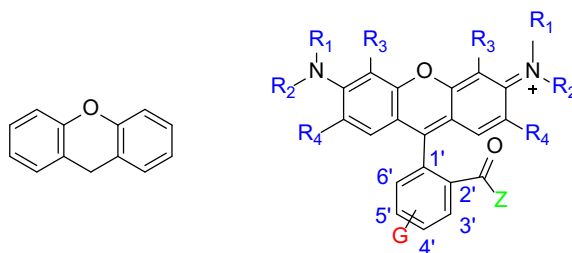


Figure 3.1: Molecular structure of the xanthene core (left) and general structure and substituent labelling of rhodamine dyes (right).

Rhodamine B (G = H, Z = OH, R_{3,4} = H and R_{1,2} = ethyl, compare Figure 3.1) is one of the most prevalent rhodamine dyes and is routinely prepared from 3-(diethylamino)phenol and phthalic anhydride in a 2:1 molar ratio via consecutive Friedel-Crafts acylation steps followed by an intramolecular Fischer esterification.³

3.1.1 The rhodamine B intramolecular cyclisation equilibrium

Rhodamine B bears an aromatic, free carboxylic acid group that can be readily functionalised and its derivatives began to be explored for their cyclisation equilibrium in 1997.⁴

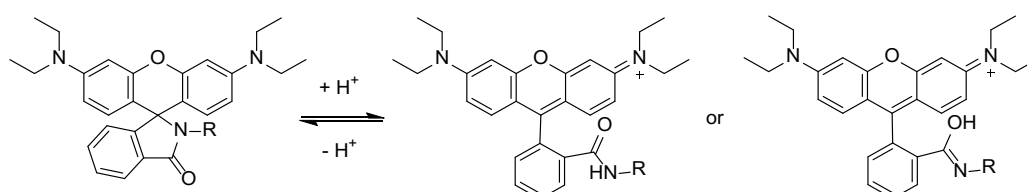


Figure 3.2: General intramolecular spirolactam ring formation equilibrium in rhodamine derivatives. The spirocyclic species (left) is non-emissive, while the ring-opened analogues (right) display characteristic rhodamine emission.

This equilibrium (Figure 3.2) is characterised by a closed spirolactam or lactone form which is non-fluorescent and almost colourless. Upon activation of the carbonyl group, for instance by acidic solutions, a strongly fluorescent and fully conjugated, ring-opened analogue is formed.⁴ This “switchable” property has been widely investigated in the sensing of various heavy metal ions,^{2,5} for preparation of pH-sensitive analytic probes⁶ and for detecting reactive oxygen species (ROS)⁷ and thiols.^{8,9} Furthermore, depending on the particular structure, some studies have shown the ring-opening to cause a fluorescence emission intensity increase of 100-fold.¹⁰

3.1.2 Photophysical properties of rhodamine dyes

Apart from the three distinct rhodamine B forms present in solution (neutral and spirocyclic, zwitterionic and ring-opened, cationic and ring-opened, photophysical properties are summarised in Table 3.1),¹¹ dye fluorescence intensity changes are also known to occur as a result of FRET processes. This is exemplified by a BODIPY and rhodamine FRET system that can be manipulated to enable ratiometric detection of Hg^+ ions.¹² Additionally, photoinduced electron transfer (PeT) processes can be observed in rhodamine-labelled DNA hairpins,¹² and lead to fluorescence quenching, as does the formation of blue-shifted, non-fluorescent rhodamine dimers (H aggregates) that are sometimes observed in aqueous solutions.¹³

	Cationic	Zwitterionic	Neutral
λ_{abs} (solvent)	553 nm (EtOH)	543 nm (EtOH)	311 nm (Et ₂ O)
λ_{em} (solvent)	572 nm (EtOH)	563 nm (EtOH)	442 nm (Et ₂ O)

Table 3.1: Absorbance and emission values of rhodamine B forms, as reviewed in reference¹¹

It is further well-known that rhodamine B derivatives comprising of two alkyl substituents on each nitrogen display temperature-dependent quantum yields and lifetimes associated with an internal conversion process occurring in the excited state.¹¹ An electron transfer from the amino group to the xanthene ring, followed by a rotation along the bond connecting them, leads to formation of a twisted intramolecular charge transfer (TICT) state (Figure 3.3).¹⁴ The TICT state, which becomes favourable in the more substituted aniline derivatives, can readily relax via nonradiative decay mechanisms leading to decreased quantum yields and fluorescence lifetimes compared to less substituted rhodamine dyes.¹⁵

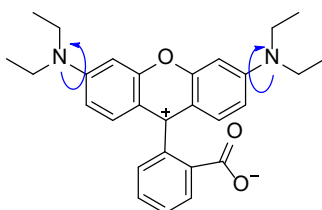


Figure 3.3: Molecular structure of rhodamine B isomer present in solution, with arrows indicating rotational movements likely occurring upon TICT formation.¹⁶

3.1.3 Rhodamine-derivatives used for cellular pH sensing

Within cells, it has been established that the intrinsic pH values of organelles vary. Lysosomes and endosomes are known to be more acidic,¹⁷ whereas the mitochondria and cytosol are slightly basic.^{18,19,20} In diseased states, the cell and specific organelles can undergo a change in pH which is often detrimental to homeostasis. For example, tumorous microenvironments and inflammatory lesions are both known to be regions of higher acidity.^{21,22,23} In inflammation, the local acidification is attributed to both the presence of short chain fatty acid by-products from bacterial metabolism and the increased production of lactic acid by neutrophils.²⁴ The ranges in pH vary although they have been found to be between about 4.5 and 7.6 for diseased tissue, compared to between pH 7.2 and 7.6 for healthy tissues.²⁵

The range in physiological pH implies, that rhodamine-based sensors relying on the spirocyclisation equilibrium can be exploited to report on cellular conditions. For pH sensor design with bioimaging applications, tight control of the pK_{cycl} values and kinetic factors is necessary. It has been highlighted that the nature of the rhodamine Z-substituent (Figure 3.1) correlates with both ring-opening rates and pK_a values and thus can be tuned to create favourable probe properties.^{26,27}

As one of the first and most notable examples of rhodamine-derived compounds investigating an acidic cellular product, Nagano and co-workers demonstrated that their novel probe could be tuned to specifically sense hypochlorous acid (HOCl) over other ROS in live cells by controlling the nucleophilicity of the 2' position substituent (Figure 3.4, **A**).²⁸ Since then, pH-switchable, rhodamine-based probes have been designed to visualise varying intracellular pH-distributions by fluorescence intensity changes (Figure 3.4, **B**) or to identify the intensely fluorescent, acidic lysosomal environments.^{10,29} Lysosomal pH-responsive compounds have been reported to quantitatively monitor stimulated pH increases (upon treatment with chloroquine) and for visualisation of pH changes during apoptosis.³⁰ Furthermore, structural modifications to rhodamine scaffolds to allow for ratiometric cell imaging approaches or FRET-based sensing of pH states have been reported (Figure 3.4, **D, E, G**).^{30,31,32}

To enable visualisation of acidic and basic pH scales, dual-emissive designs that make use of one acid and one base sensitive ring-opening equilibrium have been devised. This “two colour” strategy has been used to detect pH values within different cellular organelles by confocal microscopy and thus create a cellular pH map (Figure 3.4, **C, F** and Figure 3.5).^{20,33}

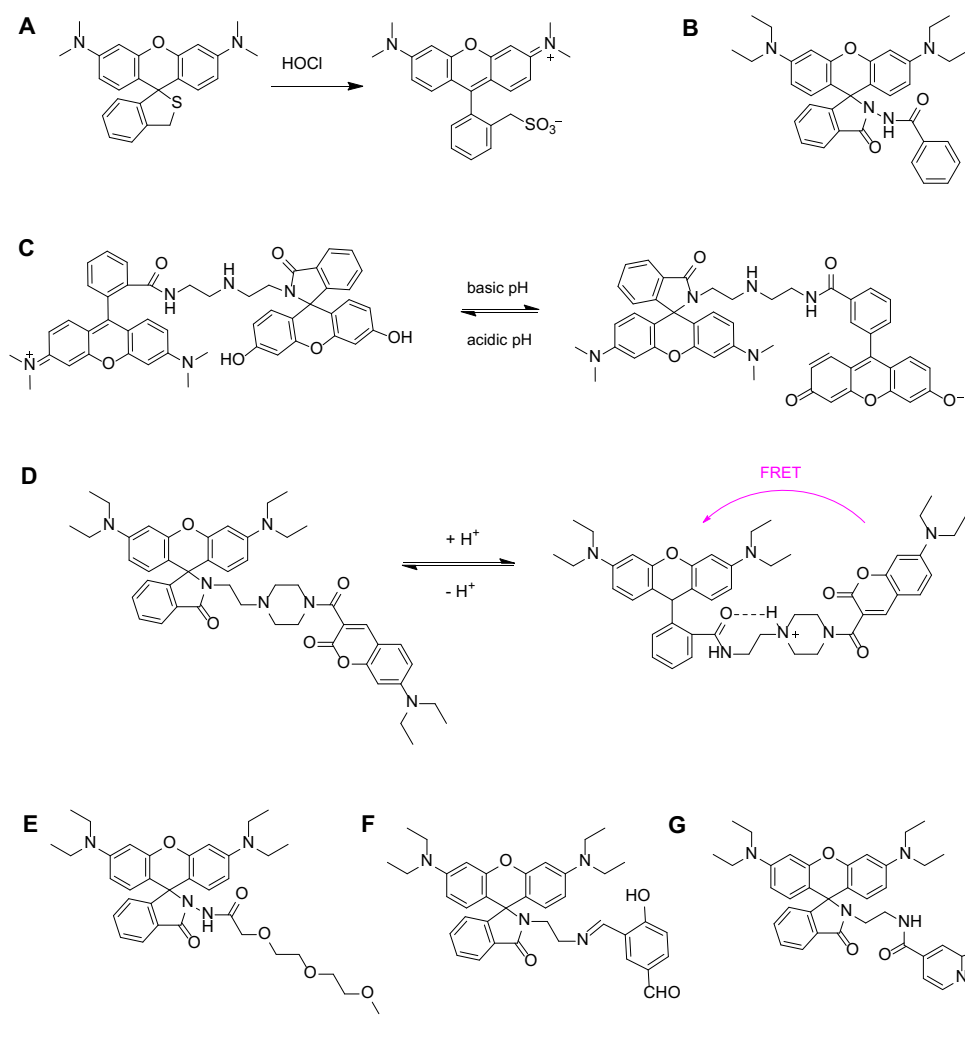


Figure 3.4: Molecular structures of pH-sensors based on rhodamine dyes for cellular intensity-based pH sensing (A, B),^{28,10} visualisation of lysosomal pH changes (D, E, G)^{30,31,32} and dual-emissive pH mapping (C, F)^{20,33}

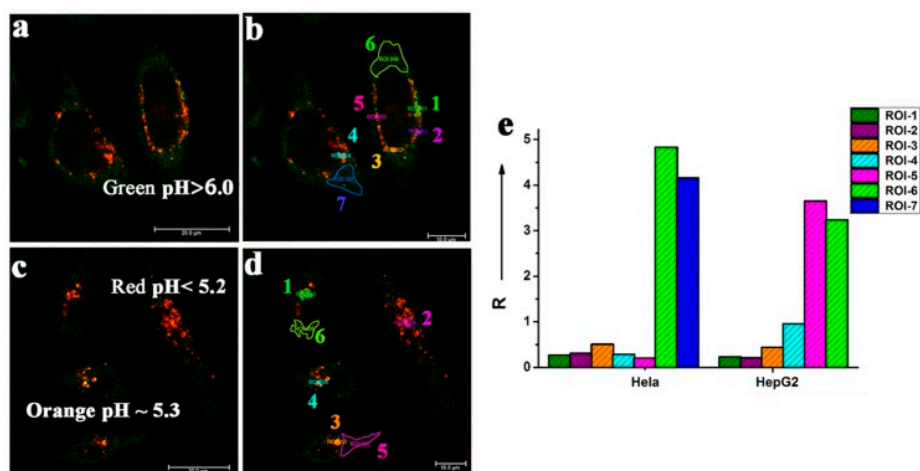


Figure 3.5: Images obtained by confocal microscopy of the dual-emissive probe **F** (Figure 3.4) in HeLa cells (a, b) and HepG2 cells (c, d). Seven regions of interest (ROI) are distinguished for cellular mapping of local pH. Figure **e** shows ratio of fluorescence intensities. Reproduced from reference with Creative Commons license (CC BY 4.0).³³

3.2 Aims

In a preclinical context, fluorescence microscopy is a valuable technique that is characterised by high sensitivity and exceptionally good sub-cellular resolution at low doses of probe.³⁴ In this chapter, the aim is to prepare three different rhodamine-derived optical probes that can specifically target FPR2/ALX in inflammation (Figure 3.6). Firstly, **17**, a rhodamine B derivative locked into its “open”, fluorescent state (via incorporation of a tertiary amine at the Z position) is linked to the small-molecule FPR2/ALX ligand, Quin C1. Secondly, the same rhodamine derivative is combined with the FPR2/ALX antagonist analogue Quin C7. Thirdly, to extend applications of this strategy, a FPR2/ALX-targeted, switchable rhodamine analogue (**13**) is prepared, to enable a readout of the fluorescence in response to changes in environmental pH values. This could both relay functional information about the pH state of the target region and thus mechanistic insights, as well as provide better sensitivity due to improved target to noise ratios.

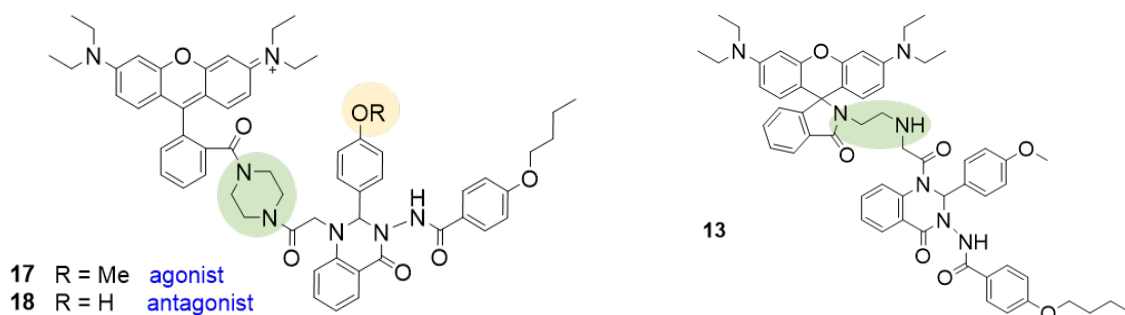


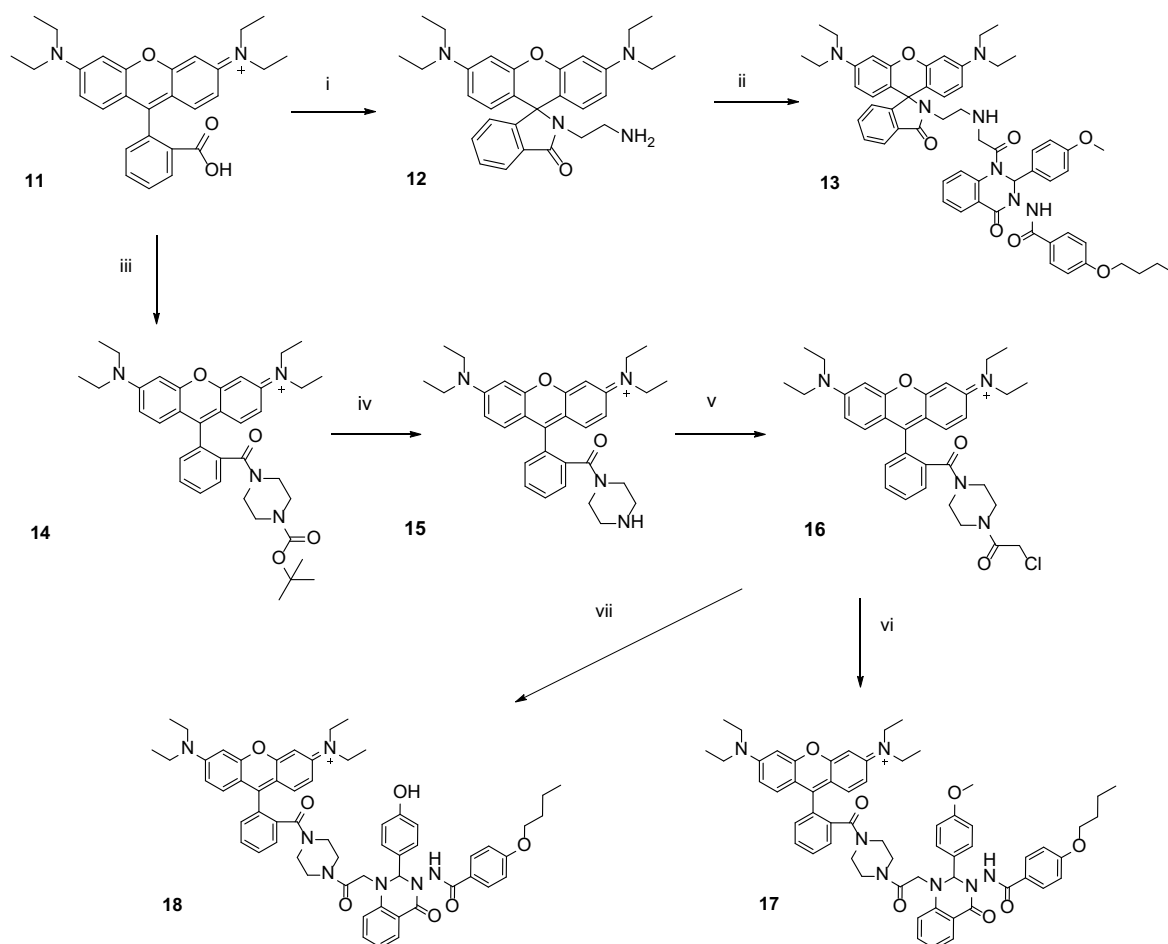
Figure 3.6: DeVised FPR2/ALX-targeted optical probes, based on Quin C1 or Quin C7 (difference denoted by yellow region), combined with a pH-responsive rhodamine unit (**13**) or conformationally locked (**17** and **18**) into the ring opened state using a tertiary amine motif (linker marked by green region).

After the compounds have been prepared and fully characterised, *in vitro* techniques are used to determine if the prepared imaging agents can bind to the relevant receptor without altering neutrophil function. Finally, if successful, these imaging agents are to be carried forward into a preclinical disease model. The following criteria should be met by the compounds prepared in this context:

1. Compounds must be water soluble, stable to decomposition and not evoke a pro- or anti-inflammatory response at relevant concentrations to be useful for imaging.
2. The compounds must localise at neutrophils/other FPR2/ALX expressing cells observed by microscopy at low concentrations.
3. Preferential uptake of the compounds in a stimulated cellular state (which mimics a diseased context) compared to at homeostasis.
4. Appropriate pK_{cycl} values to turn “on” in pathological cellular pH of the switchable compound.
5. A clear spectral change in an inflamed context compared to a healthy control visible by microscopy in order to be able to differentiate inflammatory lesions *in vivo*.

3.3 Results and discussion

The strategy for preparation of the three rhodamine-tagged FPR2/ALX-targeted moieties **13**, **17** and **18** is detailed in Scheme 3.1.



Scheme 3.1: Strategy for preparation of rhodamine-derived FPR targeted compounds: a pH-sensitive version based on Quin C1 (**13**), and two pH-insensitive versions **17** (Quin C1) and **18** (Quin C7). Reagents: *i*) ethylenediamine (excess), ethanol, *ii*) **7**, potassium carbonate, acetonitrile, *iii*) tert-butyl piperazine-1-carboxylate, triethylamine, HBTU and dichloromethane, *iv*) trifluoroacetic acid, dichloromethane, *v*) chloroacetyl chloride, triethylamine, dichloromethane, *vi*) **6**, N,N-diisopropylethylamine, acetonitrile, *vii*) **8**, N,N-diisopropylethylamine, acetonitrile.

3.3.1 Preparation of a pH-insensitive Quin C1-rhodamine derivative

To form a rhodamine B conjugate that is locked into its perpetually fluorescent, ring-opened state, thereby providing an “always on” fluorescence signal across all pH ranges, a secondary amine was attached to the rhodamine B carboxylic acid group.

This strategy has been employed within the literature and rhodamine analogues have previously been modified by reacting the carboxylic acid group with *N*-methylpropargyl amine³⁵ or a piperazine unit³⁶ which both result in products that prevent spirocyclisation. Appendage of a piperazine unit to the rhodamine B entity was originally achieved using alumina salts and harsh conditions.³⁶ More recent protocols report similar results using preferable, mild peptide coupling reactions.³⁷

Here, commercially available rhodamine B (**11**) was treated with a mono-protected 1,4-piperazine-Boc unit using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) to form **14** in a peptide coupling reaction.³⁷ HBTU is a well-known uronium salt peptide coupling reagent, that can be used in reactions proceeding under mild conditions. The reagent enables retention of chiral integrity and as a result is often the coupling reagent of choice for solid state peptide syntheses.³⁸ By-products typically generated in this reaction such as tetramethylurea can readily be separated from the product by washing steps or column chromatography.³⁹ A generalised reaction mechanism for peptide bond formation with HBTU is shown in Figure 3.7.

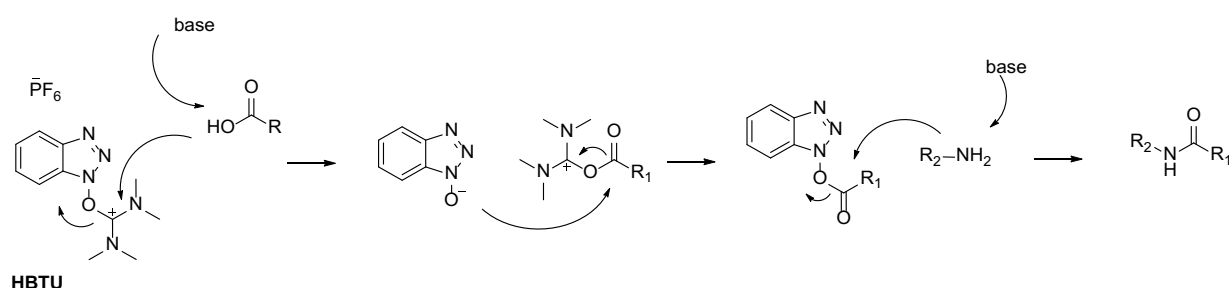


Figure 3.7: Reaction mechanism for peptide coupling of a carboxylic acid and an amine using HBTU as a reagent.

The peptide coupling was carried out in dichloromethane using triethylamine as a base at room temperature and the resulting bright pink compound was isolated by silica column chromatography eluting in dichloromethane and methanol (20:1). Spectra obtained by ¹H-NMR and mass spectrometry were in good agreement with previous reports.^{40,37} Subsequent deprotection of the piperazine Boc protecting group was carried out in trifluoroacetic acid and dichloromethane. The reaction proceeds via a protonation step followed by loss of the stabilised *tert*-butyl cation from the ester group. Next, ester decarboxylation occurs to afford the free amine. This was evidenced by loss of the 9H *tert*-butyl singlet signal at 1.42 ppm within the ¹H-NMR spectrum of **15**.

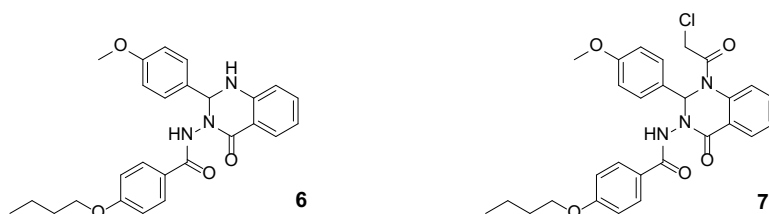


Figure 3.8: Compounds Quin C1 (**6**) and Quin C1 acetyl chloride (**7**) from Chapter 2.

To append the previously described targeting group Quin C1 (**6**, Figure 3.8), a nucleophilic substitution reaction between **15** and **7** was initially attempted. However, this reaction did not result in formation of

the desired product. To bypass this step, the terminal piperazine amine was instead functionalised via acylation with chloroacetyl chloride. Subsequent purification by column chromatography resulted in isolation of compound **16**. The characteristic protons adjacent to the chloro group appeared within the ^1H -NMR spectrum as a broad singlet resonance at 4.24 ppm integrating to 2H. In addition, piperazine-based protons were apparent as a broad resonance between 3.24 - 3.67 ppm. The typical, rhodamine-based aliphatic triplet signals (assigned to H_d , Figure 3.9) and quartet resonances (assigned to H_e) were apparent by ^1H -NMR spectroscopy at 1.33 ppm and 3.77 ppm. Aromatic resonances were assigned based on coupling patterns and previous literature (Figure 3.9).

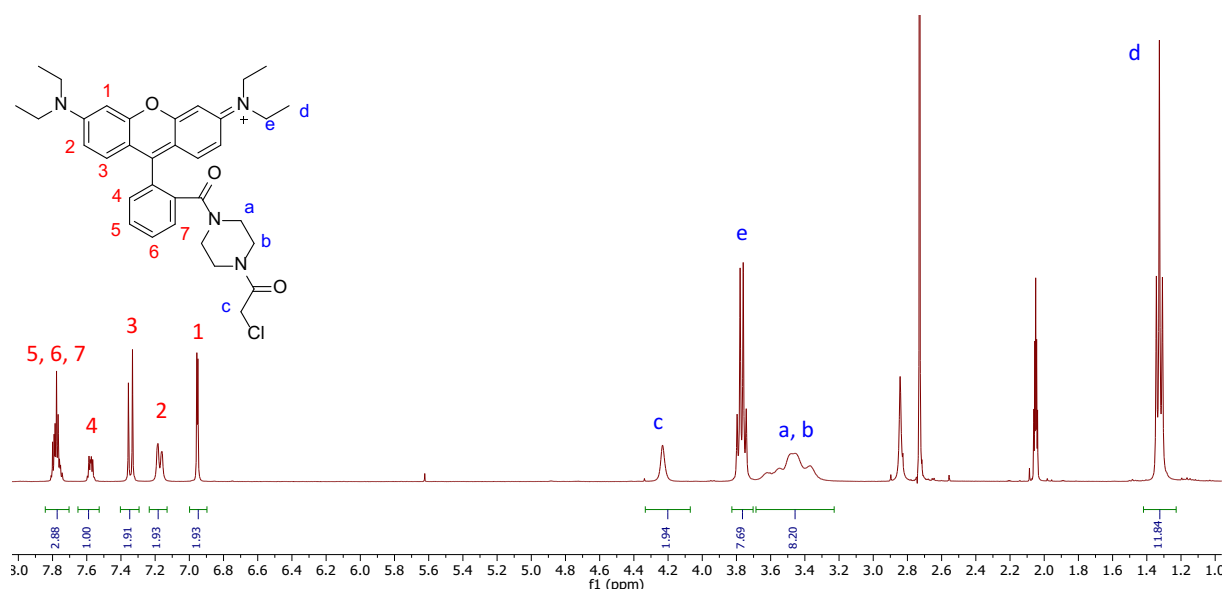


Figure 3.9: ^1H -NMR spectrum (in d_6 -acetone at 400 MHz and 298 K) with proton assignments of compound **16**.

Next, **16** was treated with **6** using *N,N*-diisopropylethylamine as a base under mild heating to produce the final targeted rhodamine construct **17**. The reaction was monitored by thin layer chromatography (TLC). After two days, a new compound was evidenced and the reaction mixture was washed with water, extracted with dichloromethane, dried over magnesium sulphate and purified. Isolation of the adduct proved challenging, as the product repeatedly co-eluted with an impurity, but after repeated silica column chromatography **17** was obtained as a deep pink solid in low yields (30 %).

The compound was characterised by HRMS and NMR spectroscopy. A $[\text{M}]^+$ ion was observed as the main signal at 996.5035 a.m.u. within the high resolution mass spectrum and purity further confirmed by LCMS. Within the ^1H -NMR spectrum, proton signals appeared broad and overlapping and could not be definitively separated or assigned. However, relative integration ratios between aromatic (22H) and aliphatic regions (42H overall, often in broad, overlapping resonances) were established as expected.

3.3.2 Preparation of a pH-switchable Quin C1-rhodamine derivative

A switchable rhodamine-analogue based on Quin C1 was formed by reacting rhodamine B (**11**) with an ethylenediamine chain. The two reagents were heated at reflux in ethanol according to a previously published method.⁴¹ Excess ethylenediamine was conveniently removed under reduced pressure and the compound was isolated in high yields without requiring further purification. Formation of the resulting compound **12** was confirmed by spectral comparisons with reported values and the tethered ethylenediamine protons appeared as 2H triplets at 2.32 and 3.16 ppm within the ¹H-NMR spectrum. Compound **12** was further reacted with Quin C1-acetyl chloride (**7**) under basic conditions to produce compound **13**. Although not an efficient reaction, purification could be achieved by repeated silica column chromatography to isolate the switchable rhodamine-C1 conjugate **13**. The resulting compound was found to be in its “off”, non-fluorescent state under basic and neutral conditions and an “on” response was observed in the presence of acidic media (pH < ~5.5). In the spirocyclic form, a characteristic signal within the ¹³C{¹H}-NMR spectrum arising from the quaternary carbon centre appeared at 68.6 ppm (a value that is comparable to literature reports of related resonances).^{42,27} The spirocyclic “switch” was observed by eye as well as formation of the ring-opened analogue caused the colour of the compound to change from very pale orange to a deep pink.

Formation was further evidenced by HRMS with an [M+H] molecular ion peak at 970.4863 a.m.u. Proton signals obtained by ¹H-NMR spectroscopy were assigned by comparison with the two starting materials and aided by 2D NMR spectroscopy. Resonances corresponding to the butyl chain (at 0.97 ppm, 1.50 ppm, 1.77 ppm and 4.08 ppm) were comparable to those of **6**. Other alkyl signals were assigned to the methoxy group (singlet at 3.72 ppm), the aniline ethyl chains (as a triplet at 1.12 ppm and between 3.26 and 3.42 ppm), the ethylenediamine connecting unit (as broad signals at 2.50 - 2.64 ppm and overlapping with other resonances between 3.26 and 3.42 ppm) and the bridge from the chloride linker (broad signal between 3.84 - 4.01 ppm). The stacked aromatic regions detected by ¹H-NMR spectroscopy for compounds **7**, **12** and **13** are shown below (Figure 3.10). Cross correlations by COSY between H_c and the resonance assigned to H_{b,a}, as well as H_c and H_d and cross peaks between H_g and H_h enabled definitive identification of resonances that had shifted from the starting materials.

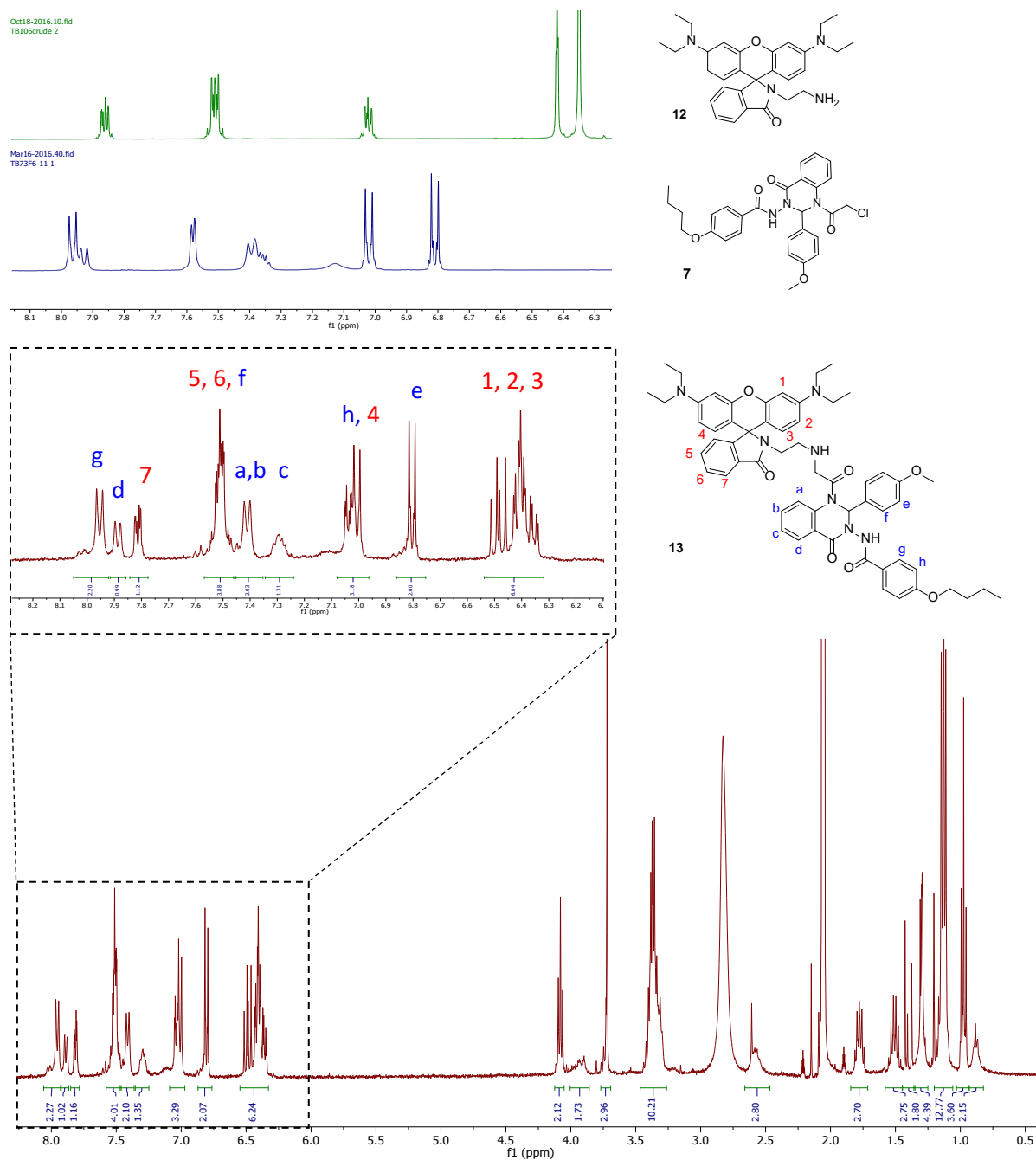


Figure 3.10: ^1H -NMR spectrum (in d_6 -acetone at 400 MHz and 298 K) and assignment for compound **13** with enlarged aromatic area shown above and proton regions labelled on the molecular structure. Inset above, the ^1H -NMR aromatic regions stacked for starting materials **12** (in green) and **7** (in blue).

3.3.3 Preparation of a pH-insensitive Quin C7-derivative

The Quin C7 scaffold was considered less suitable for imaging purposes compared to the related Quin C1 structure as a result of poorer synthetic accessibility and a biologically more active profile encountered when assessed for neutrophil function modifications *in vitro* (section 2.3.5). Specifically, increasing inhibition of normal neutrophil functions such as chemotaxis, transmigration and MPO release was observed as the concentration of Quin C7 present was increased.

Nevertheless, in order to allow comparison of arising spectroscopic properties, **16** was reacted with Quin C7 (**8**, Figure 3.11) under basic conditions to form compound **18**, the antagonist ligand-based counterpart to compound **17**. The unprotected Quin C7 scaffold contains two potentially nucleophilic sites that could attack the alkyl chloride group, the hydroxyl group and the aromatic amine, which necessitated exact assessment of the resulting structure to establish the site of functionalisation. As the phenolic group has been shown to be essential in modulating the compounds' bioactivity, attachment via this group would rule out the usefulness of the compound as an FPR-binding motif.

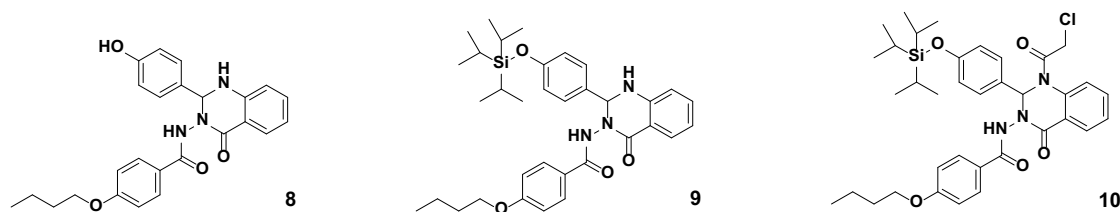


Figure 3.11: Structures of Quin C7 (**8**), Quin C7-TIPS (**9**) and Quin C7-TIPS acetyl chloride (**10**) from Chapter 2.

Once prepared, **18** was isolated by column chromatography and purity was confirmed by analytical LCMS. By HRMS a peak corresponding to an M^+ ion was observed at 982.4862 a.m.u. which is indicative of both of the possible attachment modes (Figure 3.12) that do not differ in mass.

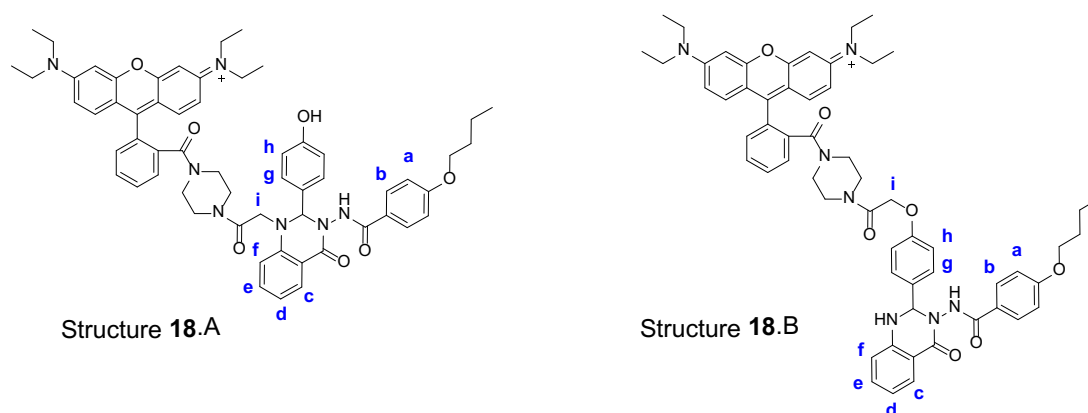


Figure 3.12: Molecular structures of compounds potentially formed via the route described to produce **18**.

Evaluation by ^1H -NMR NOESY spectroscopy revealed a cross peak between the bridging $-\text{CH}_2-$ group (H_i , Figure 3.12) and H_h , thus providing strong evidence for formation of structure **18.B**. Consequently, the utility of **18** for imaging purposes was ruled out and the compound was not carried forward for *in vitro* testing.

To synthetically circumvent these issues, a TIPS-protected Quin C7 (**9**, Figure 3.11) analogue was prepared (see section 2.3) and functionalised using chloroacetyl chloride to yield **10** (Figure 3.11), which should enable appendage via the amine functionality. Unfortunately, time constraints did not permit repeated preparation of **18**.

3.3.4 Photophysical properties

Absorption and steady state fluorescence spectra were recorded in aerated methanol or methanol/water (1:1) solutions for all three compounds. The fluorescence excitation and emission spectra for the three quinazolinone-rhodamine derivatives (**13**, **17** and **18.B**) are summarised in Table 3.2.

	Emission in nm (λ_{exc})	Excitation in nm (λ_{em})	Absorption in nm
13	457, pH-dependent peak at 581 (350)	347 (450) neutral pH	269, 313 neutral pH pH-dependent peak at 562
17	403, 428, 453, 582 (350)	315, 355, 406 (weak), 561 (580)	259 (weak), 308 (weak), 352, 560
18.B	390, 459, 581 (350)	308, 355, 405, 563 (580)	254, 308, 360 (weak), 561

Table 3.2: Summary of emission, excitation and absorption spectra of compounds **13**, **17** and **18.B** in 1:1 methanol/water mixtures.

A pH-dependent rhodamine-based emission and absorption profile was observed for compound **13** in methanol/water mixtures (1:1). No rhodamine-centred emission was visible at 581 nm (using 350 nm excitation) at pH values above about 5 and increasing fluorescence was observed as the pH value of the solution was decreased (Figure 3.14). This effect was attributed to the amount of ring-opened species present in solution, with low pH values skewing the spirolactam equilibrium towards the open, fluorescent state, while the spirocycle prevailed at high pH values. The emission band observed at higher energy (457 nm) for this compound showed no notable pH dependence except for a sudden intensity drop at pH values of 2.7 and 2.2, which could be indicative of protonation or decomposition. Similarly, absorption of **13** in 33 μM solutions in MeOH/water (1:1) was recorded at varying pH values between pH 2.8 and pH 8.8 and the band at 562 nm showed the same pH-responsive trend that was evidenced within the emission spectra (Figure 3.14). The spirocyclisation equilibrium properties are

essential to imaging applications and control whether the probe is fluorescent under the desired conditions. In order to determine the intramolecular spirocyclisation equilibrium constant, pH values between 4.98 and 2.80 were selected and absorption intensity values at 560 nm for the corresponding pH values were noted (higher pH values resulted in no observed absorption at 560 nm). The intensity values obtained by this method were normalised and plotted against pH value, as described by Urano and co-workers.⁴³ The obtained data was fitted to a curve with a sigmoidal shape using the in-built OriginPro 2017 curve-fitting function and the pH value at half the maximum absorbance was determined as $pK_{cycl} = 3.97$ (Figure 3.13).

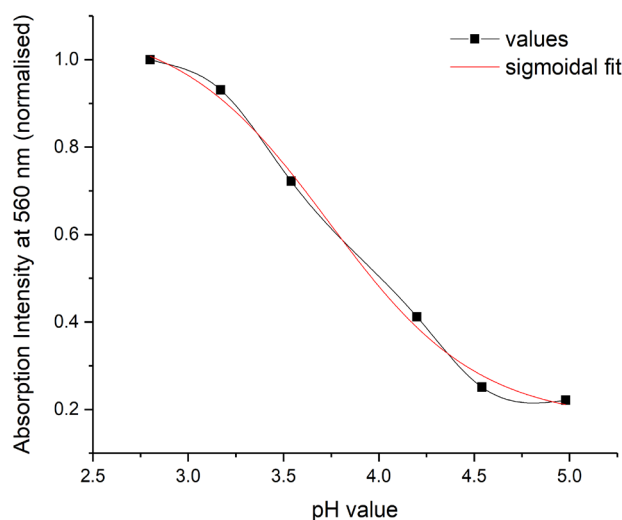


Figure 3.13: Plot displaying pH value versus normalised absorption at 560 nm of compound **13** with a sigmoidal curve fit that enabled calculation of the pH at half the maximum absorption value (pK_{cycl}).

Typically, compounds with higher pK_{cycl} values are cited as desirable for cellular imaging probes in order to ascertain the compound remains in its fluorescent and thus observable state under physiological conditions. However, in this context, the fact that the compound is expected to be predominantly non-fluorescent under cellular homeostatic conditions provides an advantage. The probe is expected to become visible only under pathological conditions, where a strongly acidic environment is observed, whereas the compound remains “dark” in physiologically normal pH states. As a result, compound **13** is envisaged to enable sensitive and highly selective detection of diseased cellular states via an “on” response.

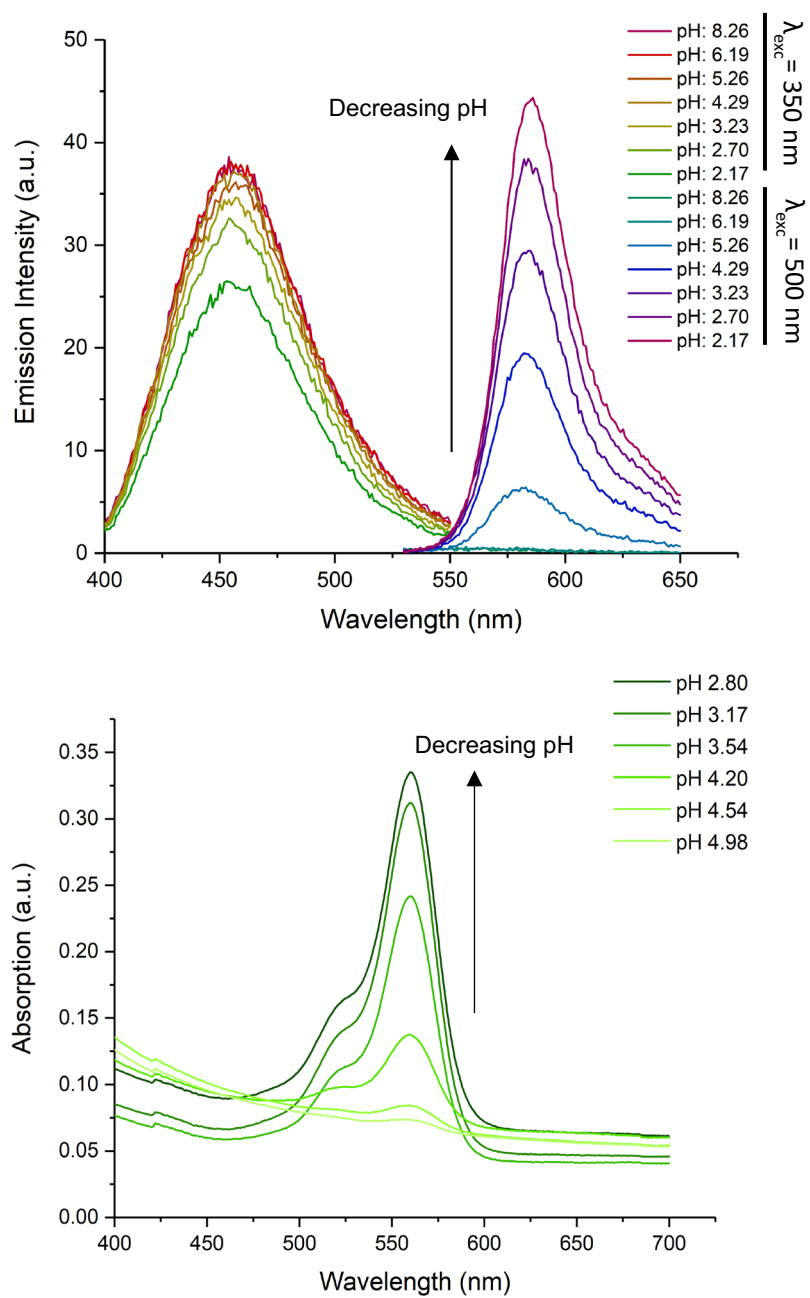


Figure 3.14: pH-dependent emission spectra of **13** (above, λ_{exc} indicated in legend) and relevant section of the absorption spectra of **13** (below).

3.3.5 Assessment of binding to Formyl Peptide Receptors

After successful preparation and spectroscopic characterisation of the compounds, the next aim was to establish whether the compounds bind to neutrophils, allowing the cells to be imaged. For this purpose, human neutrophils were isolated, seeded on poly-L-lysine sheets, incubated (one hour at 37 °C, 5 % CO₂) with **17** and then washed to remove any residual compound. Labelled neutrophils were fixed with 10 % formalin before co-staining with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using an inverted fluorescence microscope set up in the z-stack mode. As is depicted in Figure 3.15, neutrophil nuclei were stained with DAPI and their characteristic multi-lobed (two to five) structures are apparent.⁴⁴ Compound **17** appears to have been taken up by neutrophils along the cellular periphery, which could indicate binding to the membrane.

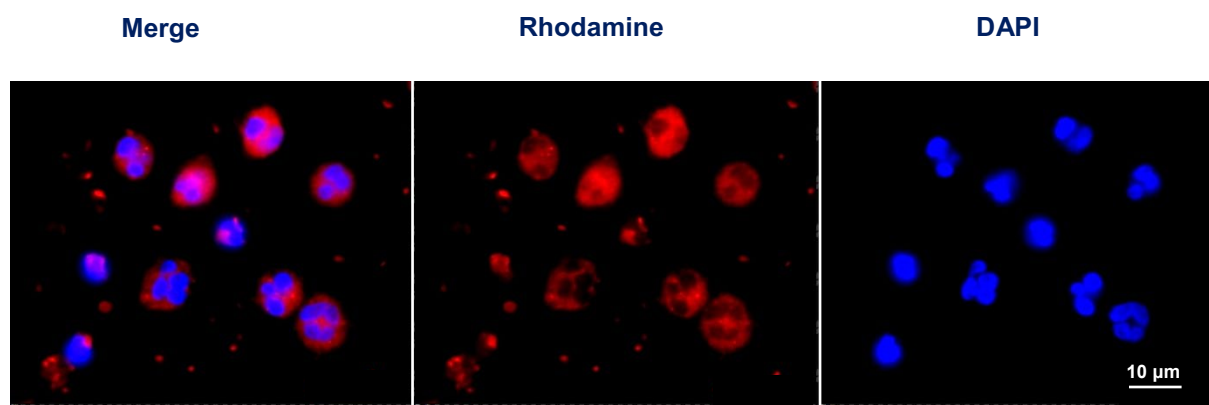


Figure 3.15: Representative image of fixed human neutrophils that had been treated with **17** and the nuclear stain DAPI, demonstrating binding of the compound to the cell periphery.

To confirm that **17** is binding via the FPR family, a pharmacological blocking study was carried out using isolated neutrophils (isolation protocols carried out by Dr Junaid Ansari and Miss Gaganpreet Kaur at LSUHSC Shreveport) from three different healthy donors ($n = 3$), who gave prior informed consent. Neutrophils suspended in Dulbecco's Modified Eagle Medium (DMEM) were pre-treated with vehicle (phosphate buffered saline, PBS) or FPR antagonists (to block the receptors), followed by incubation with **17** (10^{-6} M). After washing steps, the cells were fixed, treated with the nuclear stain DAPI and imaged under a fluorescence microscope. The images obtained were quantified by comparing emission intensity ratios between **17** and DAPI (recorded at $\lambda_{\text{exc}} = 595\text{nm}$ / $\lambda_{\text{em}} = 645\text{ nm}$ and $\lambda_{\text{exc}} = 356\text{nm}$ / $\lambda_{\text{em}} = 460\text{ nm}$ respectively) in neutrophils within each field of view. Several images per donor were quantified and averaged to obtain the final values.

To elucidate whether neutrophil binding occurs via the FPRs, prior blocking of both surface receptors (FPR1 and FPR2/ALX) using the known pan-antagonist Boc2 was carried out.^{45,5} No observable change in intensity of the DAPI stain was detected compared to the vehicle group, whereas a statistically significant fluorescence intensity decrease ($p < 0.05$) was evident at excitation and emission bands

relevant to compound **17** when the cells had been pre-incubated with Boc2. These results imply that binding of **17** is indeed occurring via the FPRs (Figure 3.16 and Figure 3.17).

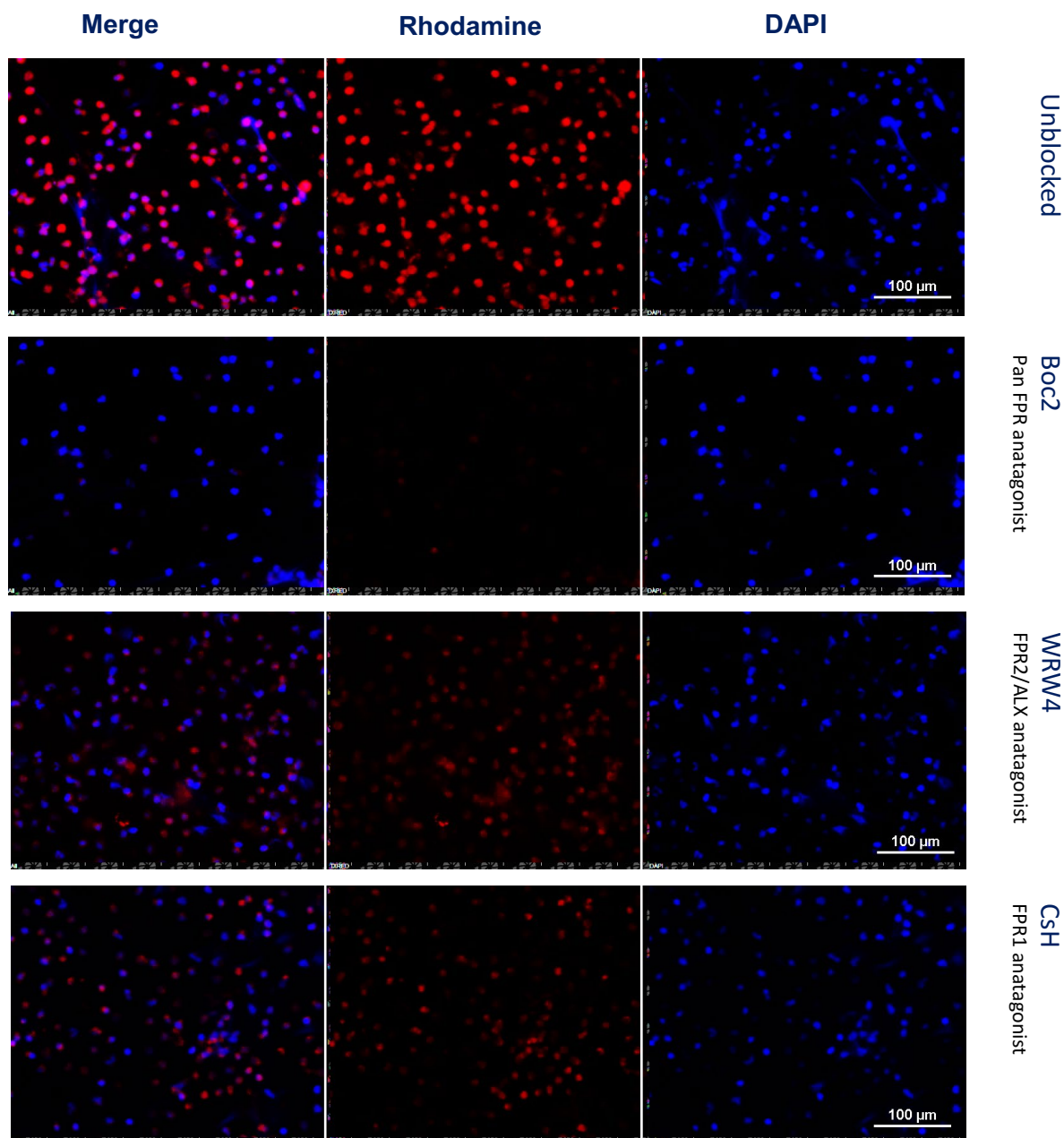


Figure 3.16: Representative fluorescent images of neutrophils pre-treated with vehicle (top row) or the respective antagonist, Boc2 (second row), WRW4 (third row) or CsH (bottom row) and incubated with compound **17** and the nuclear stain DAPI. A clear decrease in emission intensity is observed in the blocked groups ($n = 3$ independent experiments).

To assess whether binding specifically occurs via FPR2/ALX, as is reported for the Quin C1 ligand, FPR1 and FPR2/ALX specific antagonists were used. When blocking was carried out using the known

FPR1-selective antagonist Cyclosporin-H (CsH), which should not block FPR2/ALX sites, or the FPR2/ALX-antagonist WRW4 (which should block FPR2/ALX sites), no statistically significant differences compared to the unblocked control were found. Nevertheless, a trend showing a decrease in emission intensity ratios with both specific blocks was consistently observed (Figure 3.16 and 3.17).

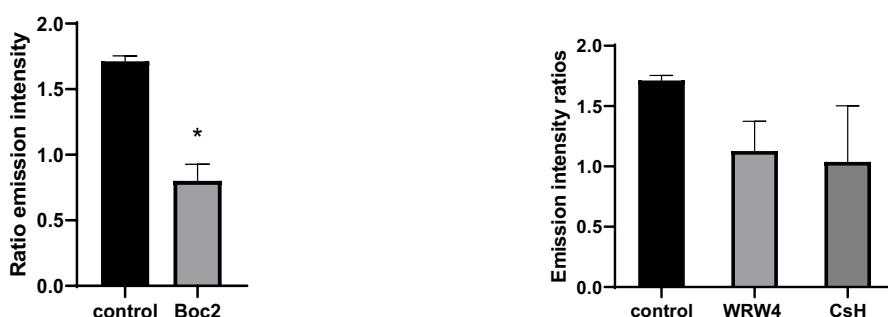


Figure 3.17: Quantification of images obtained of neutrophils treated with vehicle, Boc2 (left), WRW4 (right) or CsH (right), indicating a significant intensity difference in Boc2 blocked groups and showing a trend observed using both WRW4 and CsH. The data is expressed as $\pm$ SEM from $n = 3$ independent donors. * indicates $p < 0.05$.

Taken together, the data is suggestive of possible binding to both receptors, although blocking with WRW4 or CsH groups was not sufficient to gain statistical significance at $n = 3$. An interesting point for further investigation would be to increase n numbers and/or assess the binding of compound **17** to cells stably transfected with just FPR1 or just FPR2/ALX (such as human embryonic 293 kidney cells).⁴⁶ At present, these results illustrate that selectivity for FPR1 or FPR2/ALX could not be reliably determined yet, although repeat experiments increasing n numbers are planned in the future. The fact that binding of the compound occurs via the FPR family however was confirmed, as blocking using Boc2 showed a significant reduction in binding compared to the control.

3.3.6 Testing of the pH-sensitive probe in a cellular context

Encouraged by the promising results obtained in the binding study, the ability of the pH-switchable compound **13** to flag up acidic environments in a cellular context was investigated. Inflammation has long been known to be associated with a locally lowered pH range, ascribed to an increased metabolic acid production.^{24,47} In addition, changes to extracellular pH environments are known to modulate neutrophil activity.⁴⁸ An acidified environment was shown to result in delayed neutrophil apoptosis, changes to cellular chemotactic properties and inhibition of neutrophil respiratory bursts.⁴⁹ These studies demonstrate the key role that pH can play in determining the host immune response.

To look at the changes in local pH, a number of fluorescent dyes have been reported, many of which accumulate unselectively both on and off target, causing low signal to noise ratios in addition to losing the ability to differentiate causes of local pH changes.^{50,51} For example, a nano-platform that had been surface functionalised with both a pH-insensitive and a pH-sensitive dye (thereby enabling a ratiometric approach to fluorescence quantification to be used) was applied to *in vivo* monitoring of pH changes in

tissue acidosis during the inflammatory response.⁵² The probe, however, was not designed to attach to a cellular receptor and accumulated unspecifically. Reports that have focussed on targeted imaging of disease states such as cancerous tissue using activatable probes are rarer within the literature. One example of these is a pH-activatable BODIPY-derived fluorophore that was combined with the cancer-targeting unit trastuzumab and was visualised selectively upon internalisation in acidic lysosomes, as the process occurred in viable cancer cells.⁵³

Using compound **13**, we aim to create a probe that can selectively bind to the FPR family on neutrophils and remains predominantly in a “dark” spirocyclic state under normal physiological conditions ($\text{pH} > 5.5$). These properties are compatible with characteristics previously established by spectroscopic methods ($\text{pK}_{\text{cycl}} = 3.97$). Once the cell becomes activated and enters a more acidic environment, the rhodamine spirolactam equilibrium is shifted towards the ring-opened form and the ensuing “on” response can be detected. This strategy could, in principle, enable cells (such as neutrophils) to be tracked as they become activated.

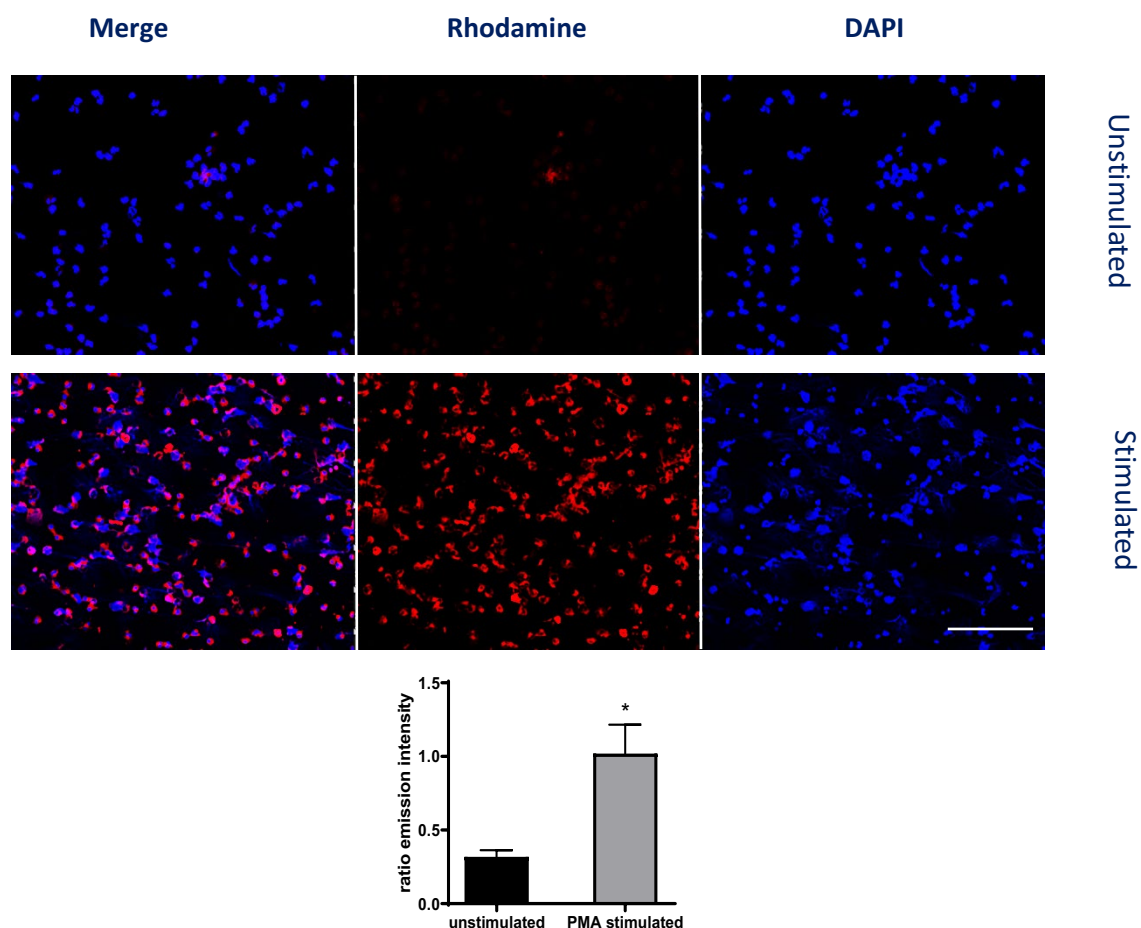


Figure 3.18: Representative fluorescent images of neutrophils pre-treated with vehicle (top row) or PMA (bottom row) to activate the cells ($n = 3$ independent experiments). Above, almost no rhodamine emission is observed (i.e. the compound is in its spirocyclic, “dark” state), whereas in the images below, it is apparent that the compound is now “on” and fluorescent. Statistical quantification of these results (inset, below) was carried out by comparison of DAPI and probe emission intensity ratios between the two groups as described in section. Scale bar is 100 μm .

To investigate whether this response can be observed in a cellular context, neutrophils were incubated with **13** and DAPI, before visualisation by fluorescence microscopy. In an unactivated state, the DAPI stain is clearly visible, whereas almost no rhodamine signal is detected (Figure 3.18). This is consistent with FPR uptake of the compound in its spirocyclic, non-fluorescent state, as the immediate membrane-proximal environment is likely to be above pH 5 to 6. Next, to simulate an inflammatory environment, neutrophils were activated with phorbol 12-myristate 13-acetate (PMA) prior to incubation with compound **13**. This group showed a significant ($p < 0.05$) increase in rhodamine-based fluorescence emission intensity, which was visualised as an “on” response (Figure 3.18). These data show that activation results in a sufficiently acidic immediate environment to induce a structural change within the rhodamine unit and skew the spirocyclisation equilibrium towards the “ring-opened”, fluorescent form of compound **13**.

3.3.7 *In vitro* validation using neutrophil functional assays

FPR2/ALX is known to produce both potent pro- and anti-inflammatory signalling cascades upon ligand-specific activation.^{54,55,56} In order for the compounds to provide a readout of inflammatory status it is imperative that binding to the receptor unit does not elicit either a pro- or anti-inflammatory response. To assess the compounds' effect on human neutrophil function and hence their suitability for imaging, three distinct functional assays were tested, which have previously been described in section 2.3.5.

The first assay, neutrophil chemotaxis, assesses the ability of the cells to migrate in response to a chemical stimulus. Neutrophils were pre-treated with varying concentrations of **13** or **17** (10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or vehicle (saline) and added to the top of a porous membrane on a 96-well plate set-up. In the bottom of the well chamber PBS control or the chemoattractant leukotriene B₄ (LTB₄) were added. The plates were incubated for three hours at 37 °C with 5 % CO₂. Analysis of the obtained data revealed that a statistically significant difference was observed between PBS control and exposure to LTB₄, while the presence of varying concentrations (10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) of both compound **13** or **17** did not have a significant impact on the ability of neutrophils to migrate through the membrane towards the chemoattractant (Figure 3.19).

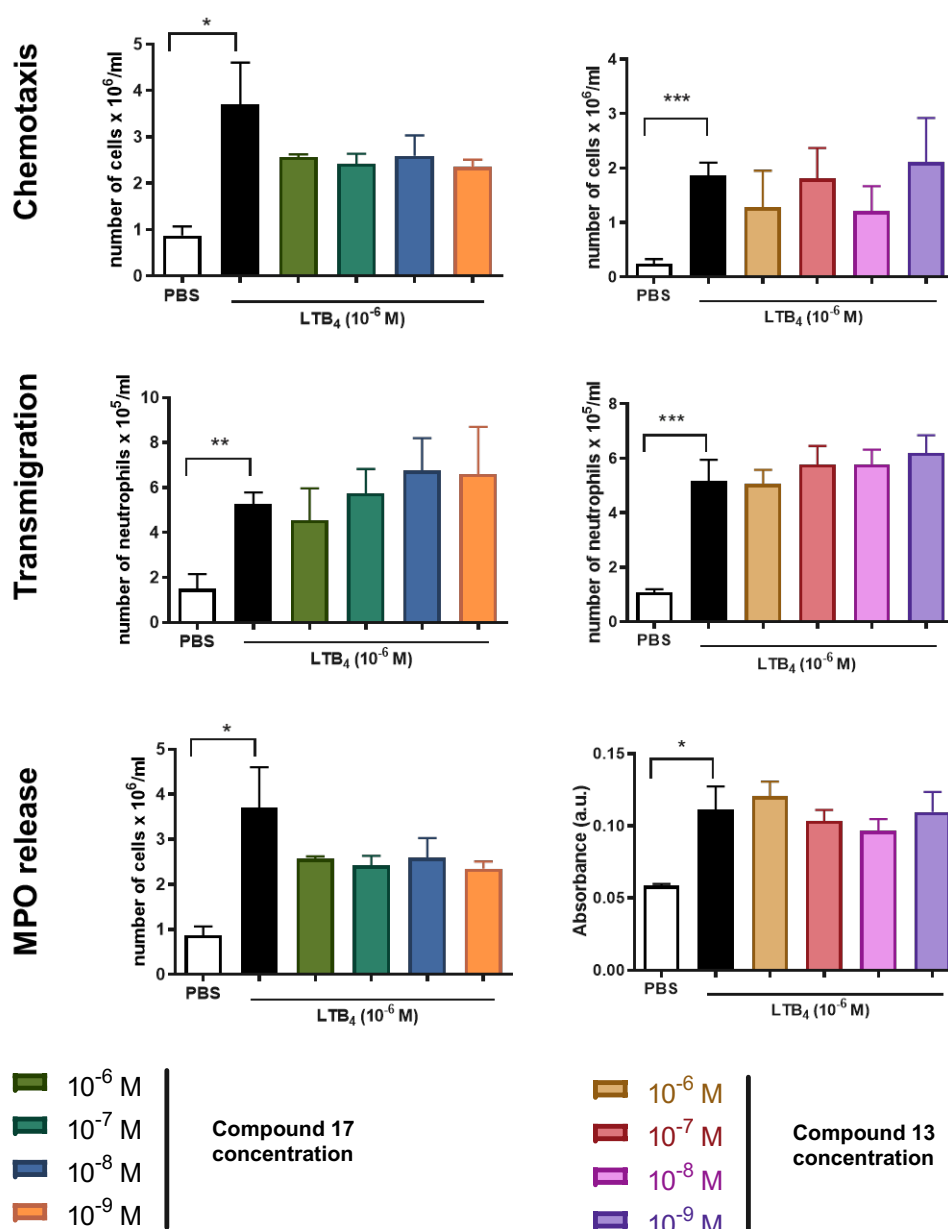


Figure 3.19: Assay data shows chemotaxis (above), transmigration (middle) and stimulated MPO release (below) in human neutrophils at varying molar concentrations of **17** (left panel), **13** (right panel) as indicated, with a constant LTB₄ concentration of 10^{-6} M. Data are mean $\pm$ SEM from cells prepared from 4 independent donors. Vehicle control is run with no compound and no LTB₄ present. * denotes $p < 0.05$ vs. corresponding vehicle group and no statistically significant difference was found within the LTB₄ groups (ANOVA and Bonferroni). Each experiment was run in duplicate or triplicate.

Similarly, the ability of neutrophils to migrate in response to a chemical stimulus (LTB₄) through an endothelial cell layer of HUVECs was tested in the transmigration assay. This assay is designed to simulate extravasation of cells out of the vessel as could occur in an inflammatory context. Again, the response was tested in cells pre-incubated with varying concentrations of **13** or **17** (10^{-9} M to 10^{-6} M) or

vehicle and results showed that at the concentration ranges assessed, the compound had no significant impact on neutrophil motility compared to the control (Figure 3.19).

Finally, neutrophil activation was measured in a myeloperoxidase (MPO) release assay and quantified spectrophotometrically relative to a TMB standard. Again, data from four independent donors showed no significant impact of the presence of the compounds at relevant concentrations on neutrophil activation (Figure 3.19).

Taken together, *in vitro* viability testing established that the compounds do not modify neutrophil functional behaviour within the parameters validated and it confirmed their potential use for the imaging of neutrophils.

3.3.8 *In vivo* model of acute inflammation

The ability of **17** to act as an imaging probe for inflammation was tested in a model of acute inflammation. *In vivo* experiments were conducted by Dr Elena Senchenkova at LSUHSC Shreveport and the set-up is schematically summarised below (Figure 3.20).

Male C57bl/6 mice were treated intrascrotally with saline vehicle or tumour necrosis factor- α (TNF α , 250 ng in 0.2 mL per mouse)⁵⁷ for 3 hours (n = 6 per group) or with TNF α and treated with BMS-470539 (to antagonize TNF α -induced neutrophil accumulation).⁵⁸ Two hours post TNF α injection, **17** was injected (15 μ g in saline 0.1 mL per mouse) and the mice were left for a further hour before treatment with a FITC-Gr-1 antibody which acts as a neutrophil-tag (administered 10 minutes prior to imaging). The cremaster muscle was visualised using intravital confocal microscopy.⁵⁹

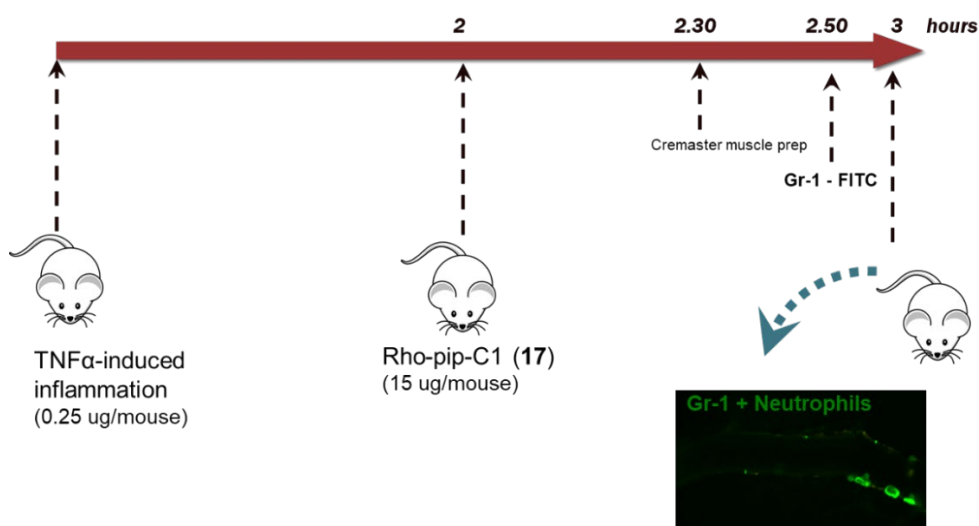


Figure 3.20: Schematic representation of experimental set-up for *in vivo* imaging carried out by Dr Elena Senchenkova

Cells were imaged and quantified in vessels of a 20 - 50 μm diameter. During the inflammatory response leukocytes are pushed towards the vessel endothelium and undergo rolling, adhesion and finally emigration outside the vessel to reach the site of tissue injury.⁶⁰ Labelled adherent, extravasated and rolling neutrophils were observed. Rolling neutrophils could not be accurately quantified in the frames taken as a result of the “rolling speed” and were consequently omitted from analysis. Other neutrophils within the vessel and extravasated cells were quantified as Gr-1 positive (to identify neutrophils) or Gr-1 and compound **17** positive cells (to identify neutrophils that had become labelled with **17**) for all groups.

The results obtained showed that within the TNF α treated acute inflammation group there is no statistically significant difference between neutrophils that have taken up Gr-1-Fluorescein isothiocyanate (FITC; Gr-1 positive) and neutrophils that are labelled with both Gr-1-FITC and compound **17** (Gr-1/**17** positive), neither for cells within the vessel, nor for cells that had extravasated out of the vessel (Figure 3.21). Additionally, within this group, the number of neutrophils that were positive for **17** amongst Gr-1 positive cells were expressed as percentages. We found that a remarkably high number of over 80 % of the Gr-1 positive population were tagged with **17** (81.68 ± 2.92 %, Figure 3.22), which is substantially higher than the 15 % of labelled neutrophils thus far reported using a peptide-based, radioisotope FPR1 probe.⁶¹

Within the saline control group, overall less neutrophils were present within the vessels. For the neutrophils that were observed, a statistically significant difference ($p < 0.05$ %) was evident between the cells within the vessel that had taken up Gr-1 (Gr-1 positive) and cells that had also taken up compound **17** (Gr-1/**17** positive). Here, only about 15 % of neutrophils had taken up the compound (15.17 ± 5.04 %, Figure 3.22). The same difference was not, however, observed in the extravasated cell group, where most of the Gr-1 population was also positive for **17**.

The results indicate firstly that compound **17** is able to tag neutrophils in an *in vivo* context and thus can be used as an imaging agent for the FPR family to track neutrophils. Secondly, the study further demonstrated that **17** is preferentially binding to neutrophils in a model of acute inflammation. One interpretation of these results is that efficient uptake of the compound is associated with an activated state of the receptor, with less binding occurring under normal physiological conditions (saline control group). This implies that compound **17** differs from a typical neutrophil tag which would unselectively label neutrophils (which are typically more abundant in inflammation and thus could still enable visualisation of inflammation) by preferentially binding to cells that have been activated.

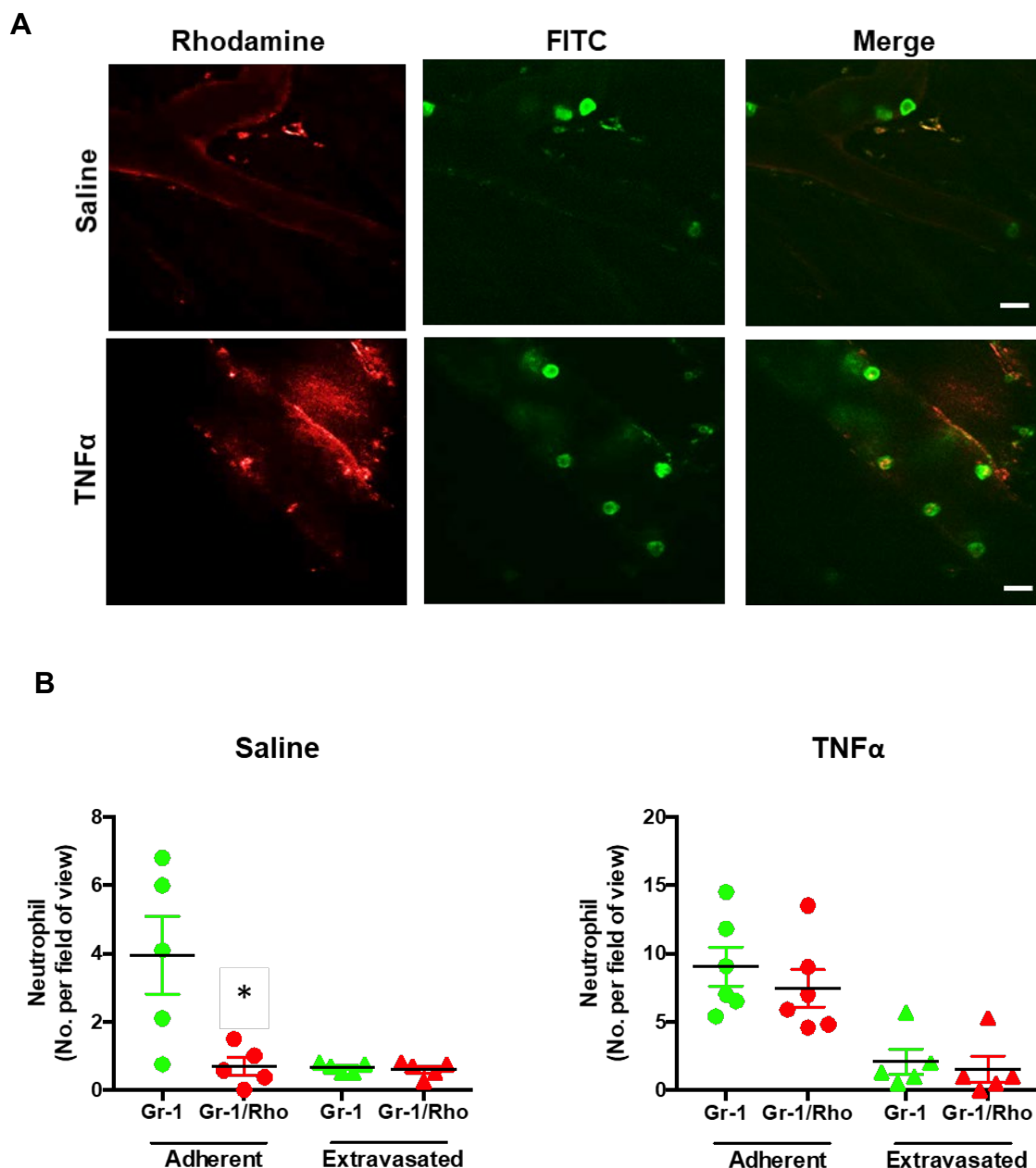


Figure 3.21: (A) Representative images of neutrophils in selected vessel of saline treated mice (above) and TNF α treated groups (below). Scale bar represents 10 μ m. (B) Analysis shows number of neutrophils within selected vessels (“adherent”) or number of neutrophils emigrated outside of the vessel (“extravasated”) in saline treated groups (left) and TNF α treated groups (right). Neutrophils were identified by their Gr-1 label and quantified as cells positive for Gr-1 versus cells positive for both Gr-1 and **17**. * Indicates $p < 0.05$ versus control. Data are overall average $\pm$ SEM of $n = 6$ independent experiments within each group. No significant difference was observed within extravasated groups, but a significant difference was observed for neutrophils within the vessels in the saline group. This indicates preferential uptake of **17** by neutrophils in TNF α -induced inflammation.

Furthermore, neutrophil emigration outside of the vessel is associated with receptor activation^{62,63} and, although fewer extravasated cells were present than within the vessel, we observed that within both the saline and the TNF α -stimulated groups there was no significant difference between extravasated neutrophils positive for Gr-1 and those positive for Gr-1/17. These results suggest that **17** is preferentially taken up when the receptor is in an activated state, independent of whether neutrophils were found in a normal physiological context or an acute inflammatory model.

Mice were also treated with an anti-inflammatory drug (BMS-470539, a melanocortin MC1 receptor agonist)²⁴ that acts to reduce TNF α -induced inflammation. Within this group, it was observed that uptake of the compound is comparable to that found within the saline control group (Figure 3.22). This implies that the agent can be used to directly monitor the therapeutic effects of an anti-inflammatory drug on the region of interest.

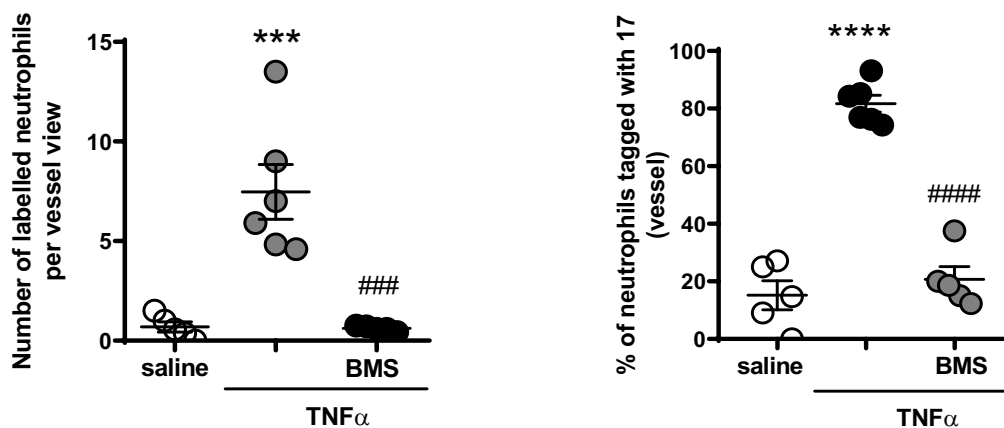


Figure 3.22: Quantification of the number of neutrophils within selected vessels in saline, TNF α -induced acute inflammation and TNF α and BMS-470539 treated groups (left) that have taken up compound **17** (Gr-1/17 positive population) and data expressed as percentage of Gr-1 cells (right). *** Indicates $p < 0.001$, **** denotes $p < 0.0001$ vs WT control, ### indicates $p < 0.001$ and ##### denotes $p < 0.0001$ vs TNF α .

3.4 Conclusions

Three related FPR probes based on a rhodamine B scaffold, **13**, **17** and **18** were prepared and characterised. Their photophysical behaviour was assessed and it was found that out of the compounds made, **17** and **18** are pH-insensitive (with the rhodamine spirolactam synthetically locked into its open, fluorescent form) and one of them (**13**) becomes fluorescent within the rhodamine-emission range upon exposure to acidic media. pK_{cycl} for the ring-formation equilibrium was determined and was found to be within the relevant range for cellular applications as a pH sensor.

Investigation into the compounds' properties in cells, enabled confirmation of binding to the FPRs by pharmacological techniques, although specificity for FPR2/ALX could not conclusively be determined. In fact, results indicate that binding is likely to occur via both FPR1 and FPR2/ALX, although this point requires further investigation. Additionally, in neutrophil functional assays **13** and **17** did not exert an effect on the neutrophils at biologically relevant concentrations. Promisingly, the pH-activatable compound was found to be “dark” in unactivated neutrophils and, upon prior activation a clear “turn on” response of the rhodamine unit was observed, indicating higher levels of acidity within stimulated neutrophils. This “switchable” behaviour could be exploited for applications in interrogating the role of activated FPRs in inflammation.

Encouraged by these results the conducted *in vivo* study on **17** established that the probe preferentially binds to adherent neutrophils in a model of acute inflammation, whereas less uptake of the compound is observed in saline control groups and mice treated with an anti-inflammatory compound. Interestingly, neutrophils that had extravasated outside of the vessels in both control and acute inflammation groups displayed similar levels of labelling with **17**, which indicates the extravasation process occurs via activation of the FPRs. Results are summarised schematically in Figure 3.23.

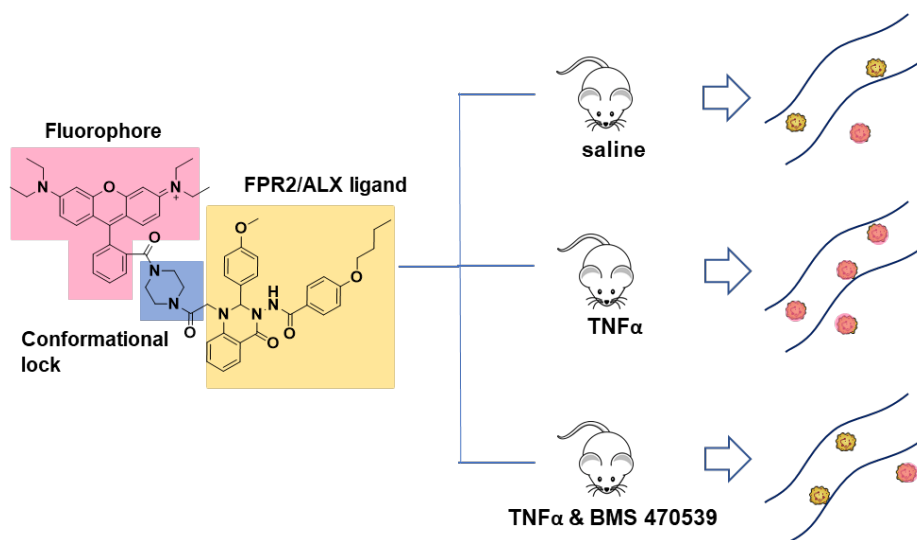


Figure 3.23: Schematic representation of design principles for compound **17** (left) and summary of *in vivo* results. Schematic depicts unlabelled (yellow) and neutrophils that have taken up **17** (in pink) within the vessel in the inflamed model, whereas extravasated neutrophils are labelled under all conditions.

Overall, the results from this chapter demonstrate that the quinazolinone scaffold provides a useful targeting vector that can be combined with fluorescent groups to mechanistically study the role of FPRs in both *in vitro* and *in vivo* settings.

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Chapter 4

Lanthanide luminescence for visualising FPR2/ALX using time-resolved microscopy

4.1 Introduction

As discussed in section 1.4.1, certain stable Ln(III) chelates have highly desirable optical properties for cellular imaging agents: their sharp, distinct peaks are almost unperturbed by ligand environment, show good resistance to photobleaching, and their luminescence profiles are characterised by large Stokes' shifts and long lifetimes.¹ As such, their emission can be well separated from cellular background signals (associated with autofluorescence from π -conjugated systems) using emission filters and time-gating techniques and they have found numerous applications in cell imaging.²

4.1.1 Terbium(III) and Europium(III) chelate luminescence

Amongst the luminescent lanthanide ions, chelates based on Tb(III) and Eu(III) are by far the most commonly utilised for cell imaging purposes. Emission wavelengths are within the visible to NIR region and Tb(III) and Eu(III) possess the largest energy gap between ground and excited state amongst the lanthanides and correspondingly highest emission intensities (Energy gap law).^{3,4}

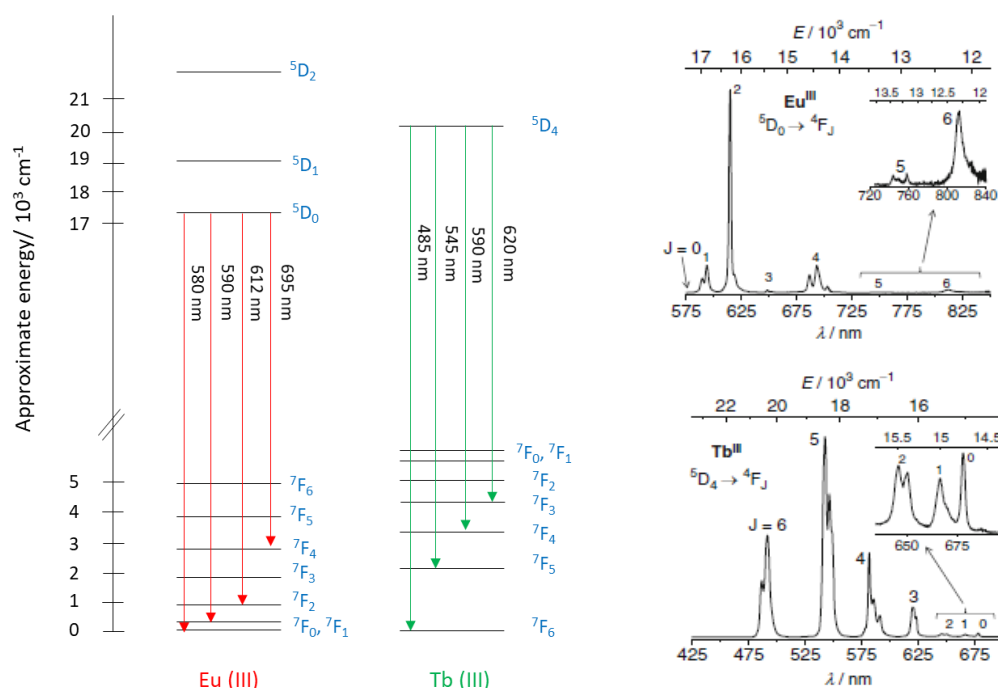


Figure 4.1: Partial approximate energy level diagram of Eu(III) and Tb(III), illustrating the emissive transitions (left) and typical emission spectra for the two lanthanide chelates, shown here for Cs₃[Ln(dpa)₃] with Ln = Eu or Tb (right). Spectra reproduced with permission from reference.⁵

Emissive terbium transitions arising from the ⁵D₄ → ⁷F_J states (J = 6, 5, 4 and 3) are observed within the green region of the visible spectrum. Amongst these, the ⁵D₄ → ⁷F₅ transition results in the most intense band while the ⁵D₄ → ⁷F_J (J = 2, 1, 0) level transitions are extremely weakly emissive, occur at longer

wavelengths and rarely observed.⁶ Under high resolution, the bands can show structures that are sensitive to the nature of the ligand environment. Eu(III) complexes emit light from a non-degenerate excited state level $^5D_0$. Transitions observed within the yellow and red region of the visible spectrum are assigned to $^5D_4 \rightarrow ^7F_J$ ($J = 0, 1, 2, 3$ and 4) transitions and further bands arising from the $J = 5$ and $J = 6$ transitions are present within the infrared region.⁷ Most notably, the $^5D_4 \rightarrow ^7F_2$ transition is considered to be a “hypersensitive transition” and its intensity is associated with the local symmetry about the Eu(III) ion. This band is more sensitive to the nature of the binding ligand and can vary in appearance depending on the complex.^{7,6} Exemplary Tb(III) and Eu(III) chelate emission spectra and associated energy level diagrams are shown in Figure 4.1.

4.1.2 Time-resolved microscopy

To selectively visualise long-lived luminescence by microscopy, a time-gated technique can be applied, where detectors switch on with a delay after the excitation pulse (Figure 4.2).^{8,9} Using this method, only signals that are sufficiently long-lived to persist after the delay time are observable and short-lived components are filtered out which can be accomplished using an intermittent light source and a gated/modulated microchannel plate intensifier with a charge-coupled device camera.^{10,11}

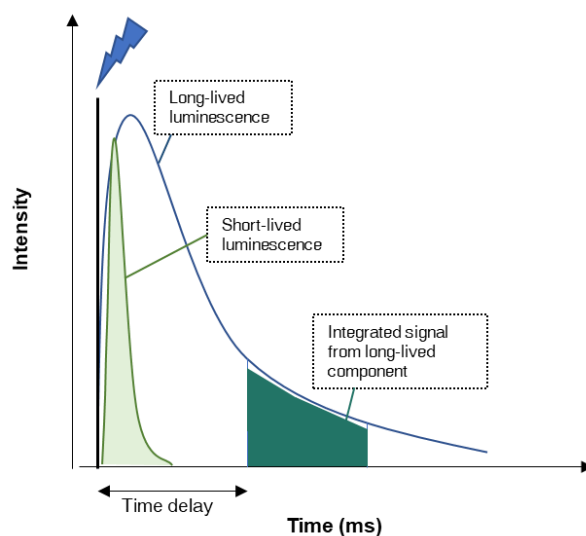


Figure 4.2: Schematic representation of the principle behind luminescence acquisition in time-gated microscopy.¹⁰

Another option for time-resolved microscopy is to monitor cellular processes via fluorescence or phosphorescence lifetime imaging (FLIM or PLIM respectively) rather than emission intensity-based detection.^{12,13} The lifetime of a given species is almost independent of concentration, can vary according to molecular environment and avoids loss of intensity through photobleaching.^{14,15} To acquire such images, time-correlated single photon counting (TCSPC) provides a read-out of the time between excitation of a sample by a pulsed laser (“start signal”) and the arrival of the emitted photon at a single photon sensitive detector (“stop signal”).¹⁶ The cycle is repeatedly acquired to account for the statistical

nature of photon emission, and the detected delays are sorted into a histogram of time after excitation pulse versus counts (Figure 4.3).¹⁷

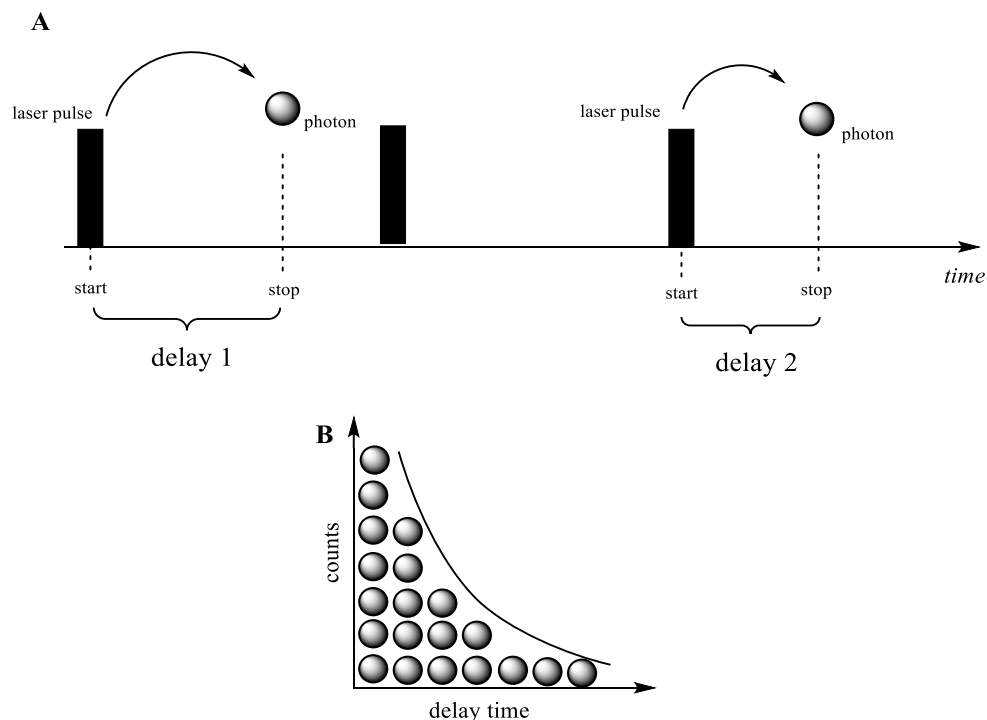


Figure 4.3: Schematic representation of the principle behind TCSPC, which repeatedly measures the time between excitation of the sample by a pulsed laser and detection of an emitted photon (A). The data are acquired as a histogram of counts versus delay time (B).

Time-resolved imaging methods provide a convenient tool to study lanthanide-based emission in the absence of short-lived, background signals. One of the major issues to overcome with this technique however, is being able to rapidly acquire high quality images despite the lower number of photons per unit time emitted by metal (lanthanide) probes in comparison to organic fluorophores.¹⁴ In recent years, a number of successful examples have been reported in live cell experiments and image signal to noise ratios were tuned to be acceptable even at lower probe concentrations.¹⁸ Furthermore, ongoing improvements of detection methods increasingly enable the advantages of lanthanide imaging technologies to be harnessed.

4.1.3 Use of lanthanide coordination compounds for cellular imaging

Initially used in biological assays as optical labels rather than for cell imaging studies (partly as a result of the UV excitation sources that are incompatible with live cells), lanthanide chelates are now commonly used in quantitative immunofluorescent bioassays as nucleotide labels, phosphate responsive tags and for monitoring enzyme activities.^{19,20,21,22} In the 1970s and 1980s, the first examples of Eu(III) and Tb(III) tags were reported to explore metalloprotein binding sites by replacing, for example, calcium with a

lanthanide to elucidate the number of bound water molecules, distances between metal ions and hence the coordination environments.^{23,24} Lanthanide ions coordinated to various ligand systems that tightly encapsulate the metal and can enable visualisation of signals in cells have since been developed. Ligand frameworks studied include, for example, helicates, cyclen-based macrocycles, cryptands, β -diketonates and metallacycles.

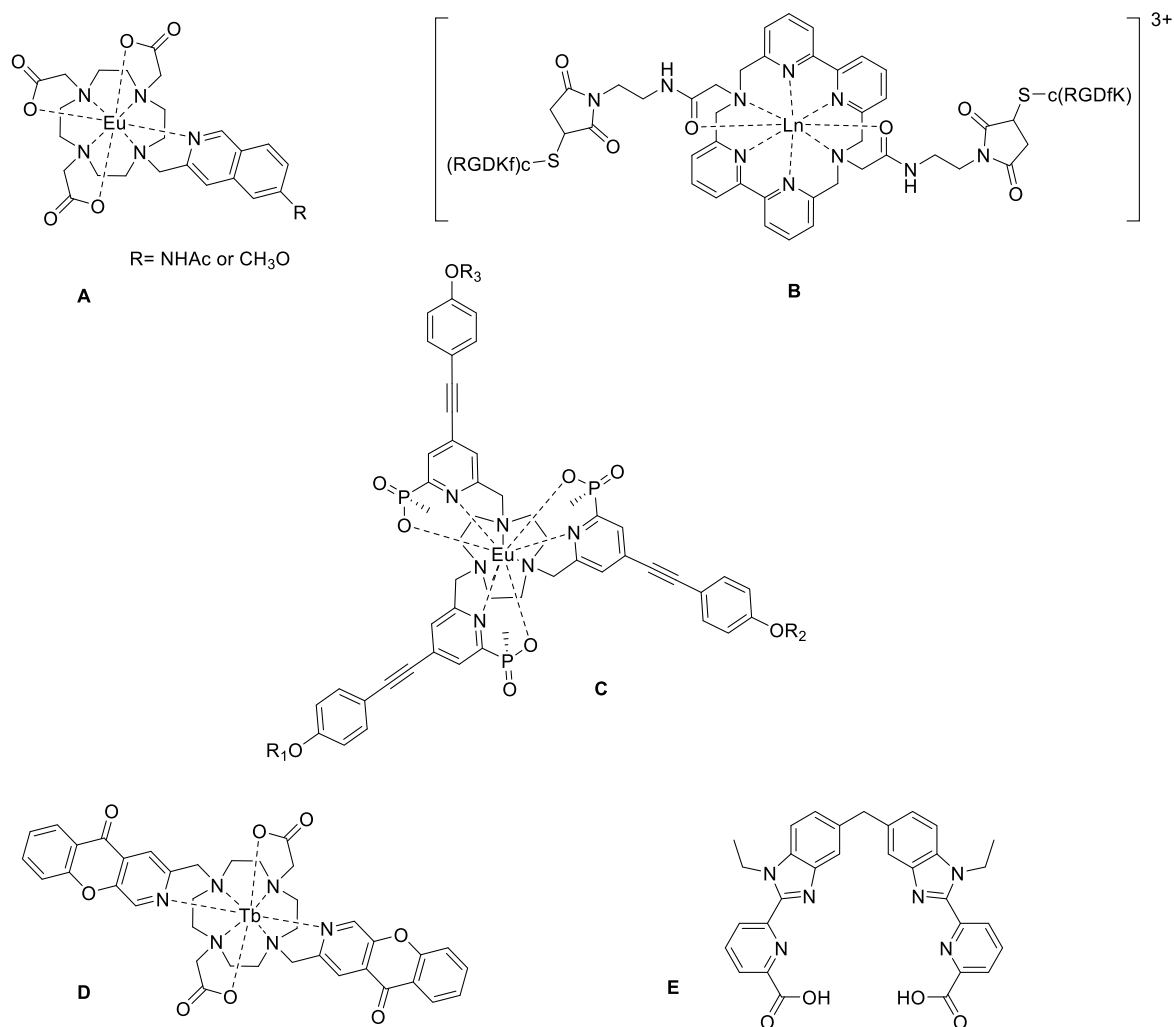


Figure 4.4: Molecular structures of lanthanide compounds and ligands used for cellular imaging.^{8,25,26,27,28,29}

Amongst these, Nagano and co-workers reported time-resolved, long-lived, luminescence imaging of Eu(III) and Tb(III) polyamino carboxylate chelates and demonstrated monitoring of intracellular Zn concentrations with a Eu(III) sensor system (structure shown in Figure 4.4, **A**).⁸ Bünzli and co-workers took a different approach in preparing Ln(III)-based optical probes by designing a helicate ligand structure capable of binding two different metals, each in a nonadentate coordination sphere. Using this design, they were able to limit the interaction with water molecules and the associated luminescence quenching. The structure was diversely derivatised (one example ligand structure is shown below, Figure 4.4, **E**)²⁶ and resulted in probes with low cytotoxicity and good cell permeability. By combining

helicate-based lanthanide complexes with bioconjugation vectors targeted to the ER and Her2/neu receptors (which are overexpressed in breast cancer), they were able to establish the potential role of Ln(III) bioprobes in cancer diagnosis.^{30,31,32,33,26} Similarly, a bipyridine-cryptand based lanthanide chelator was combined with two units of the $\alpha_v\beta_3$ -integrin moiety (c(RGDfK)) for cancer targeting and its Tb(III) and Eu(III) analogues were used to selectively visualise glioblastoma U87-MG tumour cells (Figure 4.4, **B**).²⁵ Construction of a DO2A unit with doubly substituted *trans* chromophore groups that are able to bind the lanthanide via a pyridine-derived nitrogen, afforded an octadentate ligand for Tb(III) ions. The metal complex (Figure 4.4, **D**) was assessed as a cellular probe in HeLa cells and localised within lysosomes or endosomes at high concentrations. Interestingly, at low concentrations, selective staining of chromosomal DNA of dividing cells was observed (Figure 4.5).²⁷

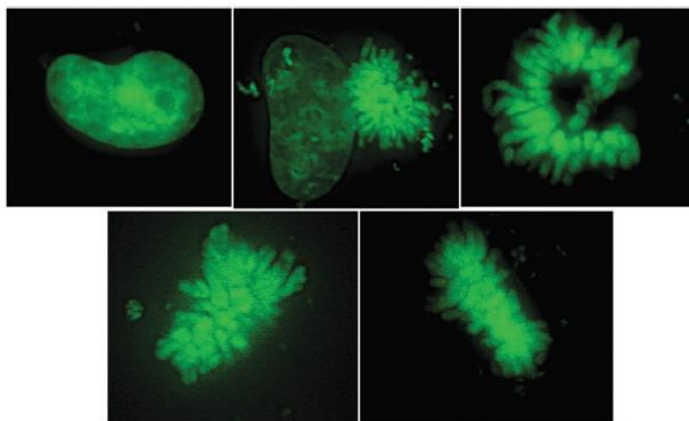


Figure 4.5: Fluorescence microscopy images ($\lambda_{exc} = 300\text{ nm}$) of cells stained with **D** in successive phases of cell division from interphase (top left), prophase, prometaphase to metaphase. Reproduced with permission from reference.²⁷

The luminescence derived from lanthanide complexes is characterised by notoriously inefficient excitation pathways and low photon fluxes, which limits their utility for microscopy. To address this issue, Parker and co-workers developed a nonadentate ligand system that, in combination with a complexed Eu(III) ion, could produce luminescence an order of magnitude brighter than values previously reported for equivalent Eu systems (Figure 4.4, **C**). The ligand design can be easily tuned to incorporate different functionalities and thereby change the compounds' cellular properties.^{28,29} Compounds derived from this structure were shown to be taken up into cells via micropinocytosis^{29,34} and localisation was observed in the mitochondria.³⁴

In an extension of purely enabling visualisation of cellular targets, lanthanide probes have been developed for sensor systems.³⁵ For example, Maury and co-workers recently reported the first molecular rotor viscosity probe based on a luminescent lanthanide complex that provides luminescence lifetime changes on a millisecond scale (Figure 4.6, **A**).³⁶ However, post-publication, Walter *et al* demonstrated that the terbium emission sensitivity observed in methanol/glycerol solutions was more likely to be a result of oxygenation levels and associated excited state quenching.^{37,38} Other examples such as probes to monitor lysosomal pH-based or cellular bicarbonate concentrations were developed

(Figure 4.6, **B** and **C**).^{39,40} Song *et al* investigated the use of Eu(III) terpyridine chelates in the context of time-gated singlet oxygen sensing and described luminescence increases within live cell images of HeLa cells that selectively occurred upon production of singlet oxygen species (Figure 4.6, **D**).⁴¹

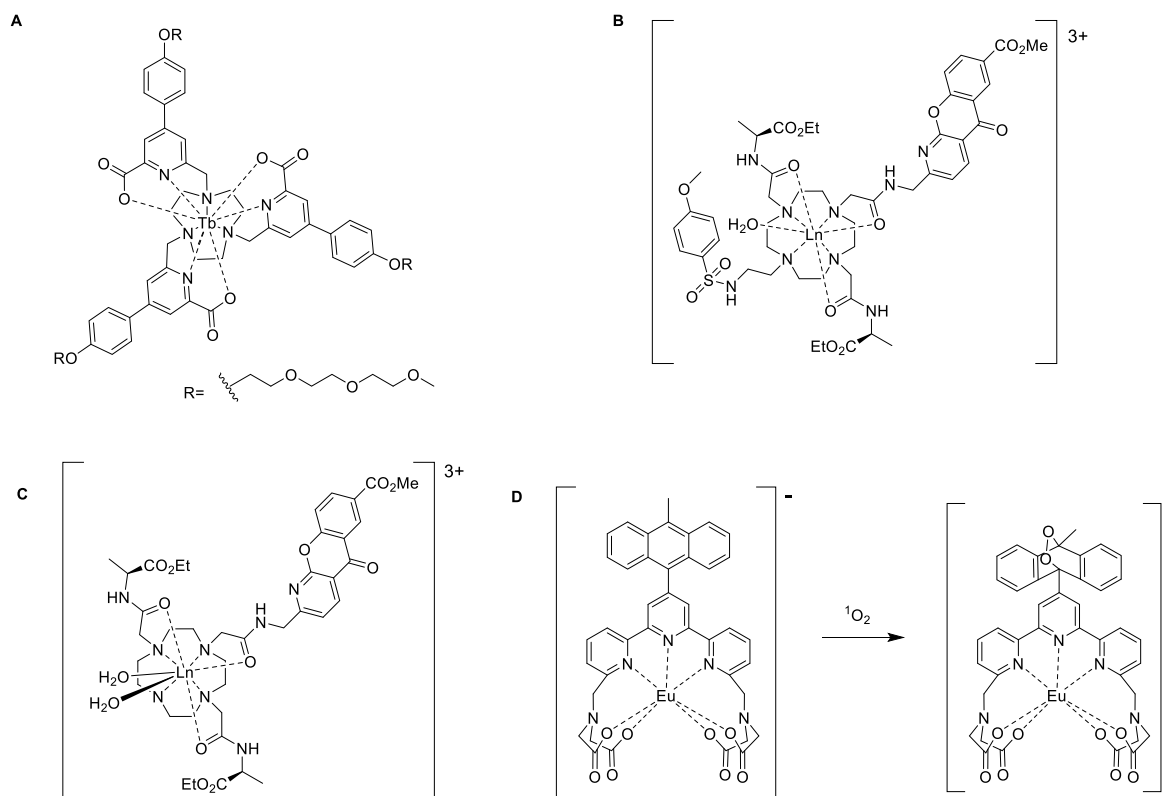


Figure 4.6: Molecular structures of lanthanide-based compounds for sensing of cellular analytes such as oxygenation levels (A),³⁶ lysosomal pH (B)⁴⁰ cellular bicarbonate (C)³⁹ and singlet oxygen (D).⁴¹

These studies demonstrate that lanthanide-based cellular probes can provide useful functional information about cellular states and, although some disadvantages compared to organic fluorophores exist, the long-lived lifetimes can provide useful, and in some cases, complementary information.

4.2 Aims

Although fluorescence microscopy is a technique widely used in cellular imaging, it suffers from inherent limitations including the difficulty in separating signals derived from an imaging probe and those occurring as a result of cellular background emission. One strategy to address this issue, is to design probes with substantially extended luminescent lifetimes compared to those arising from autofluorescence (which are in the nanosecond to 10s of nanoseconds range). Lanthanide complexes, for instance, provide micro- to millisecond range lifetimes and can be acquired using time-resolved or time-gated techniques.

Accordingly, a ligand system that reliably binds to the lanthanide series (to prevent dissociation of toxic metal aqua ions) was chosen and the complexes prepared were appended with FPR2/ALX targeting units. These constructs are designed to selectively bind to FPRs and enable time-resolved visualisation of neutrophils (Figure 4.7). As it is well-known that within the lanthanide series ligand binding is determined largely by ionic interactions, coordination properties of equivalent ligand architectures bound to different Ln(III) ions are considered equivalent. However, their respective spectroscopic properties vary, which provides an opportunity to interrogate different aspects of the coordination environment when different lanthanides are complexed. Here, the terbium, europium and gadolinium analogues were prepared and investigated as these provide information about complementary factors. Next, the effects on neutrophil functions were assessed and cellular images were obtained with the aim to:

1. Prepare and characterise water-soluble, biocompatible luminescent lanthanide compounds that can tag FPRs.
2. Establish whether the compounds adversely affect normal neutrophil functions and whether they show potential as an imaging probe.
3. Assess binding to formyl peptide receptors both in activated and unactivated cells.
4. Obtain time-resolved images of a lanthanide-based compound attached to FPR2/ALX on neutrophils that should, in principle, provide a more sensitive read-out than the previously studied organic tags.

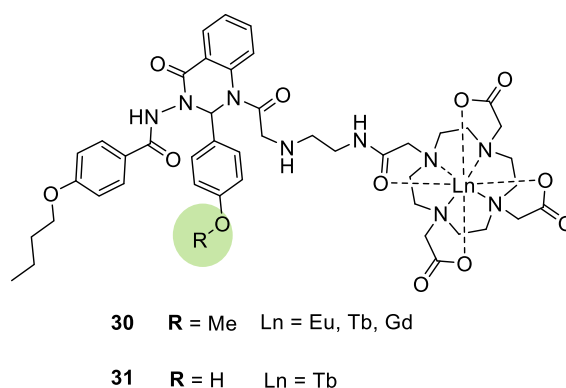


Figure 4.7: Molecular structures of lanthanide complexes for time-resolved applications based on Quin C1 (Ln. **30**) and the less stable Quin C7 (**31**) discussed in this chapter.

4.3 Results and discussion

4.3.1 Preparation of macrocyclic lanthanide-binding structures

Macrocyclic ligand frameworks based on 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid (DO3A) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) derivatives have been extensively studied in the literature as a result of their ability to form thermodynamically stable and remarkably inert complexes with trivalent lanthanide ions. This is often ascribed to effects of macrocyclic preorganisation and an excellent size match of cyclen-derived macrocycles with lanthanide ions.^{42,43} Further, a variety of clinically approved contrast agents such as ProHance, Gadovist and Dotarem are based on these ligand motifs.⁴⁴

To prepare the tris *tert*-butyl DO3A ester, *tert*-butyl bromoacetate was slowly added to a solution of cyclen and NaHCO₃ in acetonitrile and cooled at 0°C to prevent tetraalkylation.^{45,46} The resulting tri-functionalised macrocycle was isolated by recrystallisation from hot toluene. The macrocyclic structure undergoes conformational interconversions, which was evidenced within the ¹H-NMR spectrum as the characteristic, substantially broadened ring-proton signals that integrated to 16H (Figure 4.8). Additionally, two sharp singlets integrating to 9H and 18H, indicative of the two distinct *tert*-butyl proton environments were detected, as were the two acetate methylene proton environments integrating to 2H and 4H at 3.31 ppm and 3.40 ppm respectively (Figure 4.8).

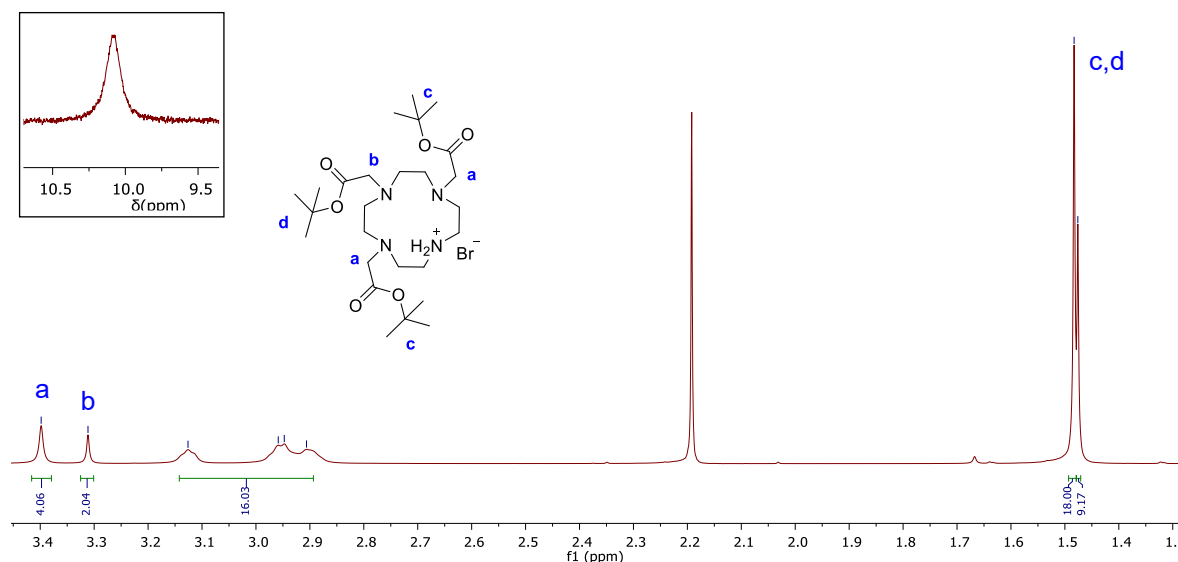
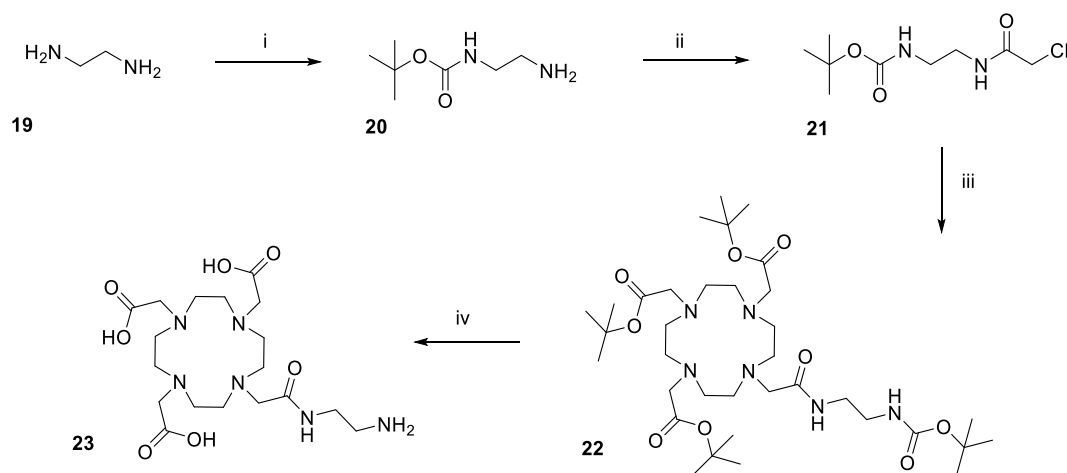


Figure 4.8: Typical ¹H-NMR spectrum of *tert*-butyl DO3A ester HBr salt in CDCl₃, run at 400 MHz and 298 K. Resonance at 2.17 ppm assigned to residual acetone. Inset NH proton signal at 10.09 ppm.

The remaining, secondary amine of the macrocycle is available for further structural functionalisation to allow the ligand system to be incorporated into larger constructs. For this purpose, two different sidearms that provide carboxylic acid or amine terminal lanthanide-complex building blocks, both providing an octadentate metal binding site, were prepared and appended.

4.3.1.1 Preparation of an amine terminal metal-binding unit

To prepare an amine terminal fourth DO3A sidearm (summarised in Scheme 4.1), excess ethylene diamine was reacted with di-*tert*-butyl dicarbonate to produce the mono-Boc-protected compound **20**.⁴⁷ The compound was obtained as a colourless oil in good yields and excess ethylenediamine could be removed under reduced pressure without requiring further purification steps. The unprotected amine site was selectively acylated using chloroacetyl chloride to form **21** with a functional site for further reaction to the macrocycle.⁴⁸

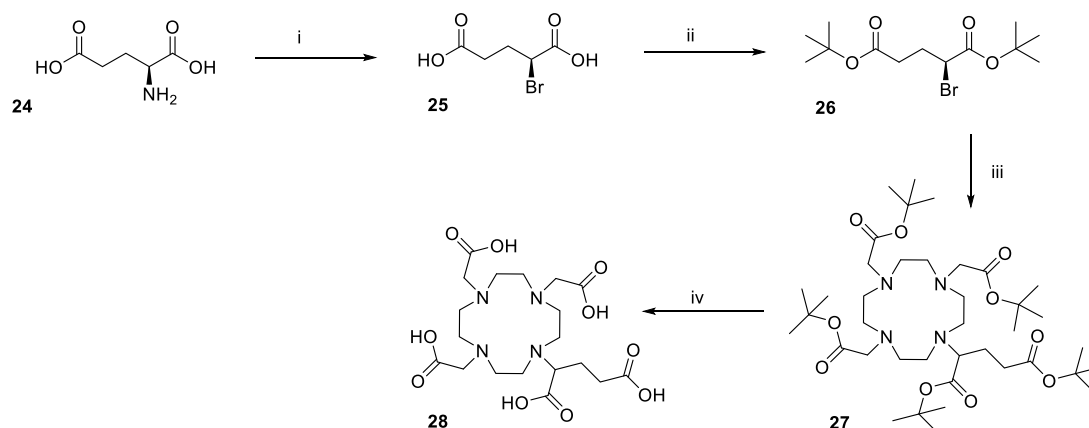


Scheme 4.1: Preparation of an amine terminal DO3A-derived macrocyclic ligand framework. Reagents: i) di-*tert*-butyl dicarbonate, ethanol, 83 % yield ii) chloroacetyl chloride, triethylamine, dichloromethane, 70 % yield iii) *tert*-butyl DO3A, potassium carbonate, acetonitrile, 33 % yield iv) trifluoroacetic acid, dichloromethane 98 % yield.

Both compounds were characterised by ¹H-NMR spectroscopy and mass spectrometry and the spectra obtained were consistent with literature results. The fourth cyclen amine was then reacted with **21** to afford the protected lanthanide-binding ligand scaffold **22** in a low yielding nucleophilic substitution reaction.⁴⁹ Isolation of **22** was achieved by silica column chromatography and yielded a pale yellow solid that was characterised by mass spectrometry and ¹H-NMR spectroscopy. Deprotection with trifluoroacetic acid and dichloromethane unmasked the amine terminal ligand scaffold to give **23** in high yields. By ¹H-NMR spectroscopy, this resulted in loss of the *tert*-butyl proton signals (observed for **22**) at 1.37 - 1.49 ppm. Taken together with appearance of the typical carboxylic acid peaks within the IR spectrum and a molecular ion peak observed at 447 a.m.u. corresponding to [M+H]⁺ within the mass spectrum, formation of the lanthanide binding ligand scaffold was confirmed.

4.3.1.2 Preparation of a carboxylic acid terminal metal-binding unit

To form an octadentate, carboxylic acid-terminal DO3A-derivative, the well-known 2-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanedioic acid (DOTAGA) motif was chosen.⁵⁰ Initially, L-glutamic acid and sodium bromide were reacted with sodium nitrite in freshly prepared dilute hydrobromic acid to form **25** via a diazotisation-bromination mechanism (Scheme 4.2 and Figure 4.9).⁵¹ Hydrobromic acid and sodium nitrite are known to lead to *in situ* formation of nitrous acid which then proceeds to form the *N*-nitrosoamine via the nitrosium ion. Subsequent combination with the glutamic acid amine and dehydration reactions leads to formation of the unstable alkyl diazonium ion.⁵⁰



Scheme 4.2: Preparation of a carboxylic acid terminal DO3A-derived macrocyclic ligand framework. Reagents: i) sodium bormide, hydrobromic acid (2 M), sodium nitrite, water, concentrated sulphuric acid, carried forward without purification, ii) perchloric acid, tert-butyl acetate, 9 % yield, iii)) tert-butyl DO3A, potassium carbonate, acetonitrile, 66 % yield, iv) trifluoracetic acid, dichloromethane 82 % yield.

The resulting nucleophilic substitution has been suggested to occur via initial anchimeric assistance from the α -carboxyl group and leads to an overall retention of configuration by double inversion (Figure 4.9) giving the brominated compound **25**.⁵²

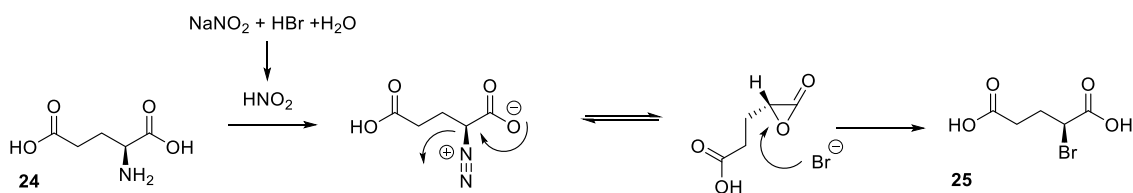


Figure 4.9: Mechanism of anchimeric assistance from the L-glutamic acid carbonyl group during the diazotation-bromination step.

Compound **25** was further reacted without purification to form the chiral, brominated diester **26**, which was isolated as a clear oil. Effects resulting from coupling of the chiral -CHBr- proton and the

diastereotopic -CH₂- group were apparent in the ¹H-NMR spectrum, resulting in a 1H doublet of doublets at 4.24 ppm and multiplets between 2.14 and 2.50 ppm respectively.

Linkage of DO3A-tBu and **26** was achieved by heating at reflux overnight, to yield a pale yellow oil. Although no studies to assess optical purity of the product were carried out, prior reports suggest that preparation via the method described above would result in a loss of optical purity.⁵³ Deprotection of **27** by removal of the *tert*-butyl units in trifluoroacetic acid and dichloromethane afforded the final structure, DOTAGA (**28**).

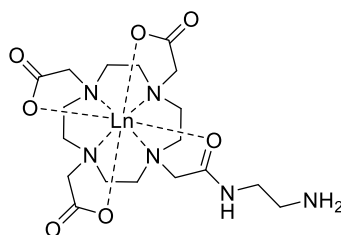
4.3.2 Formation and characterisation of lanthanide complexes

Next, **23** was chelated with a series of Ln(III) salts (terbium, europium and gadolinium). These three lanthanides were chosen to provide different structural information about the complex derived from their unique spectroscopic properties.³

Specifically, the terbium analogue was chosen to study excitation and emission spectra as well as luminescence lifetimes and the associated hydration number (*q*) that describe the number of water molecules bound to the metal centre.⁷⁵ The europium analogue provides a method to study the ¹H-NMR spectrum of the series. Paramagnetic lanthanide chelate ¹H-NMR spectra typically suffer from severe line broadening (due to long electronic relaxation times) and a wide chemical shift range. Both of these factors are minimised for europium(III) compounds compared to other lanthanide complexes, resulting in a body of literature examining Eu(III)-DOTA-derived species.⁵⁴ Gadolinium complexes on the other hand have found numerous applications in MRI imaging by enhancing the relaxation rate of nearby water protons and enable parameters related to their relaxivities to be determined.⁵⁵ The additional advantage of preparing the gadolinium analogue of the complex series, is that they can be used to model the ligand-component optical behaviour within the coordination compound, as excitation and emission directly from the Gd(III) ion fall within the UV region and thus will not be observed within the emission ranges of the ligand system.

4.3.2.1 Preparation of amine terminal lanthanide complex building blocks

Complexation of framework **23** was carried out using the terbium or europium trifluoromethanesulfonate salts or the gadolinium chloride hexahydrate in slight excess in water at pH 5.5 to produce the lanthanide complexes Eu.**29**, Gd.**29** and Tb.**29** (Figure 4.10). The resulting compounds were purified by dialysis using a 500 - 1000 Da molecular weight cut-off cellulose membrane, allowing separation of excess metal salts. Absence of free lanthanide ions was ascertained using the xylene orange indicator test.^{56,57}



Ln = Eu, Tb, Gd

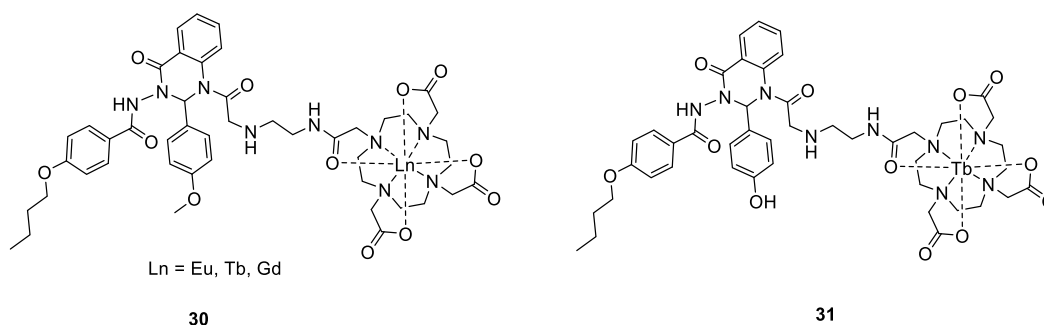
29

Figure 4.10: Molecular structure of amine terminal lanthanide building blocks for further functionalisation.⁴⁹

Chelation of the lanthanides within the macrocycle was validated by a spectral shift of the main carbonyl IR band from $\nu_{\text{CO}} = 1674 \text{ cm}^{-1}$ in the ligand to $\nu_{\text{CO}} = 1599 \text{ cm}^{-1}$ upon complexation of terbium. In addition, appearance of an amine-based band at $\nu_{\text{NH}_2} = 3472 \text{ cm}^{-1}$, which was previously swamped by the unbound carboxylic acid hydroxyl groups, was evidenced upon complexation. By HRMS, molecular ion peaks in the respective representative spectra were found at 603.1580 a.m.u, 595.1548 a.m.u. and 602.1576 a.m.u. (assigned to $[\text{M}+\text{H}]$, for Tb.**29**, Eu.**29** and Gd.**29** respectively) and confirmed formation of the desired products.

4.3.2.2 Combining lanthanide complexes with FPR2/ALX targeting units

To afford FPR2/ALX targeted lanthanide complexes, Eu.**29**, Gd.**29** and Tb.**29** were combined with the functionalised targeting units (**7** and **10**) described in Chapter 2. The resulting structures are shown in Figure 4.11.



Ln = Eu, Tb, Gd

30**31**

Figure 4.11: Molecular structures of FPR2/ALX-targeted lanthanide complexes based on Quin C1 (**30**, left) and Quin C7 (**31**, right).

Firstly, Tb.**29** was attached to **7**, under basic conditions and mild heating in acetonitrile to yield the final compound Tb.**30**. Analogous reactions were carried out with Eu.**29** and Gd.**29**, to yield the respective europium and gadolinium counterparts. The resulting compounds Tb.**30**, Eu.**30** and Gd.**30** were isolated

after dialysis and/or Sephadex size exclusion chromatography, dissolved in a minimum amount of methanol and precipitated using diethyl ether. The terbium-Quin C7 conjugate (**30**) was prepared to compare resulting photophysical properties with the Quin C1 complement. In the rhodamine-based analogues, it has already been observed that direct nucleophilic substitution using Quin C7 resulted in binding via the hydroxy phenyl group (section 3.3.3) which is, however, an essential site for receptor binding. Thus, to avoid attachment via this position, Tb.**29** was treated with **10** under basic conditions to form a TIPS protected version of the desired product. Deprotection in a tetra-*n*-butylammonium fluoride solution, followed by dialysis and precipitation from methanol using diethyl ether enabled isolation of **31**. All compounds were characterised by HRMS or conventional mass spectrometry.

4.3.2.3 Proton NMR spectroscopy

Structural characteristics of Eu.**29** and Eu.**30** were investigated using ^1H -NMR spectroscopy run by Peter Haycock of the NMR service at Imperial College London. The proton spectrum of Eu.**29** is pictured below (Figure 4.12). Resonances appear broadened at 500 MHz in D_2O , which is indicative of a bound paramagnetic metal. Further, the chemical shift range is extended compared to diamagnetic species and signals are present in the region between -20 ppm and +40 ppm, a shift range typical of Eu(III)-DOTA derivatives. One application for this phenomenon is in Eu(III)-based chemical shift reagents that enable improved interpretation of spectra by enhancing the normal chemical shift differences between proton environments by NMR spectroscopy.⁵⁴

In solution, Ln(III) DOTA structures are known to form two different diastereomers, a major SAP conformation and a minor TSAP conformation that can interconvert on an NMR timescale. Both conformations are part of an enantiomeric pair, that are, however, indistinguishable by NMR (see section 1.4.2).⁵⁸ The exact geometry ratios of the Ln(III) DOTA complexes are reportedly dependent on factors such as the metal ion size, temperature and solution ionic strength.^{59,60} NMR spectra of similar compounds have been shown to be further complicated by a variety of superimposed equilibria. Simultaneous to the conformational equilibrium between TSAP and SAP and their respective two enantiomers that are in slow exchange on an NMR timescale, a coordination equilibrium involving dissociation and association of an inner sphere water molecule is often in fast exchange for both geometries.⁵⁸

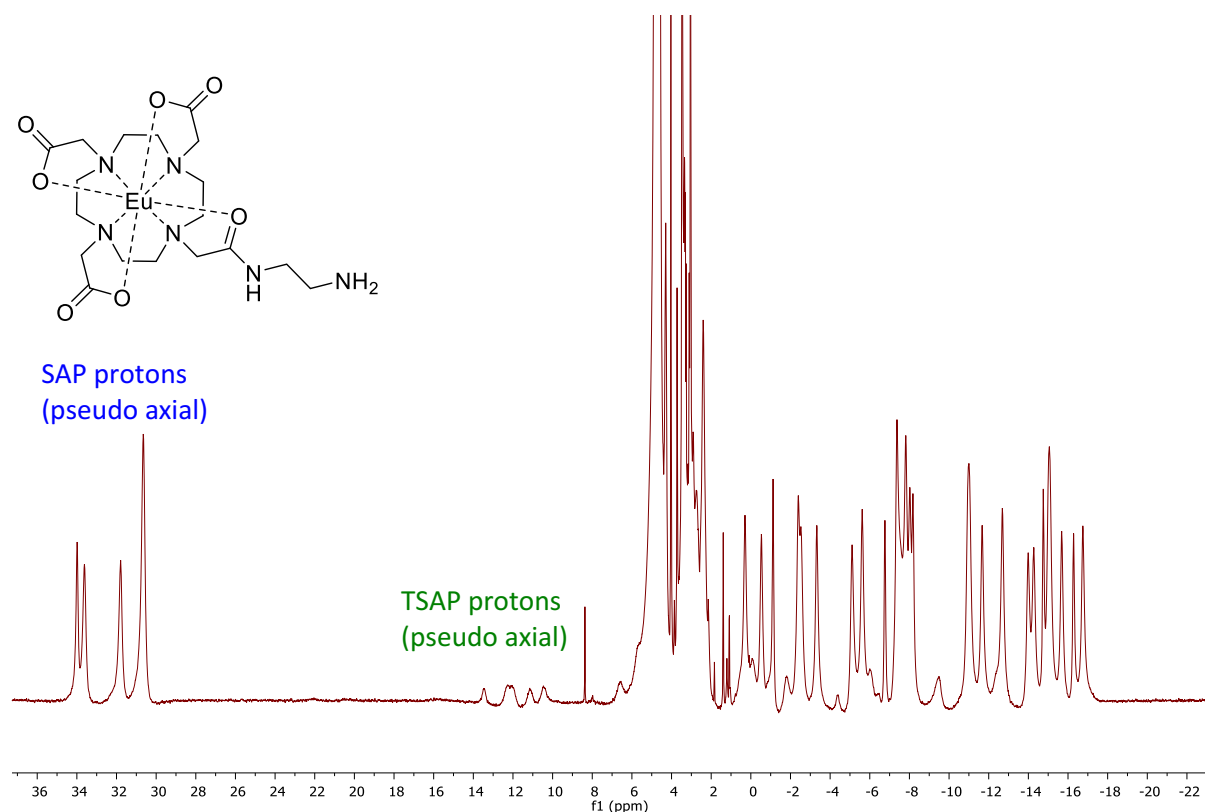


Figure 4.12: ^1H -NMR spectrum of compound Eu.29, run in D_2O at 500 MHz and 298 K, displaying the expected typical four resonances for the pseudo-axial protons in the SAP isomer (shift range 30 - 35 ppm) and the less intense TSAP isomer (shift range 10 - 14 ppm).

The parent, C_4 symmetrical DOTA-Eu complex is characterised by two sets of 6 peaks within the ^1H -NMR spectrum, assigned to the TSAP and SAP isomers.⁶¹ Derivatisation of one of the sidearms, as is the case here, leads to loss of symmetry and hence loss of proton environment equivalence. Theoretically, the C_1 symmetry of each isomer present should result in a set of 24 signals (48 in total) for the macrocyclic protons, in addition to protonation/deprotonation equilibria and resonances from nuclei further away from the metal site.⁶² The ^1H -NMR spectrum obtained for Eu.29 therefore contains substantially more resonances than the parent DOTA complex. Four resonances consistent with pseudo-axial ring protons previously reported for the dominant SAP conformation (with a larger susceptibility anisotropy and a connected greater shift range compared to the TSAP isomer) between around +30 ppm and +35 ppm could be observed.⁶¹ Four signals associated with the minor TSAP isomer were also observed in the shift range between 10 and 14 ppm and the SAP/TSAP ratio was estimated as 1:0.05 by integration, although comparatively long relaxation lifetimes of the Eu(III) complex make integrations less reliable. This ratio is however in approximate agreement with previously reported values for isomer ratios.⁶³

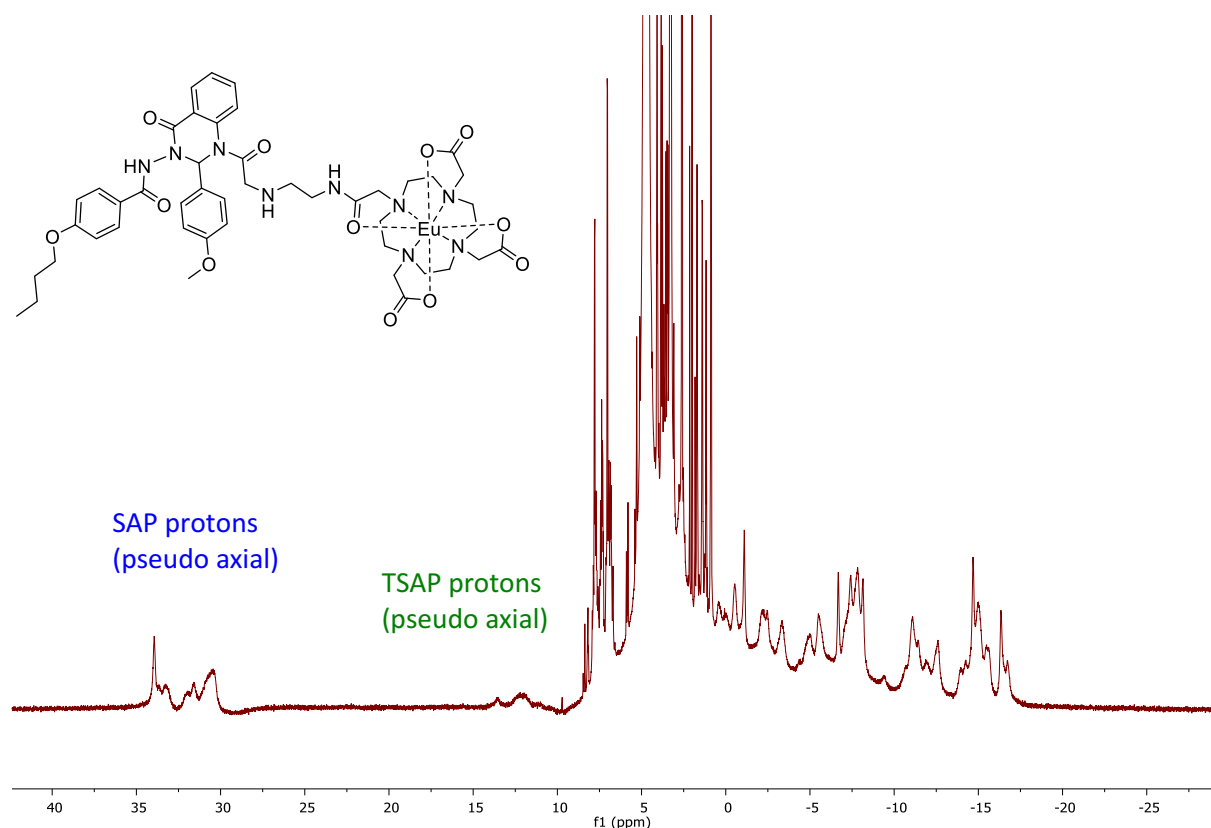


Figure 4.13: ^1H -NMR spectrum of compound Eu.30, run in D_2O at 500 MHz and 298 K, displaying the expected typical resonances for the pseudo-axial protons in the SAP isomer (shift range 30 - 35 ppm) and the less intense TSAP isomer (shift range 10 - 14 ppm).

Within the ^1H -NMR spectrum of Eu.30 (Figure 4.13), sharp signals in the region between about 6 ppm and 9 ppm appear, that were absent in the spectrum of Eu.29 and are indicative of the Quin C1-based aromatic signals. The targeting group is sufficiently far removed from the paramagnetic metal centre for the protons to only be minimally shifted. Further, the SAP pseudo-axial proton region (29 - 35 ppm) and the signals associated with the minor TSAP isomer between 10 and 14 ppm appear broadened compared to those of Eu.29 and more than four signals of each are apparent. This could potentially be indicative of further conformational equilibria occurring. To estimate isomer prevalence, the pseudo-axial proton resonances were integrated with respect to each other and the 1:0.36 SAP/TSAP ratio demonstrates an increase of the minor TSAP form compared to the starting material.

4.3.2.4 Relaxivity measurements

Relaxivity is defined as the efficiency of a compound to enhance the relaxation rate of surrounding water molecules and was first described quantitatively by the Solomon, Bloembergen and Morgan equations.⁶⁴ The value provides a means to measure how effective a complex is as a contrast agent.

Presence of a Gd(III) chelate is known to affect both T_1 (longitudinal relaxation, i.e. the time it takes for protons to realign with the external magnetic field) and T_2 (transverse relaxation, i.e. the time it takes for

the protons to exchange energy with other nuclei) times. As T_1 is usually substantially longer than T_2 , low concentrations of the gadolinium-based probes will produce a much greater shortening of T_1 and the effects on T_2 are often negligible.⁶⁵

The relaxometric properties of Gd.**30** and Gd.**29** were investigated using inversion recovery experiments conducted by Peter Haycock at Imperial College London. Relaxivity values were estimated according to a graphical procedure outlined in the literature,⁶⁵ making use of the linear relationship between $1/T_i$ and the concentration of gadolinium complex given in equation 4.1 where r_1 is the slope of the linear plot.

$$\frac{1}{T_i} = \frac{1}{T_{i0}} + r_1[Gd] \quad (\text{eq 4.1})$$

The longitudinal relaxation time (T_1) of Gd.**30** in water was determined at three distinct molar concentrations on a 400 MHz NMR spectrometer at 294 K. In addition, T_1 for just water ($T_{1,0}$) was determined under equivalent conditions. Results are summarised in Table 4.1.

	Gd concentration (mM)	Δ_χ (ppm)	T_1 (s)	$T_{1,0}$ reference (s)	r_1 (mMs ⁻¹)
Gd. 29	1.450	0.1615	0.165	1.405	4.2
	0.388	0.0432	0.637		
	0.228	0.0254	1.018		
Gd. 30	0.233	0.0260	0.787	2.881	6.9
	0.146	0.0163	1.326		
	0.134	0.0149	1.869		

Table 4.1: Measured and calculated relaxivity parameters that enabled calculation of r_1 values for Gd.**30** and Gd.**29**.

When weighing out small amounts of the compound, it was generally found that experimentally determined values over-estimate gadolinium concentrations, most likely due to the typical hygroscopic nature of Gd(III) complexes. Due to these inaccuracies, the concentration of gadolinium was both determined experimentally as well as calculated using the Evans method.⁶⁶ To achieve this, Δ_χ (defined as the shift difference between the H₂O and D₂O proton NMR peak in ppm of the compound) was determined by ¹H-NMR spectroscopy and the value was inserted into equation 4.2 to determine the concentration of Gd(III), c , and results were reported based on these values, using equation 4.2:

$$\Delta_\chi = \frac{4\pi c S}{T} \left(\frac{\mu_{eff}}{2.84} \right)^2 \times 10^3 \quad (\text{eq 4.2})$$

Where $T = 298$ K, $\mu_{eff} = 7.94$ for Gd, and $S = 1/3$. Using this method, the longitudinal relaxivity value was determined as $r_1 = 4.2$ mMs⁻¹ for Gd.**29** and $r_1 = 6.9$ mMs⁻¹ for Gd.**30**. The value obtained for Gd.**29** is

similar to typical values of complexes with a hydration number of $q = 1$ such as the clinical gold-standard contrast agent DOTAREM ($r_1 = 4.2$ at 20 MHz and 298 K, which in this case does not differ largely from the expected value at 400 MHz)⁵⁵ and the value obtained for Gd.**30** is slightly higher, indicating favourable relaxometric properties.

Parameters associated with relaxivity of a gadolinium complex can be tuned by chemical design. An interplay of hydration number (q), rotational correlation time and solvent water exchange rate are often key factors controlling the relaxivity values, of which the last is most complex to modulate via ligand design.⁶⁷ Here, the observed increase in r_1 does not occur as a result of higher hydration numbers, as the q -value was determined as ~ 1 . Both a decrease in water residence lifetimes and an increase in rotational correlation times as a result of the larger molecular size are known to increase r_1 values and therefore are likely to contribute to the relaxivity increase for Gd.**30** compared to Gd.**29**. In particular, faster water exchange rates (and associated improved relaxivity) are correlated with the TSAP geometry.⁶⁸ Considering the TSAP to SAP ratio in Gd.**30** has increased approximately 7-fold compared to Gd.**29**, this may be a major contributor to the observed r_1 value discrepancy.

4.3.2.5 Photophysical properties

Steady-state and time-gated luminescence and absorption spectra of the two formed terbium complexes Tb.**30** and **31** were recorded in aerated methanol solutions. Steady-state emission bands of the two compounds were similar, with one broad peak observed for each at 446 nm (Tb.**30**) or 443 nm (**31**), whereas excitation bands differed (Figure 4.14), with maxima at 229 nm, 260 nm and 348 nm observed in Tb.**30** and comparably red-shifted maxima at 242 nm, 321 nm and 366 nm observed for **31**. Although structurally similar, it is perhaps unsurprising that the excited states of the two compounds seem to differ, as electrostatic interactions, hydrogen bonding and steric factors of the molecule can substantially vary between a methoxy and a hydroxy substituent.

Both complexes displayed sensitised terbium emission upon application of a time gate of 0.1 ms (Figure 4.14). This demonstrates the ability of the Quin C7/Quin C1 fluorophores to populate the lanthanide emissive state and act as an efficient “antenna”. Characteristic terbium emission bands with maxima at 490 nm, 546 nm, 586 nm and 620 nm arising from the $^5D_4 \rightarrow ^7F_J$ transitions, for $J = 6, 5, 4$ and 3 respectively, were apparent within the spectrum of Tb.**30** and observed similarly for **31**.

Steady state excitation and emission spectra obtained for Eu.**30** were comparable to those obtained for Tb.**30** (with $\lambda_{\text{max}} = 451$ nm for emission when excited at 350 nm and $\lambda_{\text{max}} = 348$ nm for the longest wavelength excitation band) but only very weak europium signals were observed upon application of a time-gate. This suggests that the ligand triplet state is possibly not well enough matched in energy to provide efficient sensitisation of europium emission, whereas the terbium emissive energy state, which is located at higher energy compared to that of europium ($^5D_0 \sim 17,200 \text{ cm}^{-1}$ for Eu(III) and $^5D_4$ for Tb(III) $\sim 20,400 \text{ cm}^{-1}$), can be populated by the ligand.

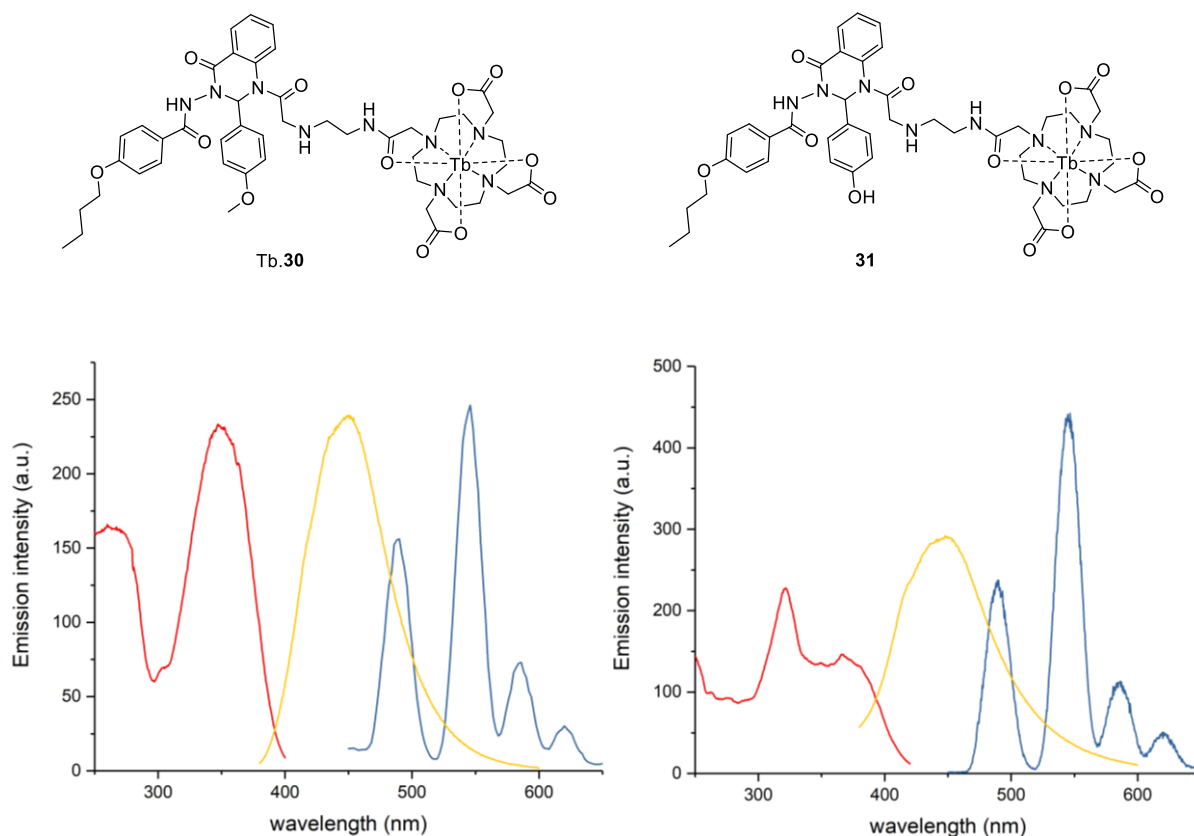


Figure 4.14: Excitation spectra (red), steady state emission spectra (yellow) and emission spectra with an applied time-gate of 0.1 ms (blue) of compound Tb.30 (left) and 31 (right) acquired in aerated methanol solutions.

To estimate the ligand-based triplet energy level T_1 , the phosphorescence ($T_1 \rightarrow S_0$) was recorded for the gadolinium complex at 77 K.⁶⁹ Measurements at low temperature are necessary as the triplet state can easily be deactivated by non-radiative processes. Emission spectra of Gd.30 were firstly obtained at room temperature and in an ethanol glass and are shown in Figure 4.15. Upon cooling, the expected lower energy maximum that is typically assigned to phosphorescence emission, however, was not observed. Instead, a higher energy band was observed at 406 nm ($24,631\text{ cm}^{-1}$), with a slight shoulder that could be indicative of the emission band observed at room temperature. To exclude the possibility that this could be a result of an artefact of the solvent/cuvette used, a blank in ethanol glass was run at 77 K under the same conditions. Comparison of the two spectra revealed that the blank did not include the newly appearing peak. One possibility is that the ligand-centred band present at room temperature originates from an intraligand charge transfer state which is known to be thermally activated, while the peak is shifted to higher energies at low temperatures or originates from a locally excited state.⁷⁰

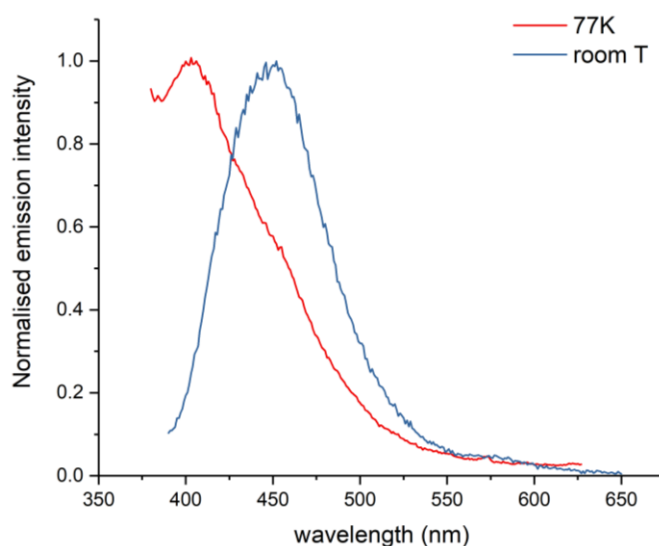


Figure 4.15: Emission spectra of Gd.30 at room temperature (in blue, $\lambda_{exc} = 350$ nm) and in an ethanol glass at about 77 K (red, $\lambda_{exc} = 350$ nm).

Comparable spectra obtained for ligand systems complexed to gadolinium that were able to sensitise lanthanide emission via an ILCT state have been previously reported. In particular, within the report by Lo *et al*, upon cooling to 77 K, a blue-shifted ILCT band appeared and the phosphorescence band was not visible when excited at 390 nm which is similar to the observation made here.⁷¹

To verify this result, the emission spectra at room temperature and 77 K were re-run using a more sophisticated set-up, that could maintain the low temperature environment of the probe throughout the experiment by Dr Lijun Jiang at Hong Kong Baptist University. At low temperature, the previously observed, higher energy shifted peak was present within the spectrum (Figure 4.16). In addition, the sustained low temperature set-up enabled detection of another faint, red shifted peak at 539 nm or 18553 cm^{-1} (marked with an arrow, Figure 4.16), which is likely to be assigned to triplet state emission.

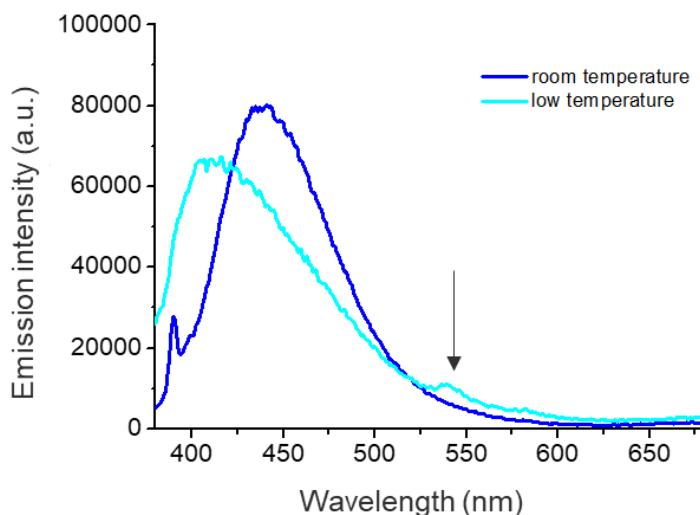


Figure 4.16: Repeat measurement of emission spectra of Gd.30 ($\lambda_{exc} = 350$ nm) at room temperature (dark blue) and 77 K (light blue). Newly appearing, red-shifted peak is marked with an arrow.

Taken together, these results led to the hypothesis that the main emission peak at room temperature results from a charge transfer band and lanthanide sensitisation may occur via the charge transfer state, which appears energetically well matched with the Tb(III) $^5D_4$ excited state ($\sim 22,173$ cm^{-1} compared to $20,400$ cm^{-1} , see above). The tentatively assigned triplet state on the other hand is weak and likely too low in energy (18553 cm^{-1}) to efficiently sensitise terbium emission. Similar sensitisation pathways have been previously noted within the literature, in particular in combination with Eu(III) complexes.^{70,72}

4.3.2.6 Determination of metal-based lifetimes and q-values

Metal-centred luminescence lifetimes were recorded in water and deuterium oxide on a Varian Cary Eclipse fluorescence spectrophotometer using the phosphorescence mode. Their decays were fitted to a single-exponential function by plotting the natural logarithm of intensity versus time (until 10 ms) and obtaining τ values from the reciprocal of the slope, assuming an ideal single exponential decay according to the equation:

$$I(t) = I(0) \exp\left(-\frac{t}{\tau}\right) \quad (\text{eq 4.3})$$

The resulting values were determined as $\tau_{D_2O} = 2.8$ ms and $\tau_{H_2O} = 1.9$ ms (Tb.30) and $\tau_{D_2O} = 2.5$ ms and $\tau_{H_2O} = 1.5$ ms (31). Decay curves and exponential fits are plotted below for Tb.30 (Figure 4.17).

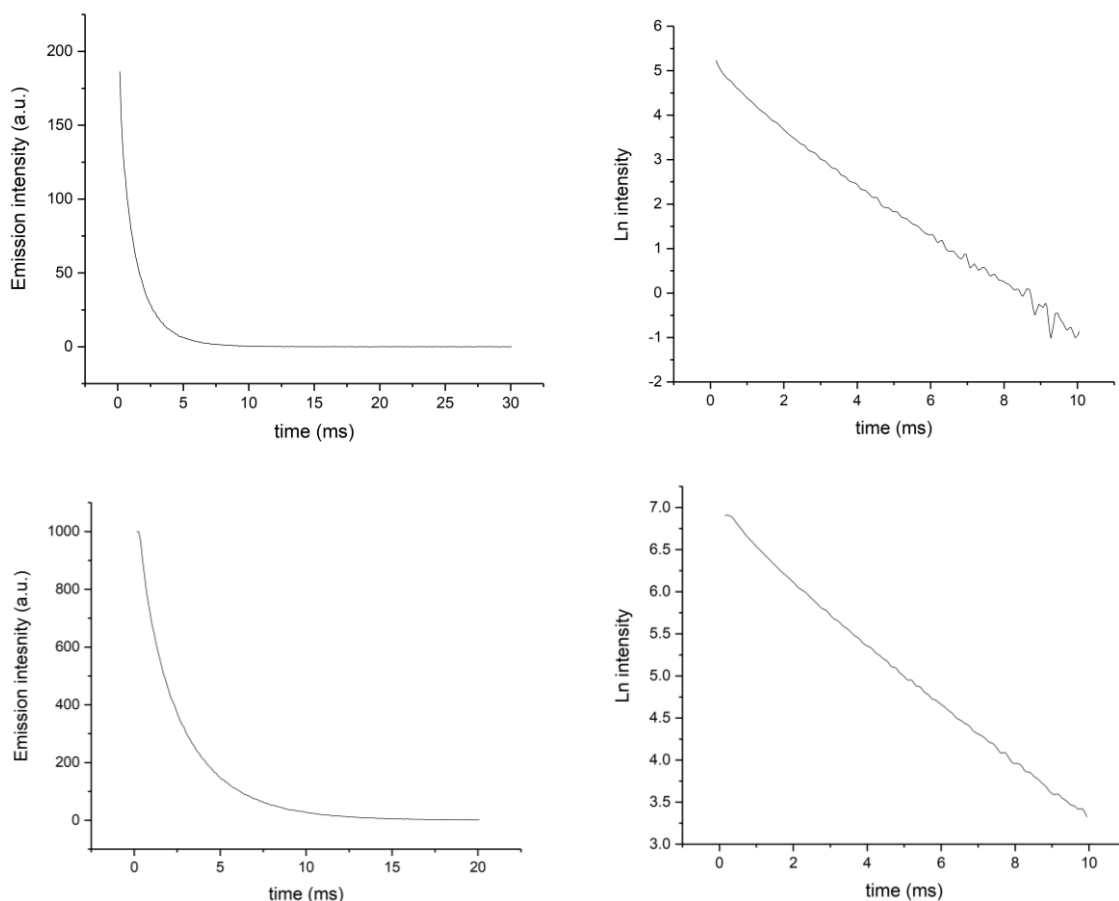


Figure 4.17: Luminescence lifetime measurements of Tb.30 in H₂O (above) and D₂O (below), depicting the decay curves (left) and a simple, single-exponential fit by plotting natural logarithm of the intensity against time to obtain τ values from the reciprocal of the slope (right).

Lanthanide excited states can be quenched via non-radiative energy transfer, which reduces emission intensity. In particular, large Franck Condon overlaps between OH oscillators and, to a lesser extent, CH and NH oscillators and the lanthanide excited states, leads to efficient luminescent quenching when these are in proximity to the metal centre.^{73,74} O-D oscillators overlap less effectively with the energy levels of the lanthanide ions and the quenching effect is significantly lower and lifetimes observed are correspondingly longer.³⁵ Although the quenching mechanisms are detrimental to the emissive properties of the metal complexes, it allows the measured lifetime values to be used to estimate the number of water molecules bound to the metal centre (q) by using an empirical relationship first proposed by Horrocks in 1981.⁷⁵ Since then, Horrocks' equation has been modified to allow for second and outer sphere quenching contributions to the luminescent lifetimes and is generally applied in the form of equation 4.4:

$$q = A(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - B) \quad (\text{eq 4.4})$$

where A and B are constants that vary for different Ln(III) ions.⁷⁶ Their numerical values account for the ability of the excited state to be quenched by nearby oscillators (A) and further describe the outer sphere solvent contributions to quenching (B). Luminescent lifetimes in water and deuterium oxide respectively

are given by $\tau_{\text{H}_2\text{O}}$ and $\tau_{\text{D}_2\text{O}}$. For terbium, $A = 5$ ms and $B = 0.06$ ms.⁷⁷ Using this methodology, q -values were determined to be $q = 0.8$ (Tb.**30**) and $q = 1.1$ (**31**) with an estimated error of $q = \pm 0.2$, suggesting an octadentate ligand environment for both compounds.

4.3.2.7 Determination of organic component lifetimes

Short-lived organic component lifetimes of Tb.**30** and **31** were assessed on a time resolved Picoquant Fluotime 300 instrument with a 375 nm picosecond pulsed laser using TCSPC. In order to fit the resulting decay curve for short lifetimes, the fluorophore response was deconvoluted using a scattering, non-emissive sample to obtain an instrument response function. A numerical reconvolution algorithm to account for the finite instrument response function (IRF) was applied to the bi-exponential luminescence response using FluoFit software. By this method, short lived emission lifetimes for Tb.**30** were determined as $\tau_1 = 10.61$ ns $\pm$ 0.12 ns (85.3 % fractional intensity) and $\tau_2 = 2.87$ ns $\pm$ 0.21 ns (14.8 % fractional intensity) with $\chi^2 = 1.10$. Fitting for **31** proved more complicated and finally, a tail fitting method was applied, by only including data from after the IRF had fully decayed. Three exponentials gave the best fit resulting in lifetimes of $\tau_1 = 11.45$ ns $\pm$ 0.16 ns (69.6 % fractional intensity), $\tau_2 = 4.64$ ns $\pm$ 0.26 ns (24.4 % fractional intensity) and $\tau_3 = 1.37$ ns $\pm$ 0.26 ns (6 % fractional intensity) with $\chi^2 = 0.96$ (Figure 4.18), which is not ideal but the best fit that was found. The obtained data indicate dissimilar excited states for the organic components of Tb.**30** and **31**.

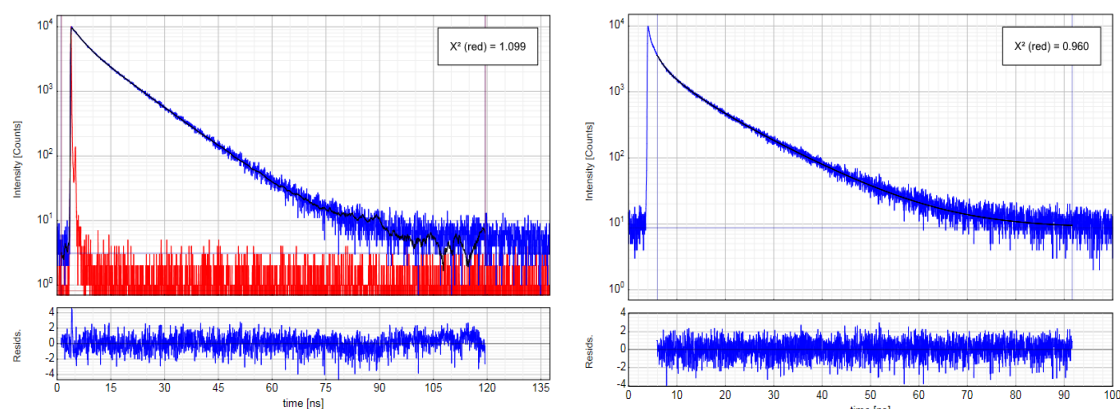


Figure 4.18: Short-lived lifetime data obtained for Tb.**30** (left) via a biexponential fit and **31** (right), using a tail-fitting method on a time-resolved Picoquant Fluotime 300 with a 375 nm picosecond pulsed laser.

4.3.3 *In vitro* viability assays

As has been previously described in section 2.3.5, the resulting lanthanide compound group was tested *in vitro* using Tb.30 as a representative example. To ensure suitability of the compound for potential neutrophil imaging applications, the effect of its presence was validated in three neutrophil functional assays that encompass model systems for the main functions the cell needs to fulfil in a physiological context:

- 1) The ability to migrate in response to a chemical gradient.
- 2) The ability to extravasate through a vessel in response to a stimulus.
- 3) To release granular contents such as MPO upon activation.

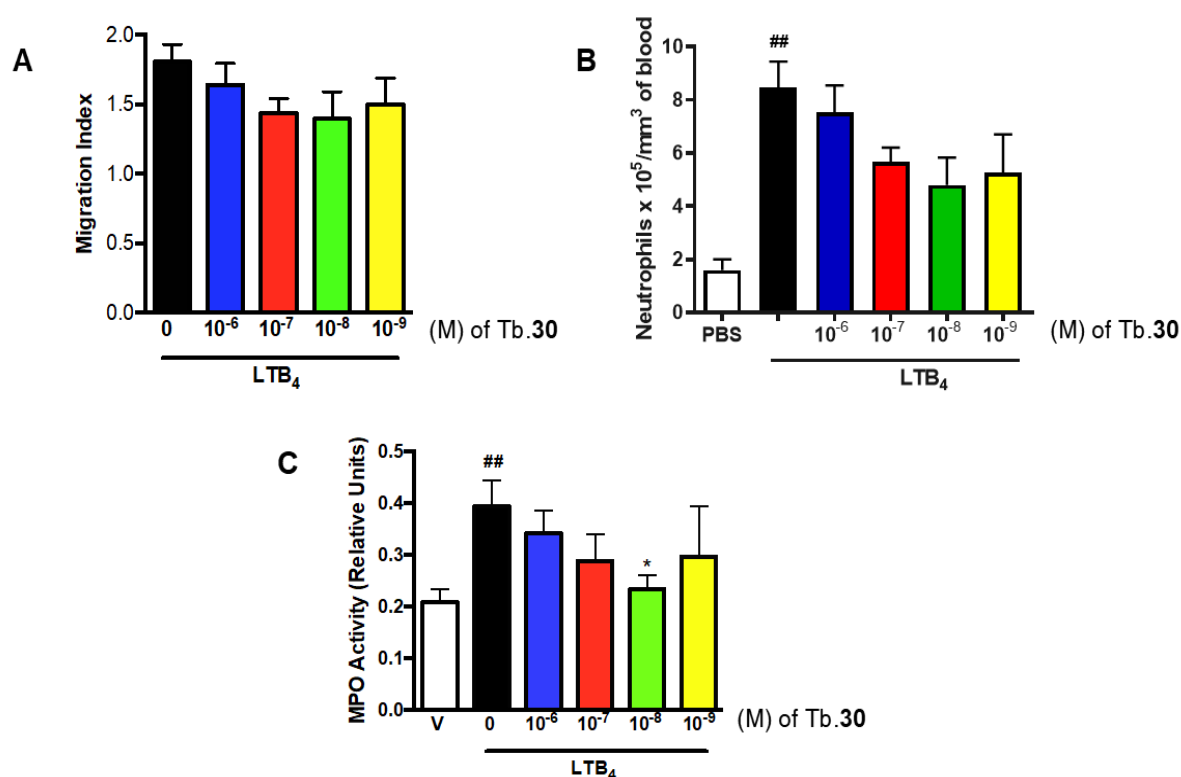


Figure 4.19: Assay data shown for the chemotactic response of human neutrophils in presence of different concentrations of the compound (**A**) and expressed as a migration index (ratio of neutrophils migrated in response to LTB₄ versus those migrated to vehicle). **B** shows data obtained for transmigration through an endothelial cell layer (HUVECs) in response to LTB₄ and **C** depicts results from the stimulated MPO release assay. Data are mean $\pm$ SEM from cells prepared from 3 or 4 different donors. Vehicle control is run with no compound and no LTB₄ present. ## $P < 0.01$ vs. corresponding vehicle control group, * $p < 0.05$ vs. corresponding LTB₄ control group.

In both chemotaxis and transmigration assays Tb.30 caused no statistically significant impact on neutrophil motility at any of the measured concentrations (Figure 4.19, **A** and **B**). This contrasts with results that have previously been obtained for Quin C1 (**6**) in section 2.3.5, where an inhibition was observed in both the chemotaxis and transmigration assays at intermediate ligand concentrations.

Although not statistically significant, there does still seem to be a trend present for slight inhibition of transmigration at intermediate concentrations (10^{-7} and 10^{-8} M, Figure 4.19). Interestingly, attaching a DO3A-based metal moiety does not alter the Quin C1 ligand's action on the ability of neutrophils to release MPO, although a significant change in neutrophil function is present at 10^{-8} M. These results highlight that, in this case, an alteration to the ligand structure results in partial loss of bioactivity at certain concentrations.

4.3.4 Time-resolved cellular imaging

Previous cellular studies conducted using fluorophores tagged to Quin C1 described in Chapter 3 highlighted the possibility that neutrophils show preferential uptake of the compound in an activated state. Lanthanide-based imaging allows for time-resolved microscopy with signal differences of several orders of magnitude between probe and cellular background, a property that could enable visualisation of differences in cellular morphology, structure and tagging between stimulated and unstimulated neutrophils.

To achieve this, neutrophils were either pre-treated with TNF α (50 ng/mL, 30 minutes) or with PBS (unstimulated group) and subsequently incubated with varying concentrations of Tb.**30** or vehicle. Cells were fixed and mounted before visualisation using a PicoQuant Microtime 200 time-resolved confocal fluorescence microscope. Following several optimisation steps, it was established that a concentration of 1 mM of Tb.**30** resulted in the best images and was required to observe long-lived luminescent signals. This concentration is high compared to effective lanthanide-based cellular probes previously reported, which are typically used at concentrations in the range of 10 - 100 μ M (see section 4.1.3) which is more suited for a physiological setting. However, these complexes are considered non-toxic even at higher cellular loads and the cells were fixed prior to imaging. It was therefore rationalised that the high concentration would be useful for this proof of concept study. Terbium-centred emission was recorded as a lifetime map using pulsed 375 nm laser excitation under the supervision of Dr Michael Coogan at the University of Lancaster. Representative lifetime maps for stimulated and control groups are shown in Figure 4.20.

In samples that had been previously stimulated, two types of pseudo-intensity were observed. Neutrophils either appeared to show uptake in a ring-like structure around the cellular periphery or, in cells that had deformed, uptake was observed in randomly speckled increased areas of intensity as well as around the plasma membrane (Figure 4.20, top row). In unstimulated samples, these ring-like structures were not observed, and cells appeared as uniformly fluorescent (Figure 4.20, below, left).

Appearance of ring-like structures in stimulated cells were postulated to relate to attachment of the fluorescent group onto activated FPRs and are comparable to images shown elsewhere.⁷⁸ For instance, it was reported that in unstimulated neutrophils, FPRs labelled with fluorescently tagged peptide-based ligands were observed to distribute randomly at the plasma membrane, whereas fluorescence was observed to concentrate along the cell periphery upon stimulation.⁷⁸ In a further subset of stimulated samples within this study, pseudopodia formation had occurred and increased amounts of “fluorescent

speckles” across the cell were noticed (FPR clusters). The existence of two different stages of stimulation was proposed, one where the receptors were tagged along the cellular edges and one that was visualised through the speckled distribution. Here, neutrophil morphologies visualised in stimulated groups labelled with Tb.30 were reminiscent of these results, providing some evidence that increased uptake of the compound in the two different stimulated cell states was observed. The expected regions of compound uptake (regions of activated FPRs) were found to contain long-lived emissive elements (fitted to $\sim 20 \mu\text{s}$) within the cell average lifetime decay curves (Figure 4.21) and for pixels chosen in the relevant areas.

Promisingly, no long-lived components could be observed under equivalent conditions in unstimulated samples, likely a result of a smaller fraction of Tb.30 bound to the cell surface. Similarly, when stimulated neutrophils were imaged without pulsed laser excitation (to eliminate terbium-based signals from the image), a loss of the characteristic ring-like fluorescence appearance was detected, further confirming the presence of terbium-based emission.

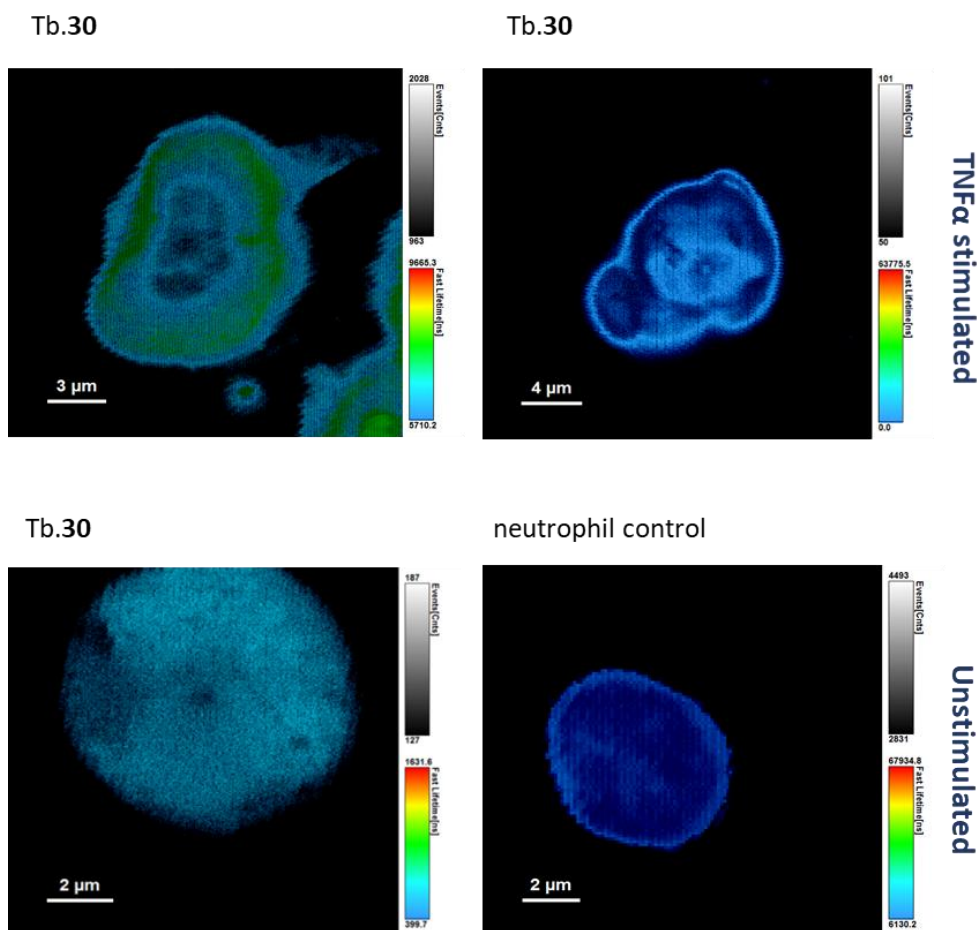


Figure 4.20: Images of neutrophils obtained using a time-resolved confocal microscope to visualise uptake of Tb.30 in stimulated cells (above). Two distinct activation states were observed. In unstimulated neutrophils (below) no long-lived decay components were detected.

However, an issue encountered within this set-up was that the obtained lifetime maps did not reflect the average lifetimes recorded per pixel of interest, despite the data from the decay curves consistently showing terbium-associated long-lived lifetimes. In a positive control experiment, where Tb.30 was imaged in solid state on a cover slip, the same issue with lifetime mapping was encountered, supporting the hypothesis that the mapped values are in fact not fitting.

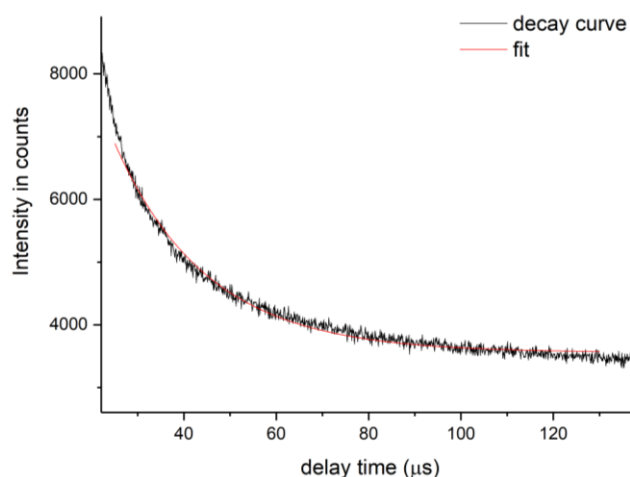


Figure 4.21: Luminescence lifetime decay and tail-fitting (shown in red) of the overall average decay in a stimulated neutrophil, demonstrating terbium-based emission and a lifetime of $\sim 20 \mu\text{s}$.

Taken together, these results provide evidence that Tb.30 is being taken up in neutrophils that had received prior stimulation using TNF α (Figure 4.21) and can be visualised in a time-resolved manner. In the unstimulated groups, neither a ring-like structure around the outside of the cell, nor long-lived emission components could be observed, indicating insufficient uptake of Tb.30 without prior activation.

Further optimisation is clearly required. During this study cells were incubated with high concentrations of Tb.30 before lanthanide emission was recorded, which is not ideal in a physiological context. The weak emission observed could be a result of insufficient complex absorbance at the higher wavelength excitation source (375 nm laser compared to the 350 nm excitation recorded in emission spectra), which means only a fraction of the lanthanide complex is excited per cycle. Further, terbium emission quenching mechanisms appear to be occurring within the cell as indicated by the shortened lifetime of $\sim 20 \mu\text{s}$ compared to 1.9 ms in H₂O solution spectra. Amounts of Tb(III) chelate uptake can vary between individual cells within the sample and in areas with low levels of probe, as would be expected within the unstimulated samples, the signal is likely to be attenuated to background levels.

Nevertheless, these preliminary results provide an informative backdrop for future work. Based on these results, further probe design iterations discussed in Chapters 5 and 6 were devised to improve the compound's luminescence properties.

4.4 Conclusions

In this chapter, preparation and characterisation of a stable lanthanide-binding framework tethered to a small-molecule FPR2/ALX targeting group were described.

Properties of the respective terbium, europium and gadolinium complexes were investigated. It was found, that the Quin C1 ligand could act as an efficient sensitising unit for terbium emission and produce long-lived luminescent signals. The europium analogue was found to exhibit typical DOTA-type signals for both the SAP and the TSAP conformations within the $^1\text{H-NMR}$ spectrum, and attachment of the targeting group resulted in an increase in the relative amount of the TSAP isomer. In addition, r_1 values of 6.9 mM s^{-1} were measured for the gadolinium complex at 400 MHz, a value that is improved from the typical $r_1 \sim 4 \text{ mM s}^{-1}$ for commercially used contrast agents based on octadentate metal-binding frameworks.

These promising results led to application of the complex in cellular studies to investigate 1) the effects of the compound on neutrophil viability and 2) binding of the compound to FPR2/ALX receptors in their respective activation states using a time-resolved microscopy set-up, building on results obtained in Chapter 3. Despite the weak cellular signals obtained from the metal during the experiments, time-resolved terbium emission could be observed, and compound uptake preferentially occurred in activated cells. In fact, no significant terbium emission was noted in the unactivated group, indicating reduced compound binding. These results confirmed previous studies using rhodamine-tagged analogues in an inflammatory context. Further, neutrophil functional assays ascertained that within controlled concentration ranges required for imaging purposes, the agent has no significant effect on the cells' abilities to perform typical physiological functions. In order to conclusively use the compound for targeted, time-resolved microscopy, the photophysical parameters would need to be optimised to increase the compounds' brightness and as such be able to reduce amounts delivered into cells. One strategy to achieve this would be to shorten the distance between metal and sensitising unit, for instance by removing the ethylenediamine bridge between the two. Another strategy towards more emissive lanthanide-based probes is to include an organic dye fluorescent acceptor which is discussed in detail in Chapter 6.

4.5 References

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Chapter 5

FPR2/ALX-targeted Re(I) tricarbonyl motif for time-resolved microscopy

5.1 Introduction

Emissive complexes based on a stable, d^6 low spin Re(I) fac-tricarbonyl core, have attracted interest for cellular imaging as a result of their luminescent properties and chemical reactivity that is considered analogous to the clinically relevant ^{99m}Tc analogues.¹ They are often combined with either bidentate polypyridyl or tridentate bisquinoline chelators with low-lying π^* orbitals and their coordinative saturation implies that they are comparably inert and robust towards ligand exchange reactions (Figure 5.1). As such, these complexes are deemed “safe” under physiological conditions, where release of toxic metal species could lead to detrimental effects.²

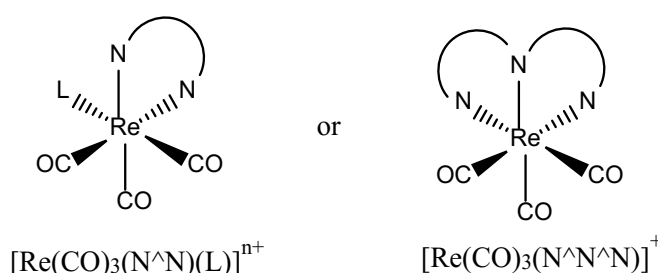


Figure 5.1: General structures of Re(I) tricarbonyl complexes based on bidentate polypyridyl ligands (left) and tridentate bisquinoline chelators (right).

5.1.1 Photophysical features of Re(I) tricarbonyl motifs

From a photophysical perspective, Re(I) compounds provide three main advantages over organic fluorophores for cellular imaging:

- 1) larger Stokes shifts
- 2) long-lived luminescent signals (up to 100s of μs lifetime ranges)
- 3) enhanced photostability (resistance to photobleaching)

Unlike Ln(III) complexes which exhibit similar properties to those named above, Re(I) tricarbonyl motifs are characterised by high extinction coefficients due to an allowed $\pi \rightarrow \pi^*$ MLCT excitation pathway, thus providing brighter images at lower concentrations.^{3,4}

Typical absorption spectra for $\text{Re}(\text{CO})_3(\text{N}^{\wedge}\text{N})\text{L}$ species consist of an intense, spin allowed ^1IL absorption band within the UV region, adjacent to a less intense, allowed $^1\text{MLCT}$ band within the visible region, that is known to display solvatochromism. In addition, a $^3\text{MLCT}$ band originating from a formally forbidden transition can usually be observed as a weak band at longer wavelengths than the other two.⁵ The $^3\text{MLCT}$ excited state is long-lived and can be exploited for time-resolved applications. Population of this state often occurs via absorption by a metal-centred d-orbital electron that is promoted to a ligand based π^* orbital ($^1\text{MLCT}$ excited state) and subsequent intersystem crossing to the $^3\text{MLCT}$ excited state. To observe efficient phosphorescence and facilitate the charge transfer process, a low oxidation

state metal is often combined with an easily reduced ligand, such as a highly conjugated aromatic system.⁶

Depending on the complex, excited electronic states of a Re(I) tricarbonyl species can also include halide-to-ligand charge-transfer (³XLCT) or ligand-to-ligand charge-transfer (³LLCT) processes, as well as mixing with intraligand ³IL ($\pi \rightarrow \pi^*$) states. Often, phosphorescence emission occurs from both the ³IL and the ³MLCT state, that can experimentally be distinguished by their k_r values (where ³IL character in the emissive state leads to unusually long lifetimes and/or low emission quantum yields).⁵ For instance, it is known that in Re(I) tricarbonyl bisquinoline-type complexes, emission was initially assigned to transitions originating from an IL state (fluorescence) and an MLCT state (phosphorescence) as described above, but a more recent and comprehensive study re-assigns these states to a fluorescent ¹LC/LLCT state with ¹MLCT admixture and a predominantly ³LC emissive triplet state.⁷

The luminescent properties of [ReN^N(CO)₃L]ⁿ⁺ complexes can be tuned to a certain degree, by controlling the energy gap between the ligand-based π^* LUMO and the metal-based HOMO d-orbitals. Stabilisation of the π^* LUMO orbital leads to a red-shift in the absorbance and can be achieved by adding electron withdrawing groups to the 4 or 4' position of a bipyridine N^N ligand.⁸ Similarly, the d-orbitals are highly stabilised by backbonding interactions with the tricarbonyl core. By introducing different ligands *cis* to the diamine ligands, the energy gap between HOMO and LUMO can be reduced. A recent study demonstrated, that a shift to longer wavelengths of the MLCT absorption band, going from L = acetonitrile to chloride followed by substitution of a carbonyl group on each complex by PMe₃, was achieved.⁹

5.1.2 Re(I) tricarbonyl motifs for cellular imaging

Rhenium fac-tricarbonyl compounds with tridentate bis(quinoline)-derived units initially attracted attention when they were investigated by Stephenson *et al* in 2004, who developed a new strategy to prepare peptide-based, biologically targeted rhenium complexes. They used automated solid-phase peptide synthesis to develop a so-called single amino acid chelate (SAAC) strategy, involving a Re(I)/Tc(I) binding amino acid mimic based on Fmoc-lysine that can be incorporated into any peptide position.⁹ This approach was combined with the FPR1 targeting sequence fMLF, to create Re(I) fluorescent complexes (Figure 5.2, **R1**). The compounds were visualised on the periphery of neutrophils when these were cooled, whereas at room temperature, the complex signals were observed to be internalised into the cells.¹⁰ The ^{99m}Tc analogue of the compound was prepared, and no metal dissociation in physiological media was observed after 24 hours when tested, indicating potential for *in vivo* use. The same group extended their strategy to incorporate the thiol-reactive maleimide group into the SAAC structure for biological applications¹¹ and the same bis(quinoline) ligand motif was biotinylated and complexed with both a Re(I) and a ^{99m}Tc(I) core. Subsequent uptake of the compound by avidin beads was demonstrated (Figure 5.2, **R2**).¹² Similar complexes were conjugated to glucosamine derivatives to target the glucose transporter (Figure 5.2, **R3**),¹³ combined with β -breaker

peptides for potential applications in Alzheimer disease monitoring,¹⁴ a folate derivative in order to target folate receptor overexpressing cancer cells¹⁵ and coupled with B12 which enables metal-complex vitamin B12 conjugates to be taken up via the cubilin receptor.¹⁶ Raszeja *et al* prepared an asymmetric ligand structure derived from the bisquinoline ligand shown in Figure 5.2 that formed complexes with improved photophysical behaviour (i.e. extension further into the NIR emission range, long-lived ³MLCT components and compatibility with aqueous media).¹⁷

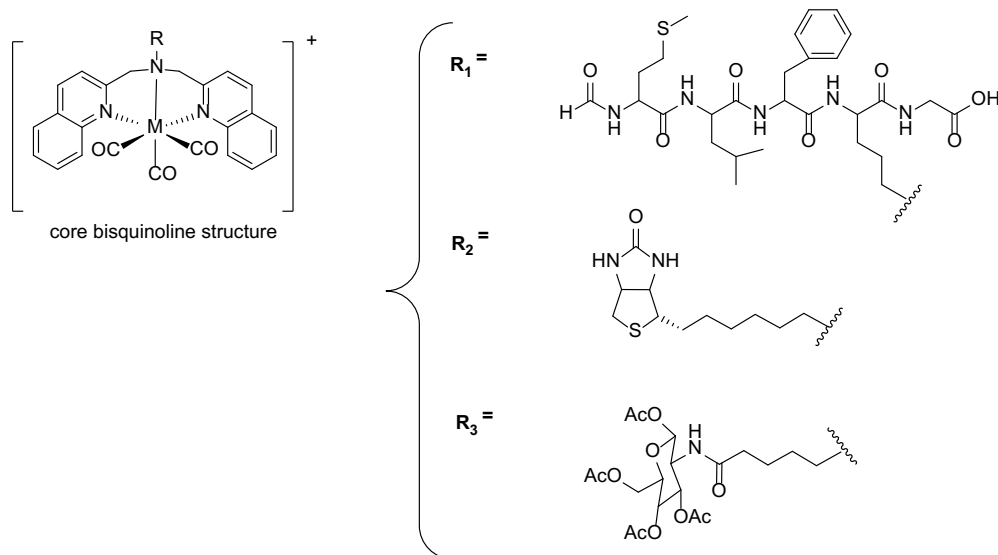


Figure 5.2: General molecular structure of the tridentate bisquinoline core (left) and examples of some *R* substituents investigated within the literature (right).^{9,12,13}

Re(I) complexes with polypyridyl ligand structures have also shown useful cell imaging properties. Amongst these structures, a diversity in cellular localisation which appears to be largely determined by compound charge and lipophilicity was found.¹⁸ For instance, Amoroso *et al* prepared a thiol-reactive Re(I) tricarbonyl chloromethylpyridyl bipyridine complex and showed that it accumulated in the mitochondria (Figure 5.3, **1**),¹⁹ whereas nuclear uptake was observed for a Re(I) tricarbonyl species that had been functionalised with acridine (Figure 5.3, **3**)²⁰ or with bombesin and an acridine fluorophore (Figure 5.3, **4**).²¹ Accumulation in the Golgi apparatus or the endoplasmic reticulum was observed by Louie *et al* for the series of biotinylated complexes they presented for binding to avidin (Figure 5.3, **2**).²² Overall, these diverse results led to systematic investigations into the principles guiding cellular localisation of rhenium tricarbonyl bisimine complexes, as conducted by Coogan and co-workers.¹⁸ They varied the charge, size and lipophilicity of the sixth metal-substituent in order to assess what effect these factors had on cellular uptake in yeast and human adenocarcinoma MCF-7 cells. Their findings suggested that cationic, lipophilic complexes preferentially tend to accumulate in cytoplasmic structures such as organelle membranes, while electrophilic species are taken up by the mitochondria. Anionic, lipophilic species on the other hand were found to be barely taken up by the cells, whereas anionic, polar compounds accumulated in the plasma membrane.¹⁸

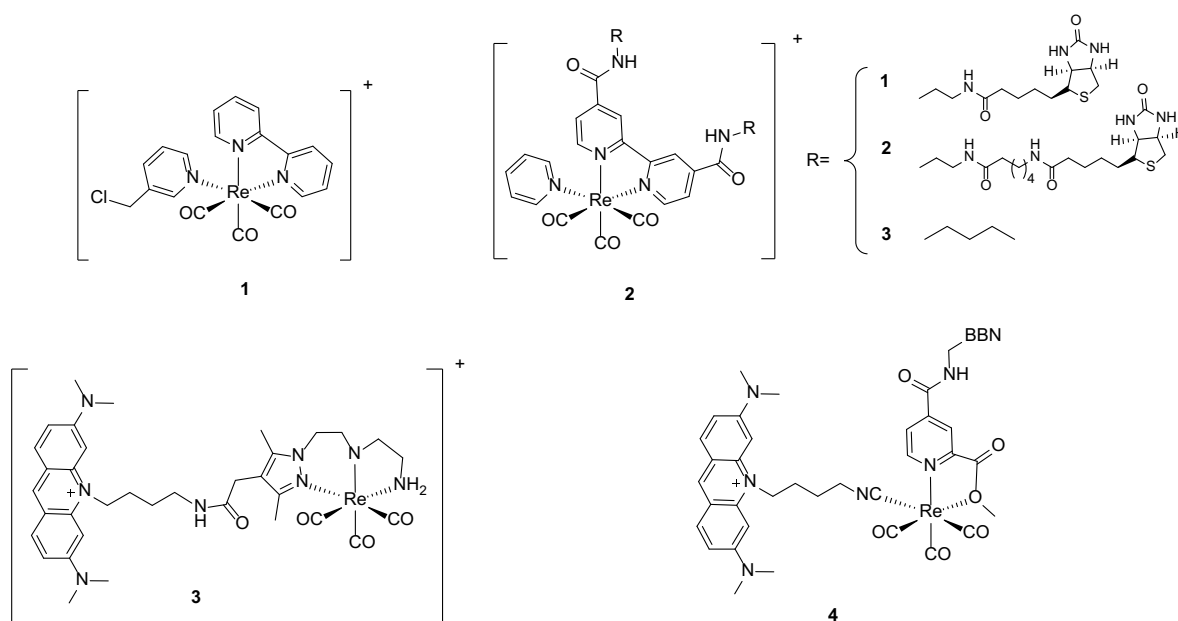


Figure 5.3: Rhenium(I)-based cellular imaging tools that localise in defined cellular organelles such as the mitochondria (**1**),¹⁹ Golgi apparatus or endoplasmic reticulum (**2**)²² or the nucleus (**3** and **4**).^{20,21}

5.1.3 Other biological applications of Re(I) tricarbonyl motifs

Apart from cellular imaging, complexes based on the Re(I) tricarbonyl motif have found numerous biologically relevant applications.

For instance, Re(I) tetrazolato complexes can act as a luminescent protein stain for SDS gels.²³ Another report details a Re(I)/^{99m}Tc(I) pair with a tricarbonyl core and a tridentate amine motif attached to a DNA-intercalating unit. The compound was used simultaneously for its radiotoxic effect which could be harnessed for therapeutic applications (when ^{99m}Tc was bound) and fluorescent imaging (upon complexation of Re(I)).²⁴ In photodynamic therapy, compounds that are non-toxic but can generate species that are toxic to malignant cells upon irradiation are exploited in order to induce cell death, usually via production of singlet oxygen.²⁵ A photocytotoxic effect has been observed for certain Re(I) tricarbonyl motifs and their anticancer activity has been shown in cells.^{26,27,28} Another therapeutic application for Re(I) complexes was introduced by means of releasing of carbon monoxide, an important signalling molecule with a variety of biological functions. The concept of carbon monoxide releasing molecules (CORMs) for therapeutic means was introduced by Motterlini *et al* in 2002, when they showed that controlled CO release from dimanganese decacarbonyl and tricarbonyldichlororuthenium (II) dimers can be explored in vascular dysfunctions.²⁹ Although less common, cytoprotective effects upon regulated CO release from Re(I) motifs have been shown in for instance cellular ischemia reperfusion models.^{30,31,32}

5.2 Aims

The aim of this chapter is to create a rhenium-based FPR2/ALX-targeted compound (**47**, Figure 5.4) that has two main purposes:

Firstly, the luminescent properties of the envisaged complex are optimised compared to the previous dye and lanthanide-based compounds. To this end a Re(I) *fac*-tricarbonyl motif was chosen, because these complexes are typically brighter than terbium-based probes (of which very high concentrations were required for visualisation in Chapter 4) due to more efficient excitation pathways. Compared to the typical lanthanide sensitisation pathway that requires short-wavelength excitation, Re(I) tricarbonyl complexes can usually be excited using lower energy sources, which makes them more compatible with cellular imaging. Furthermore, luminescence lifetimes for similar Re(I) complexes tend to be in the 100 ns to 10 microsecond ranges which is at least an order of magnitude longer than that of organic dye analogues. The lifetime scale is therefore ideal to enable time-resolved detection, but at the same time greatly increase the photon output per unit time compared to a typical lanthanide chelate.³³

Secondly, compound **47** offers an improvement from the previously described Re(I) FPR-targeted compound **R1** by introducing the non-peptide targeting group Quin C1 (Figure 5.4). The fMLF targeting group originally used is known for its potent agonist activity which is unselective for FPR receptors and internalisation of the receptor-ligand complex visualised by microscopy likely indicates GPCR activation with a downstream effect and thus unsuitability for imaging purposes.¹⁰ In addition, compound **47** should display selectivity for FPR2/ALX which would allow for more specific information on neutrophil systems to be obtained compared to using a pan-FPR targeting system.

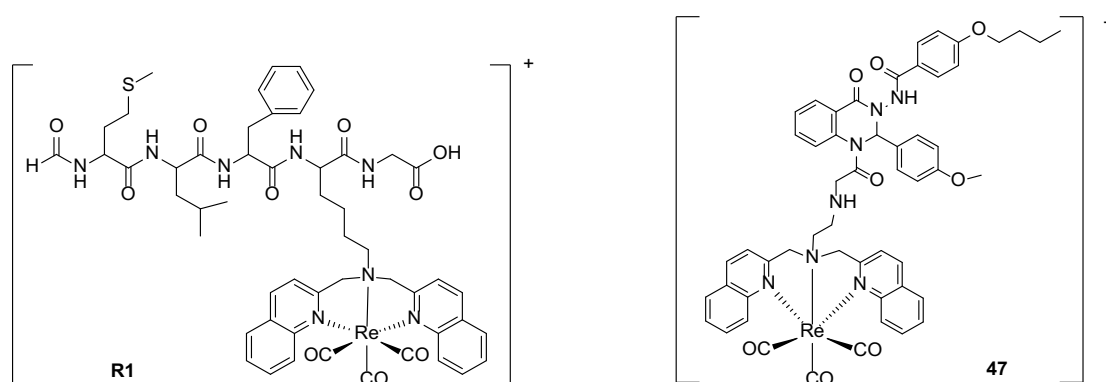


Figure 5.4: Structure of reported Re(I) FPR-targeted agent (**R1**)¹⁰ and envisaged Re(I) complex with optimised properties for time-resolved microscopy (**47**).

5.3 Results and discussion

5.3.1 Preparation of a Re(I) tricarbonyl motif for targeting the FPR2/ALX receptor

Preparation of a Re(I) tricarbonyl luminescent Quin C1- species was attempted via three distinct synthetic pathways that are summarised in Scheme 5.1:

- 1) Route 1 attempts to build up the metal-binding framework stepwise onto the targeting group (Scheme 5.1, blue).
- 2) Preparation of a halogenated metal-binding framework and combination with the targeting vector (Scheme 5.1, orange).
- 3) Combination of the metal-binding framework and targeting unit using an amine and chloroacetyl chloride bridge (Scheme 5.1, green).

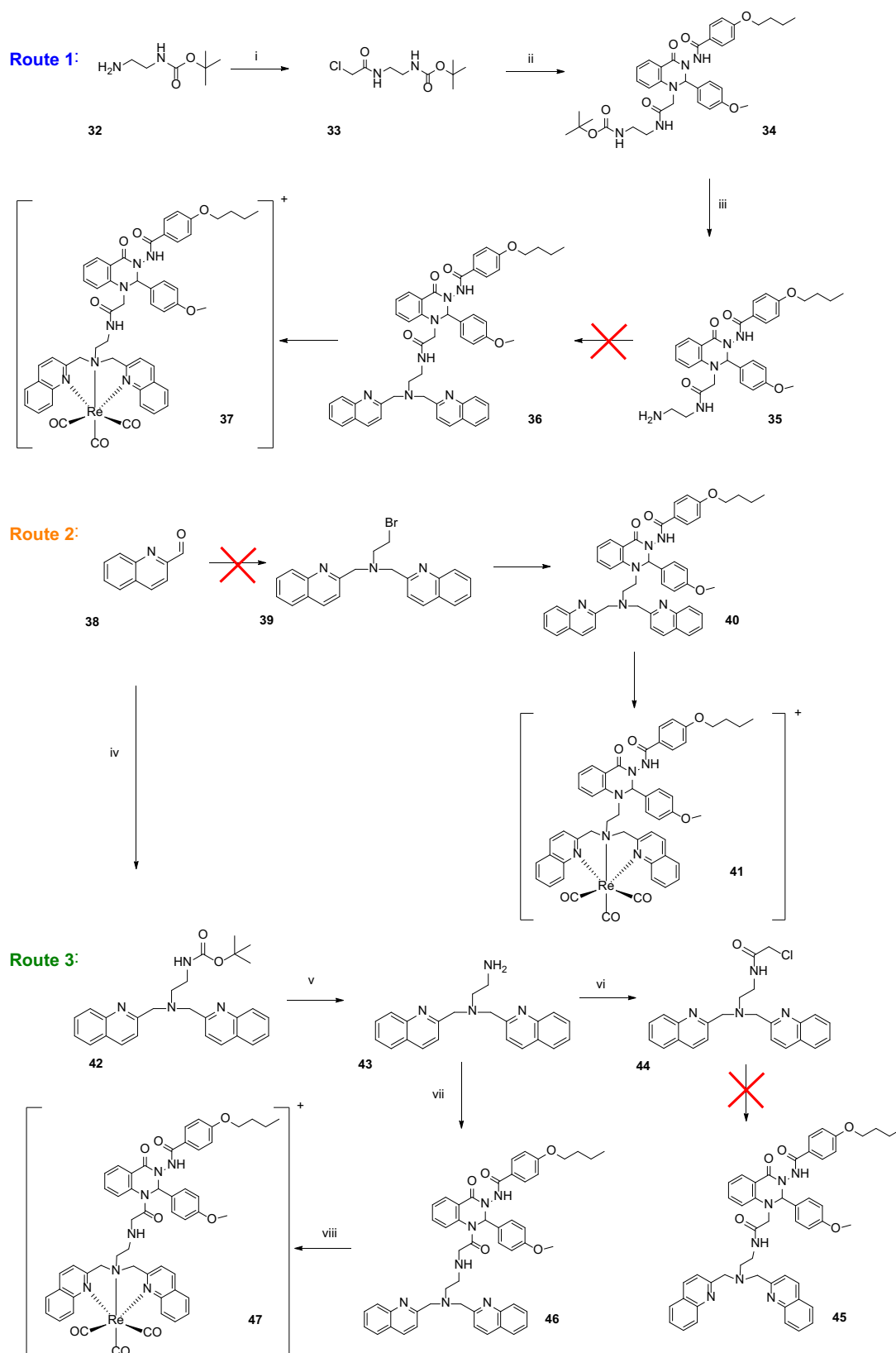
5.3.1.1 Synthetic pathway via route 1:

The initially attempted synthetic pathway was based on the strategy described in 2004 by Stephenson *et al.*¹⁰ who built up a targeting group first (in their case via peptide-based solid state synthesis) and incorporated an amine chain, followed by a reductive amination of the motif with quinoline-2-aldehyde as the final step to form the metal-binding framework.

Here, mono-BOC protected ethylenediamine (**32**) was reacted with chloroacetyl chloride to form the desired bifunctional linking group (**33**) in good yields. Next, the bridge was combined with Quin C1 (prepared as previously described), and yielded the targeting vector with an appended, protected amine group. Compound **34** could be isolated after washing steps and the terminal amine motif was subsequently deprotected using a combination of trifluoroacetic acid and dichloromethane (1:1) to yield **35**. Finally, reductive amination of **35** and **38** was attempted to form **36**. This step however resulted in numerous side products and isolation by column chromatography proved unsuccessful. Ultimately, preparation via route 1 was abandoned in favour of a different synthetic pathway, rather than optimised.

5.3.1.2 Synthetic pathway via route 2:

Instead, it was rationalised that the reductive amination step first could prove more successful than the previously attempted pathway in route 1. To this end, 2-bromoethyl amine was reacted with quinoline-2-aldehyde (**38**) in the presence of sodium triacetoxyborohydride to form **39**. Using this method, some formation of the desired compound was evident by mass spectrometry. The reaction, however, resulted in a variety of side products. Resultingly, isolation of **39** proved complicated and purification could not be fully achieved after repeated column chromatography. In hindsight, functionalising an amine arm with only one reactive functional group would have been a better choice, and the synthetic strategy was deemed not viable and revised in favour of route 3.



Scheme 5.1: Synthetic strategies towards preparation of compound **47**. Reagents: i) chloroacetyl chloride, triethylamine, dichloromethane, 70 % yield, ii) **6**, potassium carbonate, acetonitrile, 71 % yield, iii) trifluoroacetic acid, dichloromethane, 75 % yield iv) N-boc-ethylenediamine, sodium triacetoxyborohydride, dichloroethane, 77 % yield, v) concentrated hydrochloric acid, 66 % yield, vi) chloroacetyl chloride, triethylamine, dichloromethane, 24 % yield, vii) **7**, potassium carbonate, acetonitrile, 26 % yield, viii) $[\text{NEt}_4][\text{Re}(\text{CO})_3\text{Br}_3]$, methanol, 89 % yield.

5.3.1.3 Synthetic pathway via route 3:

To bypass the synthetic issues previously encountered, mono-BOC protected ethylenediamine was chosen as a side chain to enable selective sequential reactions. First, **32** was reacted on the *N*-terminus with 2-quinolinecarboxaldehyde (**38**) in a reductive amination and the desired product **42** was obtained as a pale brown oil after silica column chromatography. To achieve this, **38** and **32** were left to react for two hours, before addition of the reducing agent. Sodium triacetoxyborohydride was chosen for this purpose, as it is known to act as a mild reducing agent and the reduction of aldehyde starting material under these conditions occurs slower than the desired reduction of the iminium ion. This means, the formation of **42** from the aldehyde and the amine starting materials via the imine can be carried out in a one-pot procedure.³⁴

Characteristic ¹H-NMR signals were observed for the *tert*-butyl group (9H singlet at 1.51 ppm) and the alkyl chain at 3.33 ppm and 2.85 ppm within the spectrum of **42**, both integrating to 2H. In addition, the expected aromatic resonances were present. The two equivalent proton environments bridging the amine and aromatic groups were observed as a 4H singlet at 4.09 ppm. Finally, a peak in the mass spectrum was present at 441.2 a.m.u. which was assigned to an [M-H] ion. Deprotection of **42** was initially attempted using TFA in dichloromethane which resulted in decomposition of the compound. The method was revised towards a milder deprotection approach by reacting **42** in concentrated hydrochloric acid and a small amount of methanol (for solubility),³⁵ which yielded a pale yellow solid after washing steps. Formation of **43** was confirmed by mass spectrometry in addition to loss of the resonance at 1.51 ppm within the ¹H-NMR spectrum compared to **42**, indicating that the *tert*-butyl group had been cleaved.

Next, **43** was reacted with chloroacetyl chloride to afford compound **44**. Purification by column chromatography resulted in a pale brown solid which was evidenced by ¹H-NMR spectroscopy (see Figure 5.5). Proton signals previously observed for **42** and **43** remained comparable, although an additional proton singlet integrating to 2H appeared at 4.02 ppm, which is characteristic of the acyl CH₂ group. 2D NMR was used to further assign the protons within the aromatic region, although the correlations detected by COSY did not enable unequivocal assignment. Together with the [M+H]⁺ ion signal in the mass spectrum that displayed a typical mono-chlorinated isotope pattern, preparation of the desired product was confirmed.

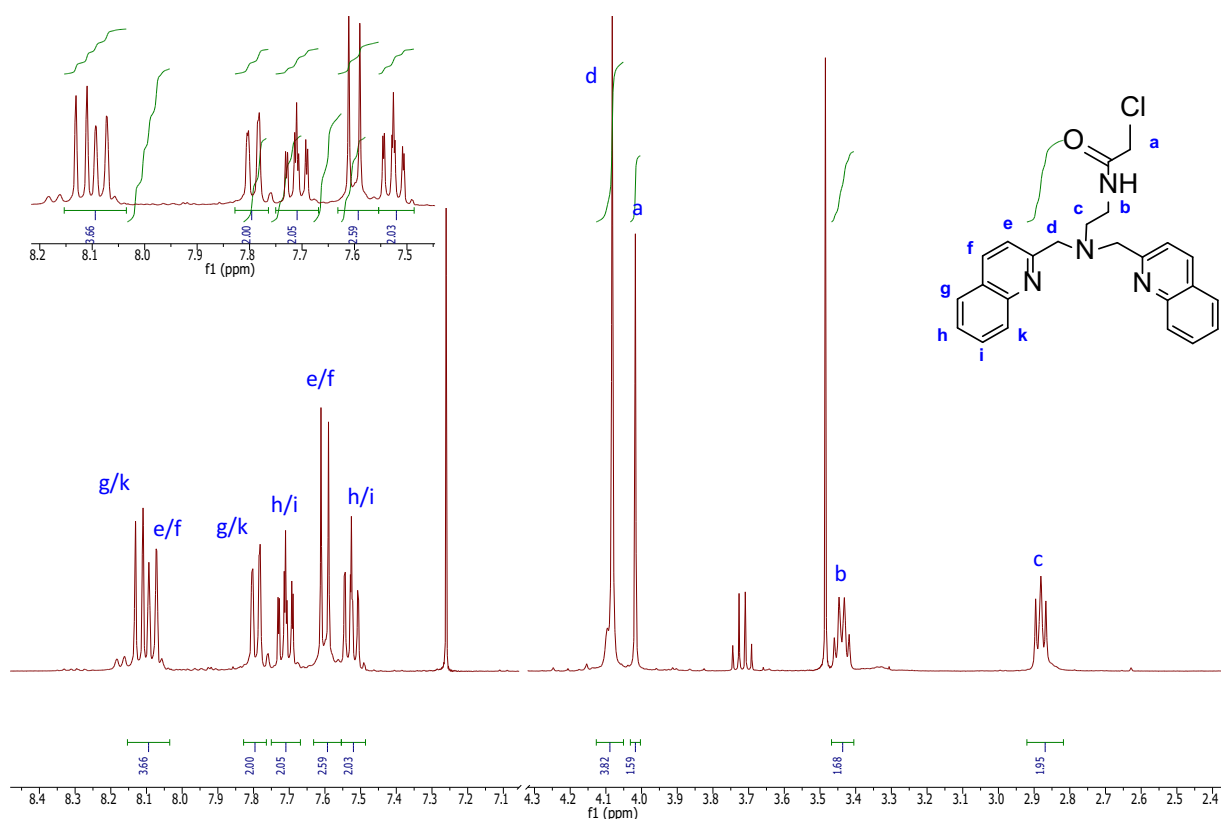


Figure 5.5: ^1H -NMR spectrum and proton assignment of compound **44** in CDCl_3 at 400 MHz and 298 K.

Compound **44** was subsequently reacted with Quin C1 under basic conditions. Following the progression of the reaction by TLC, it became evident that using potassium carbonate as a base did not result in any conversion, even after heating overnight. Instead, an excess of DIPEA was added while still heating the mixture at 60 °C. After a day, formation of the adduct (**45**) was observed by mass spectrometry (signals for the $[\text{M}+\text{H}]$ and $[\text{M}+\text{isopropyl}+\text{H}]$ ions were present at 828 and 889 a.m.u. respectively), but isolation of the product by column chromatography proved challenging and amounts eventually became too small to continue. Although not entirely successful, this route remains an option to repeat on a larger scale in the future.

As an alternative, Quin C1-chloro (**7**) and **43** were reacted under basic conditions and **46** was obtained as a yellow oil after purification by silica column chromatography. Rhenium complexation was then carried out by heating **46** and $[\text{NEt}_4]_2[\text{Re}(\text{CO})_3\text{Br}_3]$ in methanol. After leaving the mixture overnight at 60 °C, the pure complex (**47**) was isolated by column chromatography. By ^1H -NMR spectroscopy, the alkyl regions of both **46** and **47** could be assigned, and it was noted that the spectra of **47** and the uncomplexed ligand **46** appear very similar. Rhenium was bound via the quinazolinone nitrogen units which implies a shift within the proton signals of the aromatic region upon metal complexation is to be expected. However, the aromatic resonances consisted of a series of complicated, overlapping signals derived from both the Quin C1 unit and the rhenium-binding moiety and precise assignment of protons within this region was not possible. Similarly, comparison of the ^{13}C -NMR spectra showed that although most signals were similar, subtle changes within the signal shifts in the aromatic region between **46** and

47 as a result of binding to the metal were apparent. More conclusive evidence for formation of **47** was provided by appearance of a $[M+H]^+$ ion signal at 1099 a.m.u. within the mass spectrum in combination with the appearing metal-bound CO modes within the IR spectrum (Figures 5.7 and 5.8).

Vibrational $M(CO)$ modes based on the Re(I) tricarbonyl motif are typically well separated from common organic carbonyl signals and appear around the 2000 cm^{-1} region. Assuming a C_s symmetry for **47**, there are three expected, distinct $\nu(CO)$ bands, two A' modes and one A'' mode, as represented in Figure 5.6.³⁶

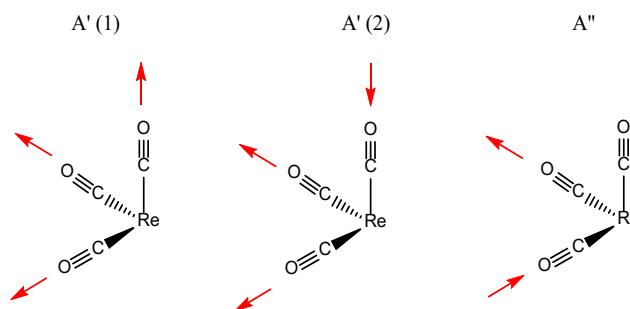


Figure 5.6: Schematic representation of IR stretching modes in a C_s symmetric Re(I) tricarbonyl species.

In higher symmetry C_{3v} $M(CO)_3$ environments, such as that of the rhenium precursor $[NEt_4]_2[Re(CO)_3Br_3]$, two $\nu(CO)$ bands (A_1 and E) are expected from group theory calculations. Comparison of IR spectra of $[NEt_4]_2[Re(CO)_3Br_3]$ and **47**, demonstrates that the three bands observed for **47** (at 1887 cm^{-1} , 2021 cm^{-1} and 2023 cm^{-1}) appear shifted to higher wavenumbers compared to the two bands observed in the rhenium starting material at 1849 cm^{-1} and 1996 cm^{-1} (Figure 5.7).

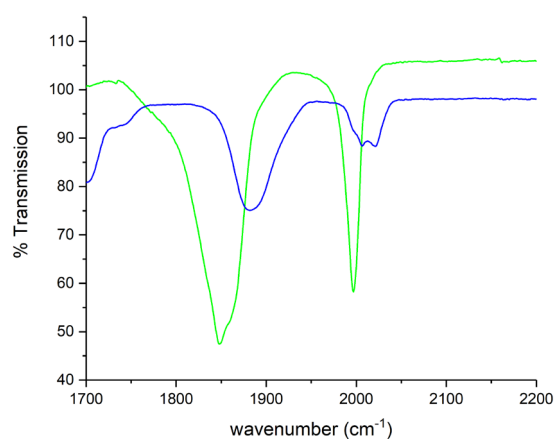


Figure 5.7: Excerpt of IR spectra of **47** (blue) and the rhenium precursor of $[NEt_4]_2Re(CO)_3Br_3$ (green), displaying three and two bands respectively.

Positions of carbonyl bands within the IR spectrum are directly related to the d-electron density around the metal centre as a result of the bonding interactions between metal and carbonyl. Increased electron

density transferred onto the metal centre by, for instance, a ligand, leads to stronger backbonding interactions, a strengthening of the metal-carbon bond and hence the associated weakening of the C-O interaction. Therefore, the shift to higher wavenumbers (and hence higher energy) is reflective of the reduced electron density on the metal centre, when bound to the bisquinoline-derived ligand compared to the three bromide donors.

The IR spectra of ligand **46** and complex **47** are overlaid and pictured in Figure 5.8. The rhenium-bound CO peaks for the complex are easily separated from organic framework-based CO signals and appear at 1887 cm^{-1} , 2021 cm^{-1} and 2023 cm^{-1} . The rest of the IR spectrum is expectedly similar to that of the ligand.

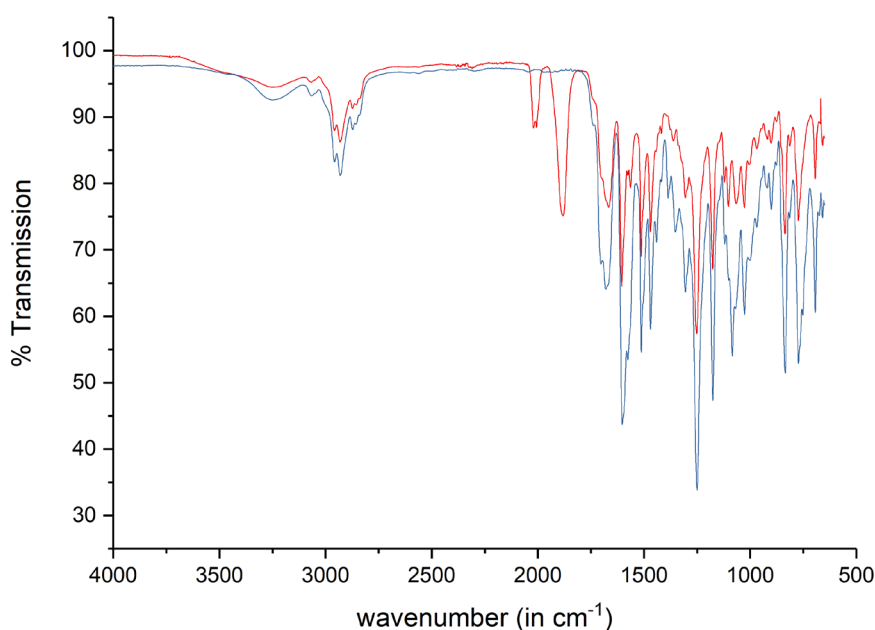


Figure 5.8: Overlay of IR spectra of **46** (blue) and **47** (red), demonstrating appearance of the typical carbonyl shifts at 1887 cm^{-1} , 2021 cm^{-1} and 2023 cm^{-1} upon complex formation.

5.3.2 Photophysical characterisation

Absorption, excitation and emission spectra of the ligand structure (**46**) and the Re(I) complex (**47**) were recorded in methanol solutions. The two absorption profiles are depicted in Figure 5.9. The spectrum of the uncoordinated ligand (**46**) is characterised by one broad, featureless absorption maximum at 269 nm that was tentatively assigned to an intraligand $\pi \rightarrow \pi^*$ transition by comparison with data reported for related complexes.¹⁷ For the rhenium complex (**47**), the most intense band was observed at 258 nm and had a similar shape to that observed for the ligand. Additional weak and overlapping absorption maxima were present within the region between 300 and 350 nm.

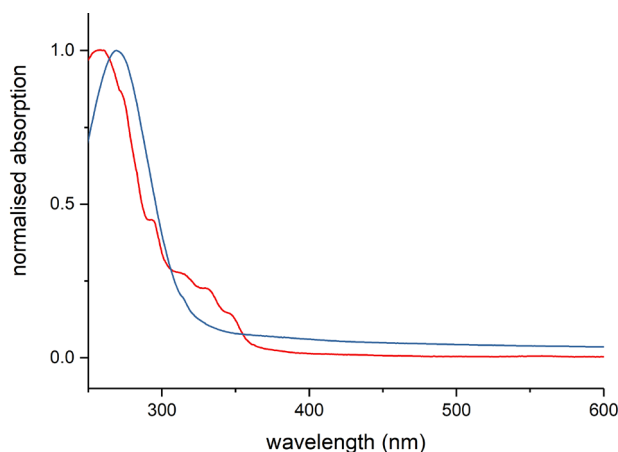


Figure 5.9: Normalised absorption spectra in aerated methanol solutions of **46** (blue) and **47** (red).

When regarding the emission spectra of **46** and **47**, it appears that the overlapping maxima within the spectrum of the rhenium-chelate (**47**) at 408 nm and 428 nm were present at similar wavelengths within the uncomplexed ligand spectrum as well, indicating these can be assigned to ligand-based transitions (Figure 5.10). Further comparison of the two emission spectra also reveals that there is a weak shoulder within the spectrum of **47** centred around 450 nm, which is not evident in the ligand-only spectrum. In combination with typical values cited in the literature and the weak nature of the band observed, this led to the hypothesis that this could be indicative of the $^3\text{MLCT}$ transition or a mixed $^3\text{IL/MLCT}$ state.⁵ In the context of cellular imaging, the lower energy, broad excitation band that extends into the visible region observed in the rhenium complex, warrants the use of longer wavelength laser (405 nm compared to the typically used 375 nm) which is less harmful to cells or leads to more efficient excitation at 375 nm compared to **Tb.30**.

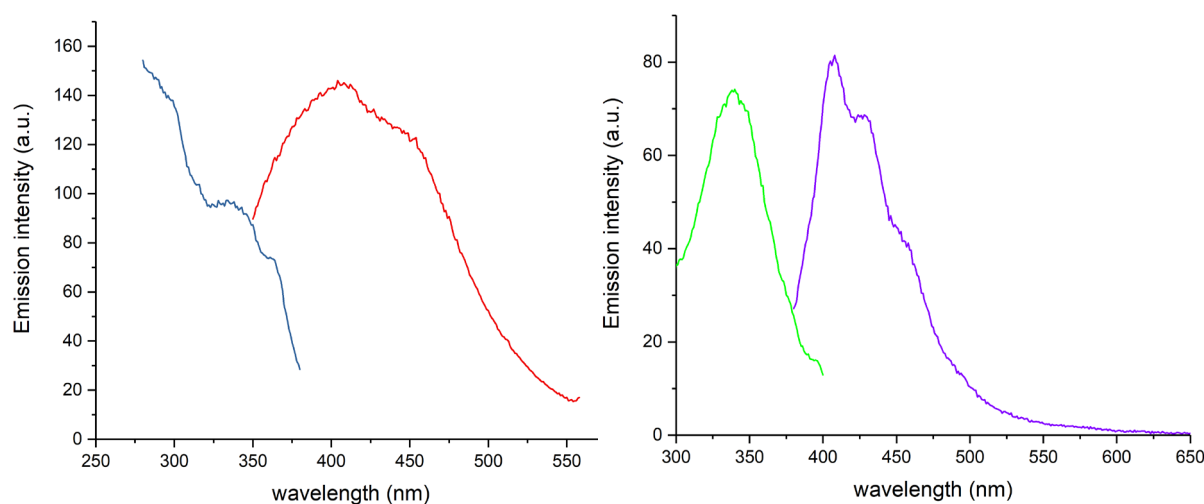


Figure 5.10: Excitation and emission spectra of **46** (left; $\lambda_{em} = 410 \text{ nm}$ and $\lambda_{exc} = 290 \text{ nm}$) and **47** (right; with $\lambda_{em} = 420 \text{ nm}$ and $\lambda_{exc} = 350 \text{ nm}$).

5.3.3 Determination of luminescent lifetimes

Long-lived excited state phosphorescent lifetimes of the Re(I) complex (**47**) were recorded in solid state and solution on the Microtime200 set-up using a 375 nm laser and a TCSPC method (with same set-up that was used for cellular lifetime measurements in Chapter 4). The data was obtained as decay curves in counts per time and fitted to a single exponential component by applying a tail fitting method. Short luminescence lifetimes are typically comparable to the instrument response and it becomes necessary to deconvolute the decay by using a numerical reconvolution algorithm to account for the finite Instrument Response Function (IRF). Here, the microscope set-up does not allow for a short-lived laser dye (in order to obtain the temporal response of the detector and account for scatter) to be used in combination with the sample and it was rationalised that the decay can be approximated to be substantially long-lived compared to the excitation source. As such, data points were only considered after $t = 0.15 \mu\text{s}$ and these could be fitted using an in-built single exponential decay function in OriginPro 8. Phosphorescent lifetimes in ethanol and solid state obtained by this method were found to be comparable. Values of $\tau = 140 \text{ ns}$ were obtained in the solid state and $\tau = 111 \text{ ns}$ in ethanol, with χ^2 values of 0.99 and 0.99 respectively. Data are illustrated in Figure 5.11.

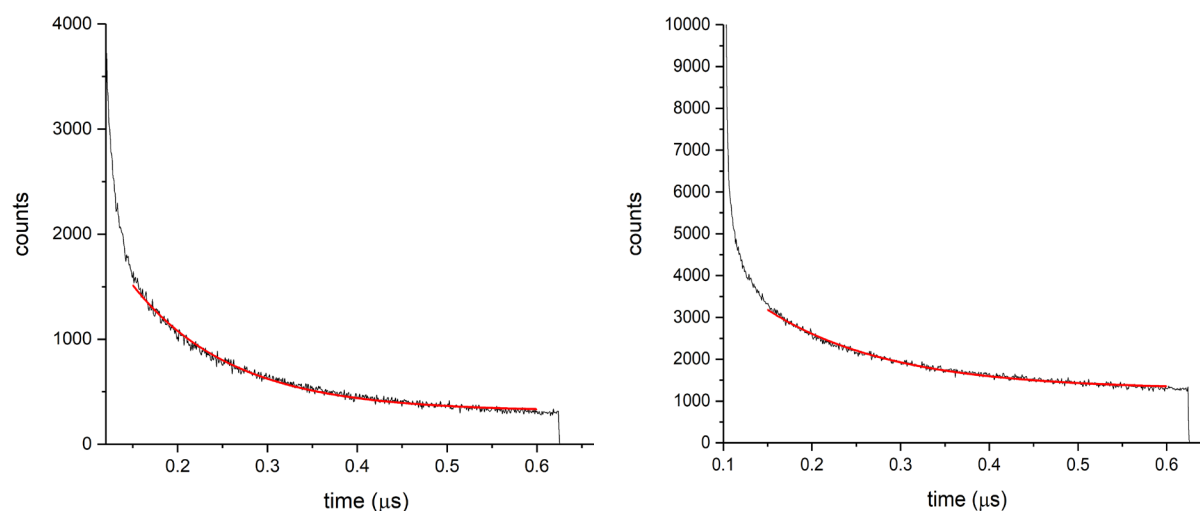


Figure 5.11: Luminescence decay data obtained for **47** in ethanol (left) and as a solid on a cover slip (right) and the single exponential fitting curves obtained (in red), enabling estimation of τ values.

5.3.4 Assessment of *in vitro* viability

Similar to the neutrophil assays described in previous chapters, the Re(I) complex was assessed for its effect on the typical neutrophil functions chemotaxis, transmigration and MPO release (see section 2.3.5 for a detailed description of the assays and quantification methods).

The data was obtained from four healthy donor samples ($n = 4$) and run in duplicate. For all three assays, a significant difference ($p < 0.05$) was observed between vehicle and LTB₄ groups. Within the stimulated groups (LTB₄ at 10^{-6} M) there is no statistically significant difference between the tested concentrations (10^{-9} M to 10^{-6} M) or the control without compound present (Figure 5.12) in any of the three assay. The data shows that the prepared Quin C1-rhenium compound (**47**) does not influence neutrophil function in any of the assays at relevant concentration ranges which is a promising basis for a cellular imaging agent.

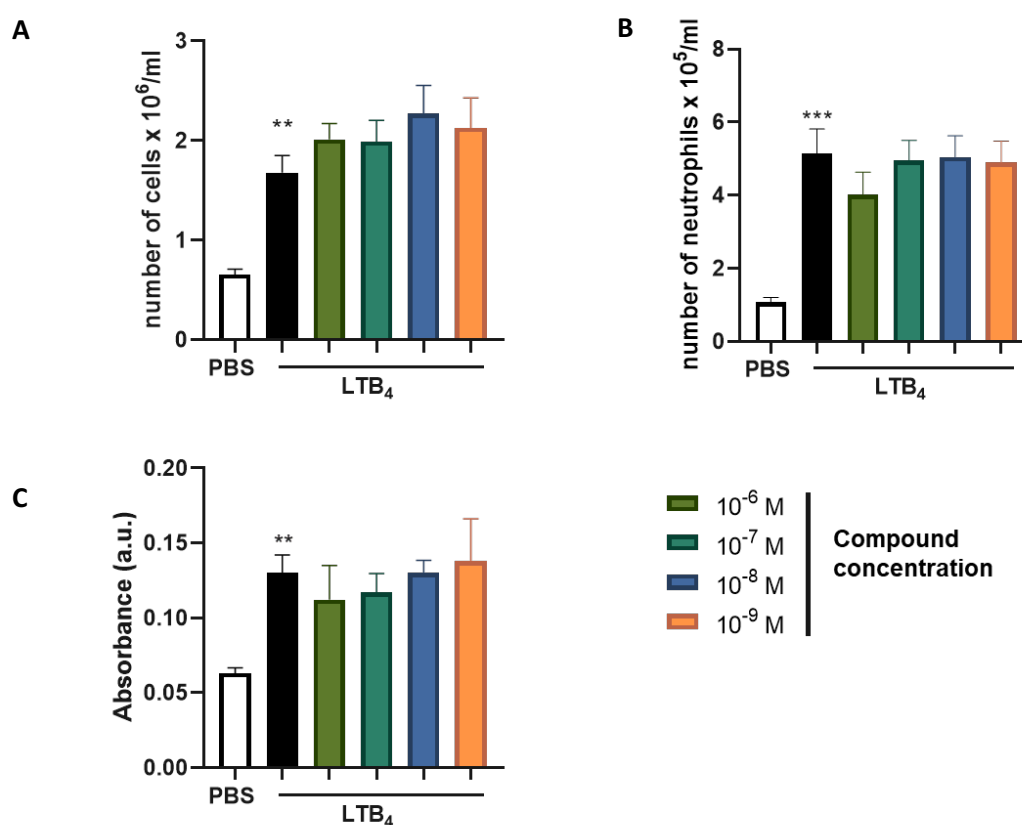


Figure 5.12: Data show *in vitro* validation using isolated human neutrophils incubated with different concentrations of **47** or vehicle. **A** shows a LTB₄ or PBS (control) stimulated chemotaxis assay. A transmigration set-up, validating migration in response to LTB₄ or PBS (control) through an endothelial cell layer is shown in **B**. Below, a stimulated (LTB₄ or PBS (control)) MPO release assay is shown that was quantified spectrophotometrically (**C**). Data are overall average $\pm$ SEM of 3 or 4 separate experiments, prepared with different neutrophil donors. Each experiment was run in duplicate. * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$ versus corresponding vehicle control group. Within the LTB₄ groups, no statistical significance was found (ANOVA).

5.3.5 Cellular imaging

Human neutrophils were obtained from healthy donor samples and pre-treated with vehicle (PBS) or TNF α (50 μ M) to induce activation. Next, the cells were incubated with **47** (at 10^{-4} M concentration) and visualised by confocal microscopy (excitation source: 405 nm laser). Representative images are shown in Figure 5.13. Cells that had been activated prior to incubation with the compound, appear to have more structure and more intense fluorescence is concentrated around the edges of the cell. This could be indicative of attachment of the fluorescent label to the cell membrane and has been previously observed with FPR2/ALX-targeted compounds in Chapters 3 and 4. The unstimulated sample appeared fluorescent with no distinct morphology and cell structures were blurred, suggesting that the signal in the unstimulated group may be due to autofluorescent background. Overall the fluorescence intensity observed was very low, possibly a result of inefficient excitation at 405 nm.

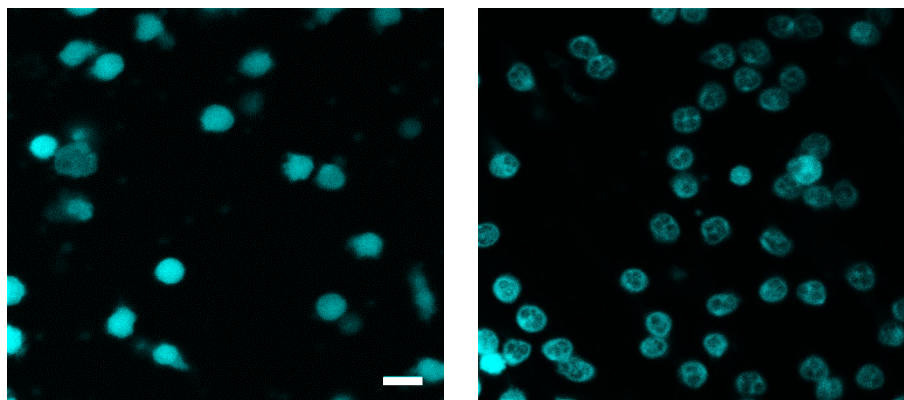


Figure 5.13: Representative images from confocal microscopy of the unstimulated neutrophil group incubated with 10^{-4} M of **47** (left) and TNF α -stimulated samples incubated with 10^{-4} M of **47** (right). Scale bar 10 μ m.

To obtain time-resolved cellular images, murine neutrophils were pre-treated with TNF α or vehicle as above and incubated with **47**, before visualisation using a PicoQuant Microtime 200 time-resolved confocal fluorescent microscope. Images were run at the University of Lancaster by Dr Michael Coogan. Compound **47** was designed to increase the brightness of the complex compared to Tb.**30** by optimising absorption properties (making the compound more compatible with the 375 nm excitation setup) and increasing the extinction coefficient. The magnitude of the rhenium-centred lifetime obtained in solution (~ 100 ns, see above) suggests that a clear separation from autofluorescence should be possible, while the photon flux per unit time has increased compared to a typical lanthanide complex with lifetimes in the ms range. For these reasons, in this preliminary study cells were incubated with 10^{-4} M concentrations of **47** which is a tenth of the amount used for the lanthanide complex in Chapter 4 and therefore a lot more compatible with a cellular context.

Murine neutrophil samples that had been stimulated with TNF α were visualised using TCSPC methods according to luminescence intensity in counts (Figure 5.14, **A**) as well as lifetime mapped (FLIM). Representative images recorded of the same cell are shown in Figure 5.14.

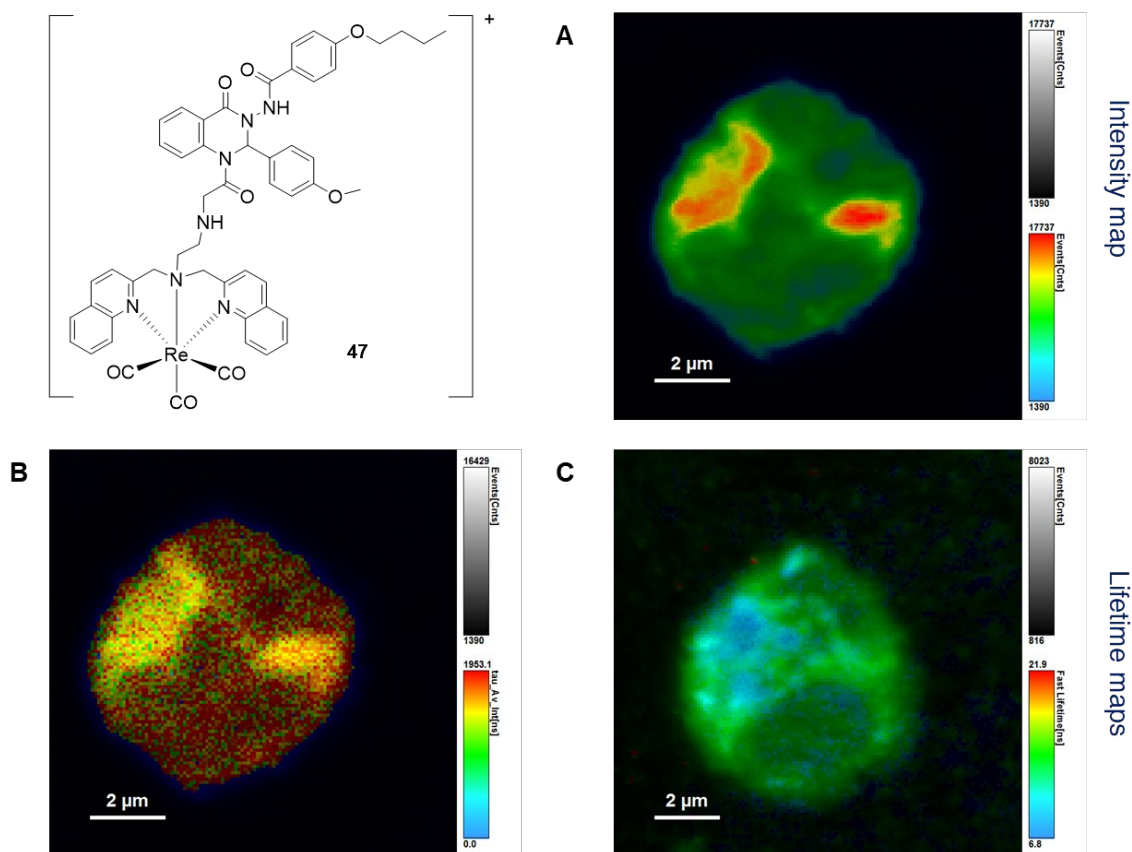


Figure 5.14: Molecular structure of compound **47** and representative images of a murine neutrophil obtained using a time-resolved confocal microscope to visualise uptake of **47** in stimulated cells as an intensity map (**A**), as lifetime maps in burst mode (**B**) and using normal excitation (**C**). Excitation source was a 375 nm laser.

In the intensity map (Figure 5.14, **A**), fluorescence emission is observed throughout the cell, with two areas of concentrated signals appearing in the centre of the neutrophil. In the lifetime maps, data acquired without a laser burst mode contained no significant long-lived luminescence components and autofluorescence dominated (Figure 5.14, **C**, average pixel lifetimes in the 0 – 20 ns range). Upon application of a pulsed laser in burst mode, emission in the 100 ns - 2 μ s range, indicative of rhenium-derived luminescence, was observed. As a control to ensure that the burst mode did not create long-lived artefacts, neutrophil samples that had not been incubated with **47** were visualised with the same settings and no long-lived emission was found. Interestingly, the metal-based lifetime observed in the cell is longer than that observed in the solid state or in ethanol solutions. In image **B**, long-lived components seem to be equally distributed throughout the cell, indicating no specific cellular localisation

of **47**, which is in contrast to observations previously made in TNF α -stimulated human neutrophils using Tb.**30** (see Chapter 4).

Samples that had not been activated prior to incubation with **47** were found to contain less observable long-lived decay signals, confirming increased uptake of the Re(I) compound in activated cell samples, which has previously also been demonstrated for Tb.**30**. Overall, this proof of principle study suggests that **47** is the more appropriate probe to generate time-resolved images of neutrophil FPR receptors.

5.4 Conclusions

Preparation of a Re(I) *fac*-tricarbonyl species chelated to a bifunctional molecule incorporating both a bisquinoline-derived unit for binding rhenium and a small-molecule FPR2/ALX targeting group is described. The compound was fully characterised and analysed for its abilities to act as an imaging agent. Long-lived phosphorescent emission in combination with excitation and emission wavelengths within the biocompatible range (375 nm laser excitation) makes it a promising candidate for cell imaging studies. Neutrophil functional assays reinforced the usefulness of **47** for FPR imaging applications, as the presence of the compound did not alter cellular properties at relevant concentrations.

Preferential uptake of the targeted compound **47** in neutrophils that had been activated using TNF α compared to neutrophils in an unactivated state was observed. By confocal microscopy, regions of higher luminescence intensity were observed on what appears to be the cellular membrane of human neutrophils, consistent with observations made in Chapter 4.

Time-resolved microscopy of murine neutrophils using a TCSPC setup produced images that contained long-lived phosphorescent signals corresponding to the metal components. Uptake was only visualised in stimulated cells, where the compound appeared evenly distributed throughout the cell with no specific localisation observed. In this aspect, the study differs from what was previously demonstrated using human neutrophils. Another notable point is that cellular-based Re(I) emission lifetimes were recorded up to around 2 μ s, which is an order of magnitude longer than for the same compound in solution. These results provide a promising basis for FPR2/ALX-targeted, time-resolved applications of the compound, which can be used to study the receptor roles in disease states.

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Chapter 6

Towards FPR2/ALX-targeted complexes for LRET and dual-modal applications

6.1 Introduction

Fluorescence imaging techniques that make use of energy transfer processes between a donor and an acceptor molecule have become increasingly important in the literature.^{1,2,3} One huge advantage of such techniques is that they allow for optimisation of the imaging process by tuning photophysical behaviour, in addition to providing information on nanometer scale distances between the lumiphore pair.⁴

6.1.1 Lanthanide-based luminescence resonance energy transfer

Using the same principles described for FRET (Chapter 1, section 1.3.2) but substituting the dye donor segment with a lanthanide chelate that provides long-lived signals has several advantages over using two organic fluorophores. Lifetimes of lanthanide complexes are in the millisecond range and hence can be separated from background and acceptor-only signals using time-gating techniques.⁵ Further, unpolarised donors with long lifetimes, such as luminescent terbium chelates, are likely to rotate within an emission lifetime and thus enable the error factor introduced into FRET pairs by κ^2 to be minimised. Taken together, these factors make the distance determination via lanthanide-based luminescence resonance energy transfer (LRET) more accurate and thus more suitable for biological applications than FRET.^{4,6}

In lanthanide-based LRET systems the total emission is a combination of two energy transfer processes: Firstly, the sensitisation process of an organic antenna unit to a lanthanide and secondly the energy transfer from the lanthanide donor to an organic acceptor unit. The factors involved in this process are summarised in Figure 6.1.

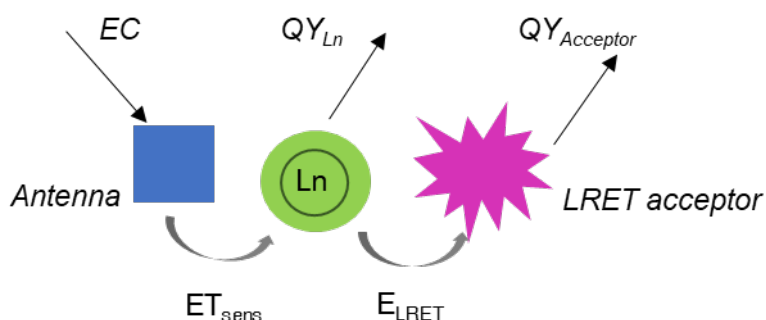


Figure 6.1: Schematic representation of processes determining emission in lanthanide-based luminescence resonance energy transfer systems. Total emission consists of EC = extinction coefficient, ET_{sens} = efficiency of lanthanide sensitisation energy transfer, E_{LRET} = efficiency of LRET, QY = quantum yield.⁷

Another way to look at the energy transfer process is to consider the changes in luminescence lifetimes. Typically, the donor lifetimes are within the millisecond range, whereas the organic acceptor molecule fluorescence lifetimes are within the nanosecond regime. The presence of an energy transfer process

can be detected by a reduction in donor excited state lifetime, but also by long-lived, sensitised acceptor emission, as illustrated in Figure 6.2.^{8,1}

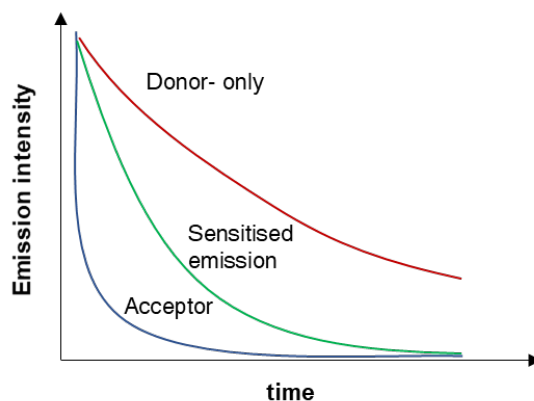


Figure 6.2: Schematic representation of donor-only (red), acceptor-only (blue) and sensitised emission (green) lifetimes in an LRET system.^{8,1}

6.1.2 LRET processes for distance determination

As discussed above, lanthanide-based LRET can be a powerful technique that has been previously applied to various distance measurements within biological systems and as such can provide insights into their mechanistic details.

Equations to describe distance dependence of donor and acceptor molecule (R) were introduced in Chapter 1 and are given below.^{8,4}

$$R = R_0 \left(\frac{1}{E} - 1 \right)^{-6} \quad (\text{eq 6.1})$$

$$R_0 = \left(\frac{8.785 \times 10^5 \kappa^2 \Phi_D J}{\eta^4} \right)^{\frac{1}{6}} \quad (\text{eq 6.2})$$

Where E is the efficiency of the energy transfer, R_0 is the Förster radius, κ^2 expresses the orientation factor between the lumiphores, Φ_D is the donor quantum yield, J defines the spectral overlap between donor emission and acceptor absorbance and η is the refractive index of the medium.^{8,4}

In 1999, Cha *et al* reported a method to measure voltage-induced distance changes of potassium ion channels and were able to establish a link between electrical charge and movement within the channel using lanthanide-based LRET. To achieve this, cysteine-reactive maleimide terbium chelates (as the donor fluorophore) or fluorescein-maleimide (as the acceptor fluorophore) were introduced into specific sites within the channel. Based on measured distance changes they were further able to suggest that a region associated with one of the segments within the transmembrane protein undergoes a rotation and a possible tilt in response to the electrical stimulus.⁹ Led by Selvin and co-workers, the S4 segment

movement within these ion channels was characterised via distance measurements obtained from LRET pairs and inconsistencies within one of the existing models of voltage sensors were exposed, suggesting an entirely different mechanism to what was initially thought was occurring.¹⁰

Previously, Selvin *et al* had already demonstrated the use of LRET pairs to elucidate the geometry of DNA double-helix formation, by including a rhodamine acceptor unit and a terbium-DTPA chelator unit in one strand each and measuring lumiphore lifetimes and emission intensities (Figure 6.3).¹¹

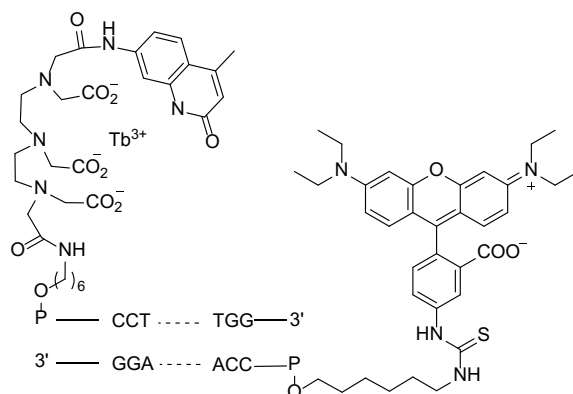


Figure 6.3: Molecular structure of terbium-DTPA chelator donor system combined with a rhodamine-derived acceptor included in DNA strands.¹¹

The study of protein-protein interactions is essential to a number of cellular processes which makes understanding their patterns and characterising the occurring interaction in detail a subject of ongoing interest. In this context, a luminescent terbium complex (TMP-Lumi4, Figure 6.4) was delivered intracellularly and observed to bind to a particular protein (eDHFR). The study made it possible to confirm interactions between the bound terbium probe and a C terminal protein motif fused to green fluorescent protein (GFP), with a six-fold intensity increase compared to the control group with non-interacting mutants (which is huge compared to the ~10 % increase expected from organic-based FRET interactions).¹² Along the same lines, application of LRET sensing in proteins genetically encoded to express acceptor and donor units was investigated. Specifically, terbium-luminescence was used to excite green or red fluorescent proteins (in a “multicolour” LRET) and applied to molecular chaperone pairs to demonstrate the impact of single point mutations on interactions *in vitro*.¹³ Reports of measuring protein synthesis via LRET reporters in real-time,^{14,15} detecting ligand binding to cell surface receptors¹⁶ and monitoring GPCR oligomerisation events¹⁷ have further extended the scope of this technique to relay functional information on cellular processes.

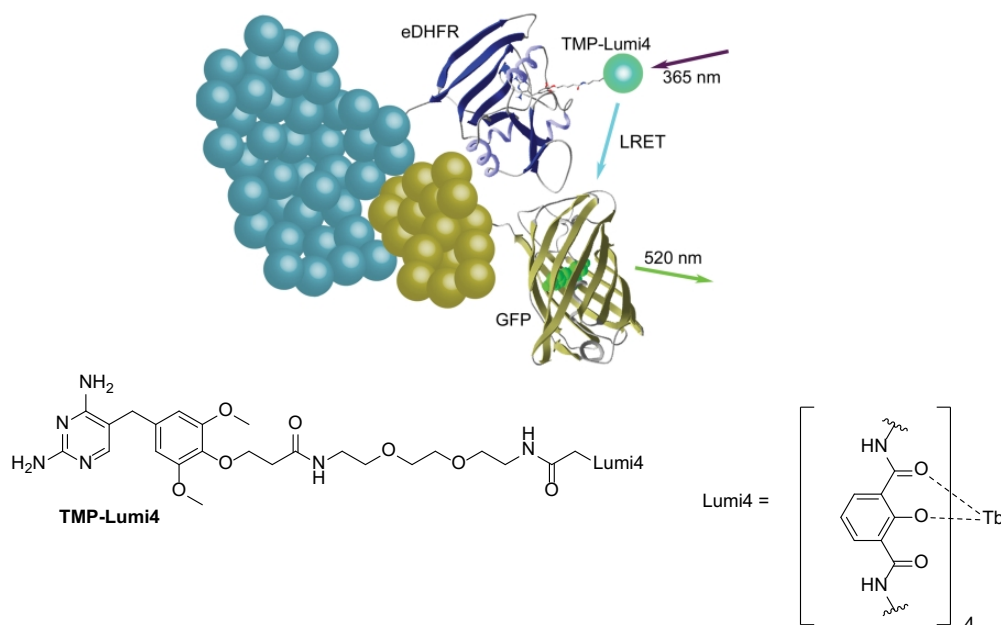


Figure 6.4: Molecular structure of TMP-Lumi4 (top) and schematic of how this compound was used to establish protein-protein interactions by determining LRET to GFP (below). Image was reproduced from reference with permission.¹²

6.1.3 Improving optical imaging of lanthanide-lumiphores in live cells via LRET

The idea behind using lanthanide chelate millisecond range emission lifetimes, is to enable extremely sensitive luminescence detection by time-resolved microscopy techniques in biological systems that are otherwise swamped by diverse autofluorescent background signals. In practice however, little or no improvement over conventional fluorescent techniques is often observed and lanthanides are still not frequently used for biological imaging.^{7,18} Two of the main factors that have severely limited the expected boost in lanthanide-based cellular microscopy are the low photon flux, resulting from the long lifetimes and associated low emission rates and inefficient excitation pathways with conventional, cell-compatible excitation sources (i.e. above ~350 nm).¹⁹ These factors have led to the need for various imaging cycles that are impractical for the scales of biological processes.^{20,21}

To address these issues, a new modality for time-resolved lanthanide detection was recently developed.⁷ The technique provides an improved optics-derived background factor and illumination source (by utilising Q-switched laser illumination). In addition, Cho *et al* report the use of LRET to circumvent the parity forbidden f-f transition to the lanthanide ground state. Such behaviour was achieved by using spectrally matched acceptor molecules to provide a faster radiative deactivation pathway and thus attain an overall 170-fold increase in lanthanide-based signal intensity. Specifically, a Eu(III) ATBTA complex (Figure 6.5) was combined with an organic dye (Atto 610, Figure 6.5), which resulted in a substantial reduction of emission lifetimes. As a result, the luminescence intensity was greatly increased and LRET enhanced images were reported to have a 50-fold increase in pixel intensities when compared to time-resolved microscopy with just the lanthanide. The combined

lanthanide/fluorophore method was therefore shown to enable removal of background fluorescence via time-gating, while at the same time the shorter excited state lifetime and alternative radiative deactivation pathway greatly increased the compounds' brightness.

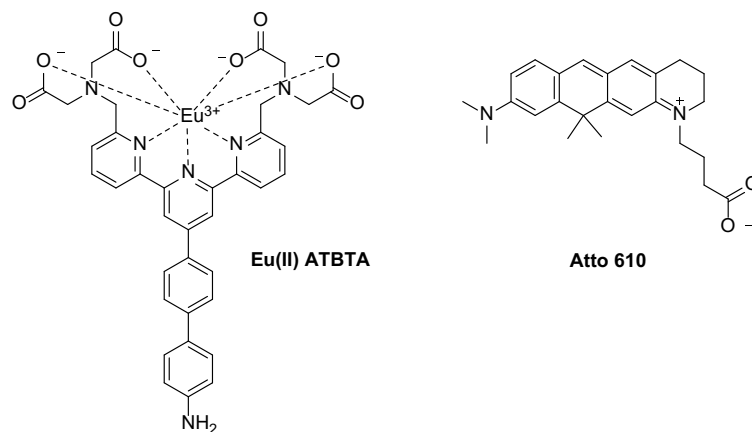


Figure 6.5: Molecular structure of Eu(III) ATBTA (left) and the complementary organic dye Atto 610 (right) reported by Cho et al.⁷

Conceptually, exploiting an LRET to increase brightness of a lanthanide-based probe for images is very different from previous reports investigating distances between the donor and acceptor unit and provides an innovative approach to circumvent practical issues and provide more useful lanthanide-based cellular probes.

6.2 Aims

The aims of this chapter are to provide a synthetic strategy to incorporate three components into one molecule: a targeting group and two distinct imaging moieties. This strategy brings together the previous chapters by incorporating an organic fluorophore, a lanthanide ion as well as the investigated FPR2/ALX targeting unit. Rhodamine B, the selected fluorophore, is spectrally well-matched with terbium as an LRET-pair. It is however not ideal for energy transfer in combination with europium due to the metal excited state energy.

Preparation of the resulting complexes incorporating different lanthanide ions are expected to provide a synthetic pathway towards targeted, dual-emissive probes that can give rise to interesting energy transfer processes upon incorporation of terbium ions. Complexation of Eu(III) allows assessment of proton environments, while gadolinium complexes can be utilised as potential dual-modal (MRI and optical) imaging agents (as illustrated in Figure 6.6).

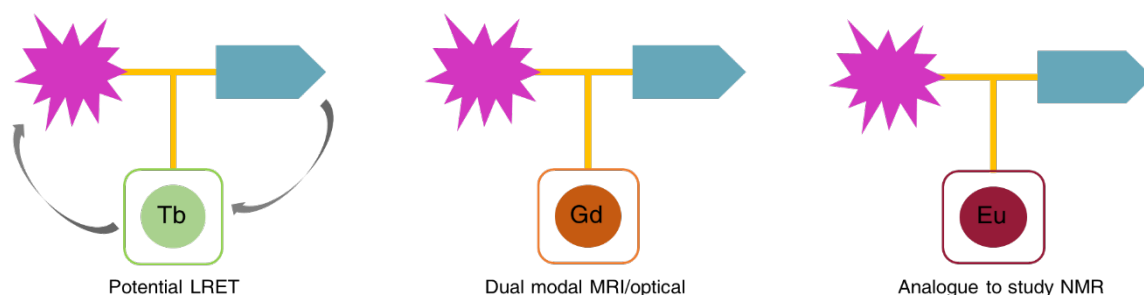


Figure 6.6: Schematic representation of the three envisioned, tri-functionalised compounds this chapter aims to prepare by complexing terbium, gadolinium and europium and their potential functions.

In addition, the construction of the terbium compound is informed by issues previously encountered when attempting time-resolved imaging in cells (i.e. low compound brightness) and thus this iteration of FPR-targeting lumiphore is expected to provide long-lived emission that is brighter than the lanthanide-only analogues.

6.3 Results and discussion

Several molecules combining a terbium donor lumiphore and an acceptor rhodamine unit were prepared to investigate factors controlling LRET processes. Initially, variation of distance between donor and acceptor molecules was examined by using different linker chain lengths between rhodamine and terbium sites and the effect on the resulting energy transfer was assessed. Next, two donor fragments within the same molecule, to increase the amount of donor-emission, were introduced.

6.3.1 Dual luminescent compounds

The Förster resonance energy transfer efficiency is characterised by a distance dependence (see section 6.1.2) and typically decreases as the distance between donor and acceptor lumiphore increases. This statement holds true for intensity measurements using a pair of short-lifetime fluorophores but can be more complicated when looking at resonance energy transfers using time-gated detection. For instance, exceptionally efficient energy transfer could imply that the process is completed prior to emission intensity detection in a time-gated measurement.²² Accordingly, the energy transfer process was investigated with varying proximity of the lumiphore pair.

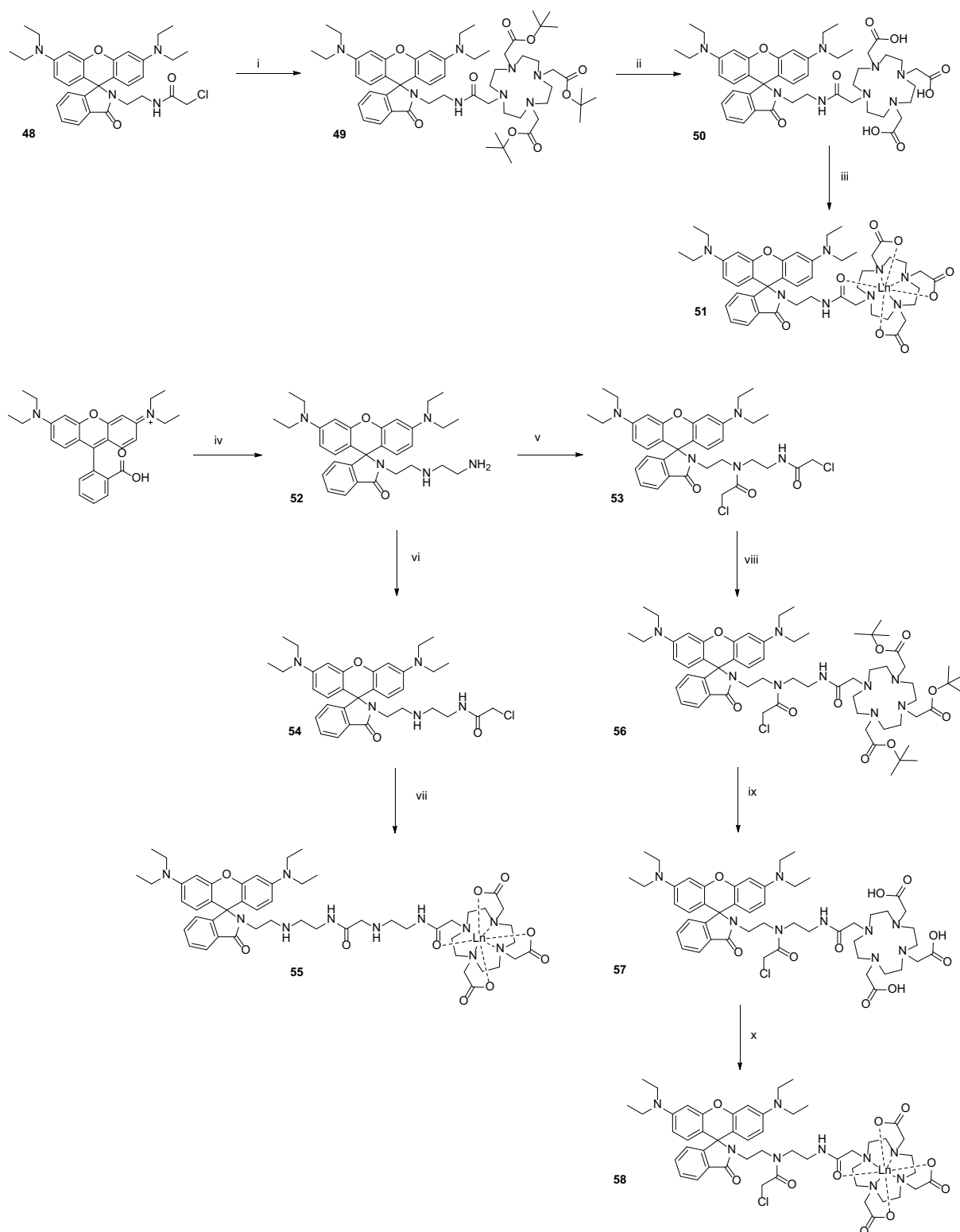
The pH-switchable rhodamine B fragment was chosen to gain an insight into the LRET processes, as this provides a means to study the same system with the acceptor fluorophore either in an “on”, ring-opened state at low pH, where absorption around 560 nm prevails, or in an “off”, cyclic state under neutral and basic conditions, where no absorption occurs at 560 nm. This allows for a unique method to distinguish donor signals from donor-excited acceptor luminescence. A further point to note within this LRET system is the excitation pathway of the terbium donor which typically requires sensitisation via an antenna unit. In Chapter 4, it was demonstrated that the targeting vector Quin C1 is able to sensitise a terbium ion. However, in systems containing just the rhodamine acceptor fluorophore and terbium chelates, it remains to be determined whether efficient lanthanide sensitisation arises. The lowest energy triplet states, which are considered most relevant for sensitising lanthanide emission, have been reported for rhodamine B both in its “open” and “closed” form in the literature. The fluorescent form has a T_1 state energy value recorded as $13,953\text{ cm}^{-1}$, which is considered too low in energy to efficiently sensitise terbium emission from the $^5D_4$ state at $20,500\text{ cm}^{-1}$.²³ The spirocyclic form has a significantly higher triplet-state energy level of $27,100\text{ cm}^{-1}$ which is expected to lead to more efficient terbium sensitisation.^{23,24}

Three distinct rhodamine B-terbium complexes were prepared, one with a short distance between the two lumiphores (**51**, Scheme 6.1), one with an intermediate chain length (**58**), and a compound with a long linker connecting the two units (**55**). It is notable in this context, that compound **51** provides no sites for further functionalisation along the chain and thus is not available as a fragment for the tri-functionalised compound, whereas the longest chain provides the most straightforward synthetic route towards a molecule incorporating all three functionalities.

6.3.1.1 Preparation of complexes with different linker lengths

The synthetic pathway towards each of the donor/acceptor compounds is summarised in Scheme 6.1. The terbium and gadolinium analogues of the shortest chain length conjugate (**51**) have been previously prepared within the Long group and the gadolinium-based version was used as a dual-modal tumour imaging probe.²⁵ The report by Rivas *et al* illustrated that at pH 7.4, efficient terbium-sensitisation occurs via the spirocyclic rhodamine species (as predicted by reported triplet state energies) and further demonstrated that a small change in lanthanide-rhodamine linker length upon going from structure **51**, to a heptadentate DO3A complex directly attached to the ethyl linker, substantially altered sensitisation efficiency. However, no lanthanide-based energy transfer to the acceptor rhodamine was observed, which is unsurprising at the reported pH values of 7.4, as the rhodamine unit is predominantly in its cyclic, non-fluorescent form and hence does not absorb at the relevant wavelength. Here, the same molecule is investigated at pH 3, which results in a significant LRET between the complexed terbium and the rhodamine unit, but possibly compromises efficient lanthanide sensitisation. Preparation of **51** was conducted according to published protocols.²⁵

In order to form the two longer linkers, diethylene triamine was combined with rhodamine B in ethanol to produce a chain containing two amine sites for further functionalisation.²⁶ The desired products were obtained in high yields and conveniently no purification was required as the volatile side products were removed by aqueous washes and under reduced pressure. Compound **52** was acylated using chloroacetyl chloride to produce two different products- the mono-chlorinated species (**54**) and the doubly chlorinated species (**53**). Reaction conditions could be tuned to favour the doubly chlorinated species over the mono-chlorinated species by adjusting stoichiometries and repeated preparation of **53** was treated that way. In this case, both products proved useful and were separated by column chromatography to afford **54** in 58 % yields and **53** in 24 % yields. Compounds **53** and **54** were observed by HRMS with the expected chlorination isotope patterns.



Scheme 6.1: Synthetic pathway towards preparation of rhodamine-terbium constructs with different chain lengths. Reagents used: i) *tert*-butyl DO3A, triethylamine, acetonitrile, 54 % yield, ii) trifluoroacetic acid, dichloromethane, 65 % yield, iii) Ln(III) trichloride hexahydrate, water pH 5.5, iv) ethylene triamine, ethanol, 76 % yield v) and vi) chloroacetyl chloride, triethylamine, dichloromethane (58 % mono-addition, 24 % double addition) vii) Ln.**29**, potassium carbonate, acetonitrile (Ln = Eu 43 %, Ln = Tb 17 % yields), viii) *tert*-butyl DO3A, triethylamine, acetonitrile, 79 % ix) concentrated hydrochloric acid, 48 % yield x) Ln(III) trichloride hexahydrate, water pH 5.5 in ~90 % yields respectively.

To identify exact sites of substitution of the chloroacetyl chloride species on the amine backbone, ^1H -NMR spectroscopy in combination with correlations detected by NOESY were utilised. In the doubly-substituted species, correlations between the amide hydrogen and one of the $-\text{CH}_2\text{-Cl}$ singlet resonances (at 3.90 ppm) were observed, as seen in Figure 6.7. At the same time, a cross-peak between the second singlet at 3.78 ppm and the proton environments assigned to H_b and H_c established that the two groups had substituted onto distinct amine functionalities rather than both attached onto the terminal amine. For the mono-substituted chlorination product (**54**), attachment to the terminal, primary amine was ascertained by the absence of correlations detected between the chloroalkyl group and H_b or H_c within the NOESY spectrum.

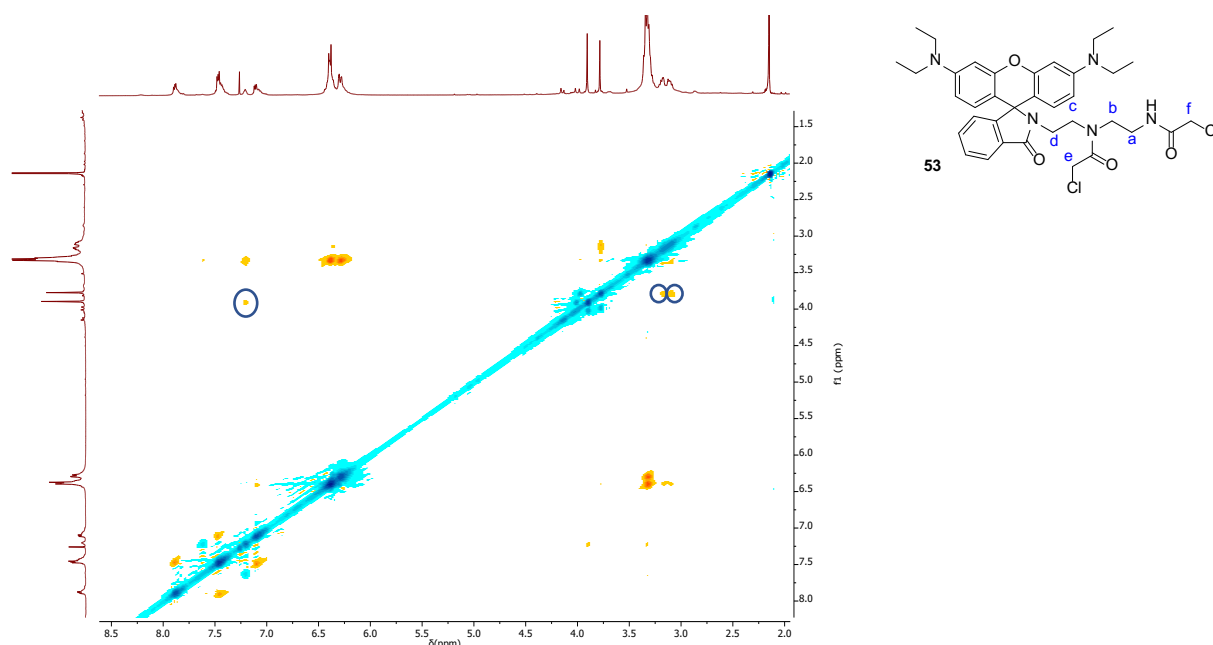


Figure 6.7: NOESY spectrum and molecular structure of **41** in CDCl_3 run at 400 MHz and 298 K. Relevant cross-peaks that enabled structural assignment are circled.

The doubly chlorinated product was used to prepare compound **58**. Addition of DO3A *tert*-butyl triester to **53** under basic conditions and reflux overnight resulted in two major products: the macrocycle was either appended to one of the functional sites to form the mono addition product, **56**, or substituted both chlorinated sites to form the double addition species (**59**, Scheme 6.2). The two compounds were both isolated by column chromatography and assigned by high resolution mass spectrometry. By ^1H -NMR spectroscopy, the resonances within the alkyl region appeared broadened and could not be resolved. The expected rhodamine-based signals were identified within the aromatic region and their relative proton integrations enabled comparison with overall integrations obtained for the broad alkyl resonances for **56** and **59**. Overall, a relative integration of 81 H was detected in compound **56**, which is indicative of attachment of one macrocyclic unit, whereas the relative integration of 130 H identified for **59** suggests two protected macrocycles were present. To form the intermediate chain length **58**, compound **56** was dissolved in a minimum amount of methanol and deprotected in concentrated hydrochloric acid to yield **57**. Loss of the *tert*-butyl protecting units was followed by ^1H -NMR spectroscopy and evidenced as an

overall reduction in relative integration of the alkyl region consistent with the lack of 27 protons and further confirmed by HRMS. Lanthanide complexation was carried out in slightly acidic aqueous media (pH 5.5) as has been previously described, which afforded the terbium, gadolinium and europium complexes after purification by dialysis.

The mono-chlorinated diethylenetriamine species (**54**) was used to prepare the compound with the longest linker between donor/acceptor systems, **55**. This was achieved by direct combination of **54** with the lanthanide building block **29** (described in Chapter 4) via nucleophilic substitution. Due to the large distance between rhodamine acceptor and terbium metal ion donor units in compound **55**, a low energy transfer efficiency is expected. On the other hand, this fragment is the easiest to prepare and retains a free secondary amine site that could in principle be used to further append the Quin C1 targeting unit.

6.3.1.2 Photophysical characterisation

The molecular structures and steady state photophysical properties of **51**, **58** and **55** at pH 3, when the rhodamine fragment exists mostly in its fluorescent state, are summarised in Table 6.1:

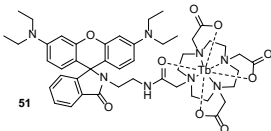
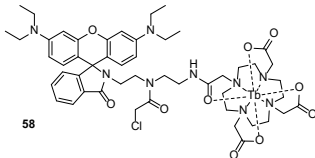
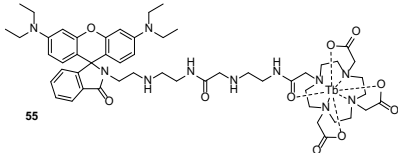
Molecular structure	Emission ($\lambda_{\text{exc}} = 350 \text{ nm}$)	Excitation ($\lambda_{\text{em}} = 580 \text{ nm}$, > 300 nm)	Absorption
	582 nm	307 nm, 357 nm, 406 nm, 561 nm	242 nm, 336 nm (shoulder), 559 nm
	581 nm	308 nm, 356 nm, 406 nm, 427 nm, 561 nm	245 nm, 273 nm, 320 nm (shoulder), 560 nm
	485 nm, 579 nm	310 nm, 356 nm, 406 nm, 426 nm, 558 nm	Broad, featureless increase 400-200 nm, 547 nm

Table 6.1: Summary of molecular structures of terbium-rhodamine conjugates with different chain lengths and their photophysical properties run at pH 3 in 1:1 water/methanol solutions.

To determine whether the rhodamine/linker unit was able to sensitise lanthanide emission and to what extent lanthanide emission translated to LRET, a time-gated set-up after excitation at 350 nm with a 0.2 ms delay was used to filter out short-lived fluorescence signals from the acceptor fluorophore. The detected luminescence is hence derived either from the long-lived lanthanide-centred states, which prevail at neutral and basic pH, or originates from a combination of long-lived donor-excited rhodamine luminescence and lanthanide emission (shown in Table 6.2). Results obtained at pH 11 were equivalent to those at pH 7 and are thus omitted for clarity. The three complexes were compared to gain insight into the effect of increasing linker size.

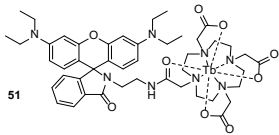
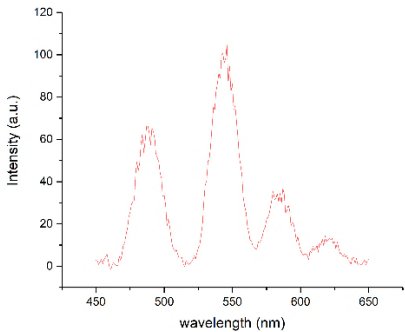
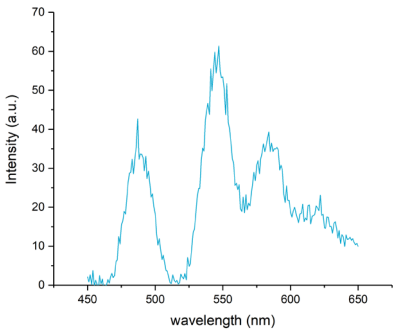
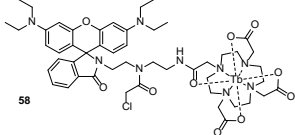
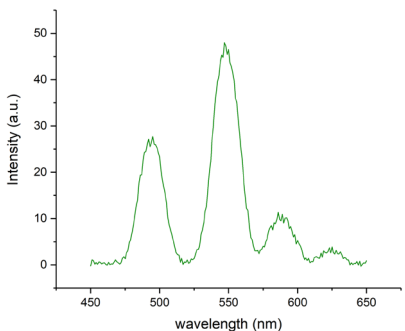
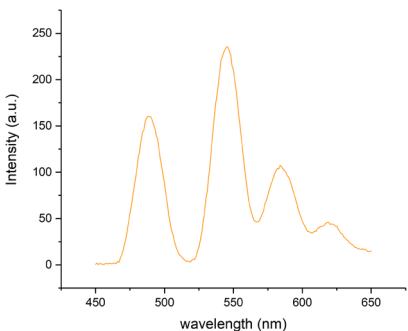
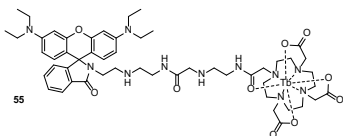
	pH 7	pH 3
 51		
 58		
 55	No significant emission	No significant emission

Table 6.2: Summary of pH-dependent time-gated emission spectra ($\lambda_{exc} = 350$ nm, delay = 0.2 ms) at pH 3 and pH 7 of compounds **51**, **58** and **55**.

In samples evaluated at high pH values (above ~5), the rhodamine moiety was found in its spirocyclic, non-emissive state and no visible light absorption at 560 nm was observed. As a result, the probe cannot absorb at the donor emission wavelengths and as such no LRET can occur. At neutral and basic pH, the spectral shape obtained for time-gated measurements resembles characteristic terbium metal-derived emission in compounds **51** and **58**, whereas for compound **55** almost no signal was observed, indicating the terbium ion is not being sufficiently sensitised. At pH 3, the spirocyclic equilibrium is shifted towards the predominantly ring-opened, fluorescent state and the rhodamine unit absorbs at 560 nm. As such, the organic fluorophore can act as an acceptor and absorb metal-based emission, resulting in donor-excited, long-lived luminescent states. The predicted band appears superimposed on the terbium-based emission and leads to an increase in emission intensity at 580 nm, where small amounts of metal-derived luminescence is observed. As is to be expected, the emission spectra show a drop-off in donor-excited rhodamine emission with increasing linker length, in both compounds **51** and **58**, whereas compound **55** displayed no notable long-lived signals as a result of the large distance between the lumiphore pair.

6.3.1.3 Assessment of LRET characteristics

Quantitative comparison between the two complexes that exhibited LRET properties was conducted by measuring the ratios of emission intensities at 580 nm (the emission maximum of rhodamine luminescence) and 490 nm (one of the emission maxima resulting from terbium luminescence), to minimise variability arising from concentration-dependence.²⁷ Further, the intensity ratios determined at pH 7 (where no LRET occurs) were subtracted from those measured at pH 3 (where LRET occurs), to eliminate donor-only signals from the analysis. For quantification, equation 6.3 was used, where I_x is the emission intensity at the specified wavelength and pH and $\Delta ratio$ is the difference in time-gated emission intensity ratio between the ring-opened and the ring-closed rhodamine acceptor:

$$\Delta ratio = \frac{I_{580\text{ nm}}(pH\ 3)}{I_{490\text{ nm}}(pH\ 3)} - \frac{I_{580\text{ nm}}(pH\ 7)}{I_{490\text{ nm}}(pH\ 7)} \quad (\text{eq 6.3})$$

Using this method, $\Delta ratio$ was determined as 0.457 for compound **51** and 0.289 for **58** respectively. Consistent with the expected trend, a clear drop-off in energy transfer magnitude was observed going from the short to the intermediate chain length, whereas no significant long-lived components were detected in compound **55**. Despite this fact, the data also shows that the intermediate chain length retains sufficient proximity between the donor and acceptor system to observe a transfer occurring and thus provides a suitable candidate to construct a larger compound. In addition, it was previously demonstrated that the 2,3-dihydroquinazolinone-derived targeting unit (**6**) is able to sensitise terbium emission, thus potentially enhancing donor emission.

6.3.2 Proton NMR spectroscopy in Eu(III) systems

The analogous europium complexes, Eu.51, Eu.58 and Eu.55, enable structural characteristics to be studied using ^1H -NMR spectroscopy. The proton spectra were all obtained at 400 MHz at room temperature in D_2O at pH 7 and are depicted in Table 6.3. The broadened proton resonances and extended shift range (about -20 ppm to +40 ppm) in all obtained spectra are typical for the presence of a paramagnetic metal and indicative of the bound europium species.

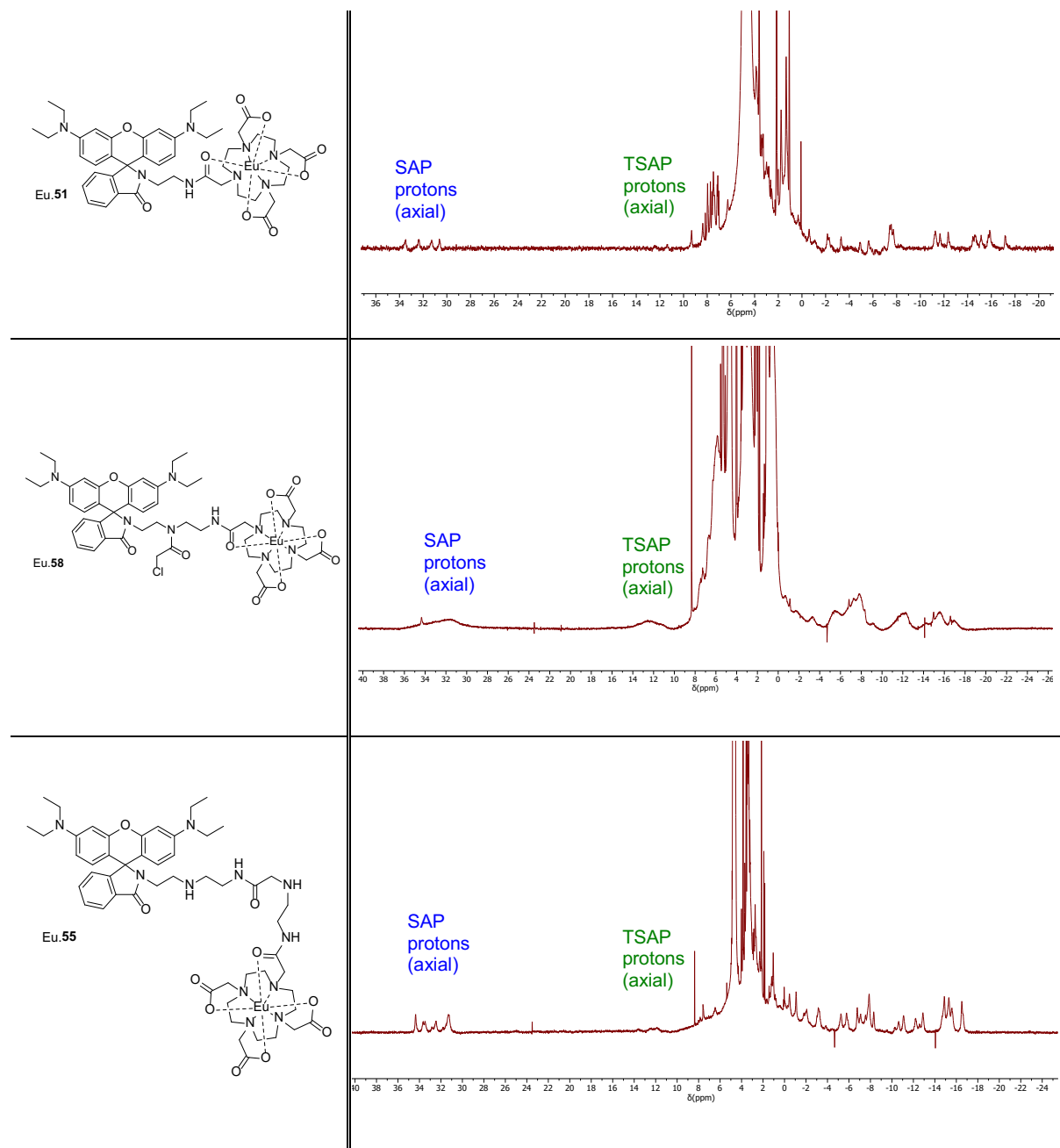


Table 6.3: Summary of ^1H -NMR spectra run in D_2O at pH 7, 298 K and 400 MHz of compounds Eu.51, Eu.58 and Eu.55.

All three proton NMR spectra contain signals that are consistent with the macrocyclic SAP conformation pseudo-axial ring protons (compare Chapter 4) in the shift range between +30 ppm and +35 ppm.^{28,29} For Eu.**51** and Eu.**55**, the resonances appear as a set of four or seven relatively sharp peaks respectively. This suggests a loss of symmetry compared to the C₄ symmetric DOTA complex, brought about by derivatisation of one of the sidearms and is expected to result in four different resonances, as observed within the spectrum of Eu.**51**. Additional peaks observed within the spectrum of Eu.**55** suggest a further underlying equilibrium, for instance involving protonation and deprotonation events.³⁰ The ¹H-NMR spectrum collected for Eu.**58** contains just one extremely broadened range of proton signals within the area of +30 to +35 ppm, and resonances generally appear substantially broadened compared to those observed for the other two complexes. This could imply that faster conformational changes are occurring within this structure.³¹ Signals associated with the TSAP isomer pseudo-axial ring protons are similarly apparent between approximately 10 ppm and 14 ppm in all three spectra.

Intensity ratios between the SAP and TSAP conformations were estimated by relative integrations of the corresponding signals. For Eu.**58** and Eu.**55**, an unusually large proportion of TSAP isomer was present in solution with ratios of 1:0.57 and 1: 0.41 respectively. For Eu.**51**, the resonances were less intense and reliable integration of the TSAP isomer signals was not possible and thus ratios could not be established.

6.3.3 Bimetallic lanthanide LRET systems

As lanthanide emission is notoriously weak, the speculation that factors limiting the LRET-derived acceptor emission intensity were more likely arising from donor excitation and emission pathways rather than the acceptor absorbance, seemed plausible. Thus, to increase donor signals, bimetallic compounds containing two of the same lanthanide donor centres and one rhodamine-based acceptor lumiphore were prepared. It was hypothesised that using this strategy, an enhancement of donor emission and hence of the resulting energy transfer process would occur. The spectral overlap between europium-based donor emission (with the most intense band at 612 nm) and rhodamine-based acceptor absorption at low pH ($\lambda_{\text{max}} = 561$ nm) is considerably poorer than that between terbium and rhodamine as a donor/acceptor pair, as is illustrated in Figure 6.8. Using the bimetallic complexes, it was rationalised, that increased emission from two donor centres should facilitate observing the weak transfer within the europium complex.

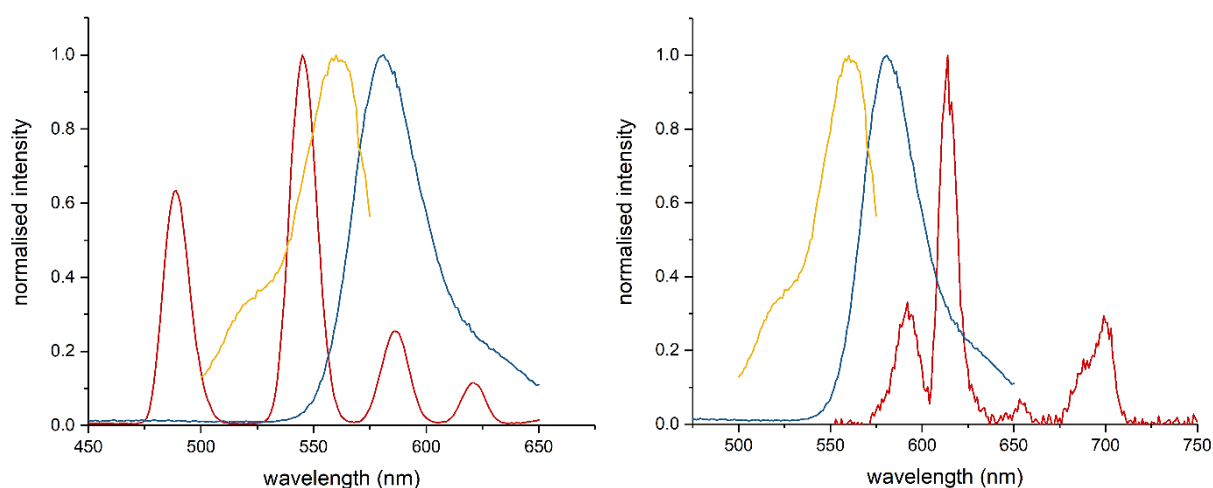
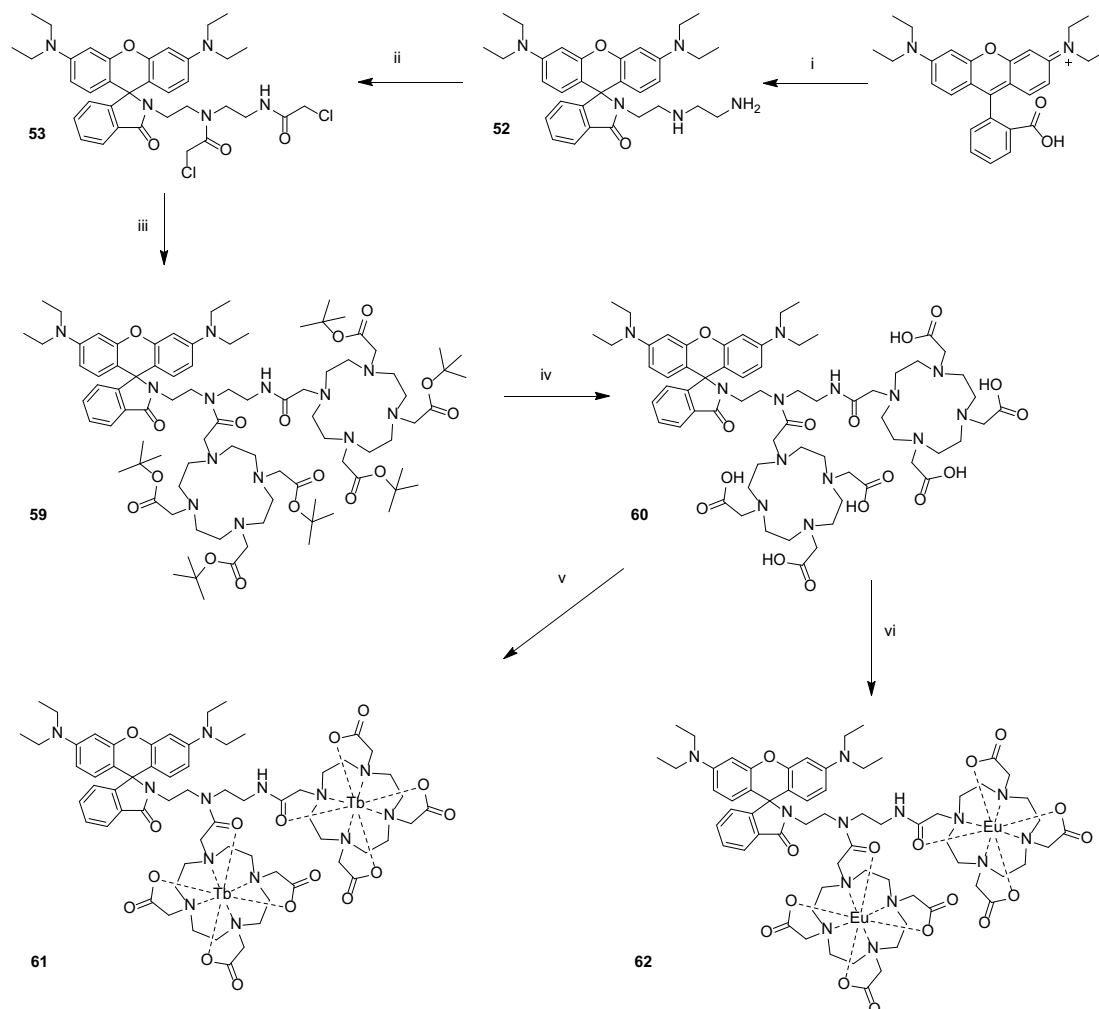


Figure 6.8: Typical steady state excitation (yellow, $\lambda_{em} = 580$ nm) and emission spectra (blue, $\lambda_{exc} = 500$ nm) of rhodamine-B derivatives and time-gated emission spectra ($\lambda_{exc} = 350$ nm, 0.1 ms delay) displaying typical terbium (left) and europium (right) emission. The spectra demonstrate good overlap between donor emission and acceptor excitation for the terbium species and poor overlap with europium species.

The synthetic pathway towards preparation of the bimetallic lanthanide complexes **61** and **62** is summarised in Scheme 6.2. In brief, **53** was reacted with 2.1 equivalents of protected DO3A and purified by column chromatography to yield **59**. Deprotection using concentrated hydrochloric acid resulted in formation of the ligand framework **60** in moderate yields (46 %) and was established by loss of the *tert*-butyl resonances within the ^1H -NMR spectrum and confirmed by presence of an $[\text{M}+\text{H}]$ species within the HRMS.

Complexation with either terbium or europium salts in aqueous conditions at pH 5.5, followed by purification by dialysis using a 500 - 1000 Da cut-off membrane afforded **61** or **62**. Molecular ion peaks assigned to $[\text{M}+\text{Na}]$ or $[\text{M}+\text{H}]$ for **61** and **62** respectively were evidenced by HRMS. Furthermore, the ^1H -NMR spectrum of **62** was found to contain highly broad, structureless resonances. This is likely a result of increased line broadening due to two paramagnetic europium centres.



Scheme 6.2: Synthetic pathway towards preparation of bimetallic-rhodamine architectures for improved LRET efficiencies. Reagents: i) ethylene triamine, ethanol, 76 % yield, ii) chloroacetyl chloride, triethylamine, dichloromethane, 24 % yields, iii) tert-butyl DO3A, triethylamine, acetonitrile, 9 % yields, iv) concentrated hydrochloric acid, 46 % yield, v) Tb(III) trichloride hexahydrate, water pH 5.5, 39 % yield vi) Eu(III) trichloride hexahydrate, water pH 5.5, 79 % yield.

6.3.3.1 Photophysical characterisation

The steady state excitation, emission and absorption spectra at varying pH values were comparable to those observed above for the respective mono-metallic compounds (Table 6.1) and are dominated by the rhodamine-based signals at pH 3. Using a time-gated set-up, donor-only and donor-excited acceptor luminescence emission spectra were recorded in neutral and acidic media respectively and are depicted in Table 6.4.

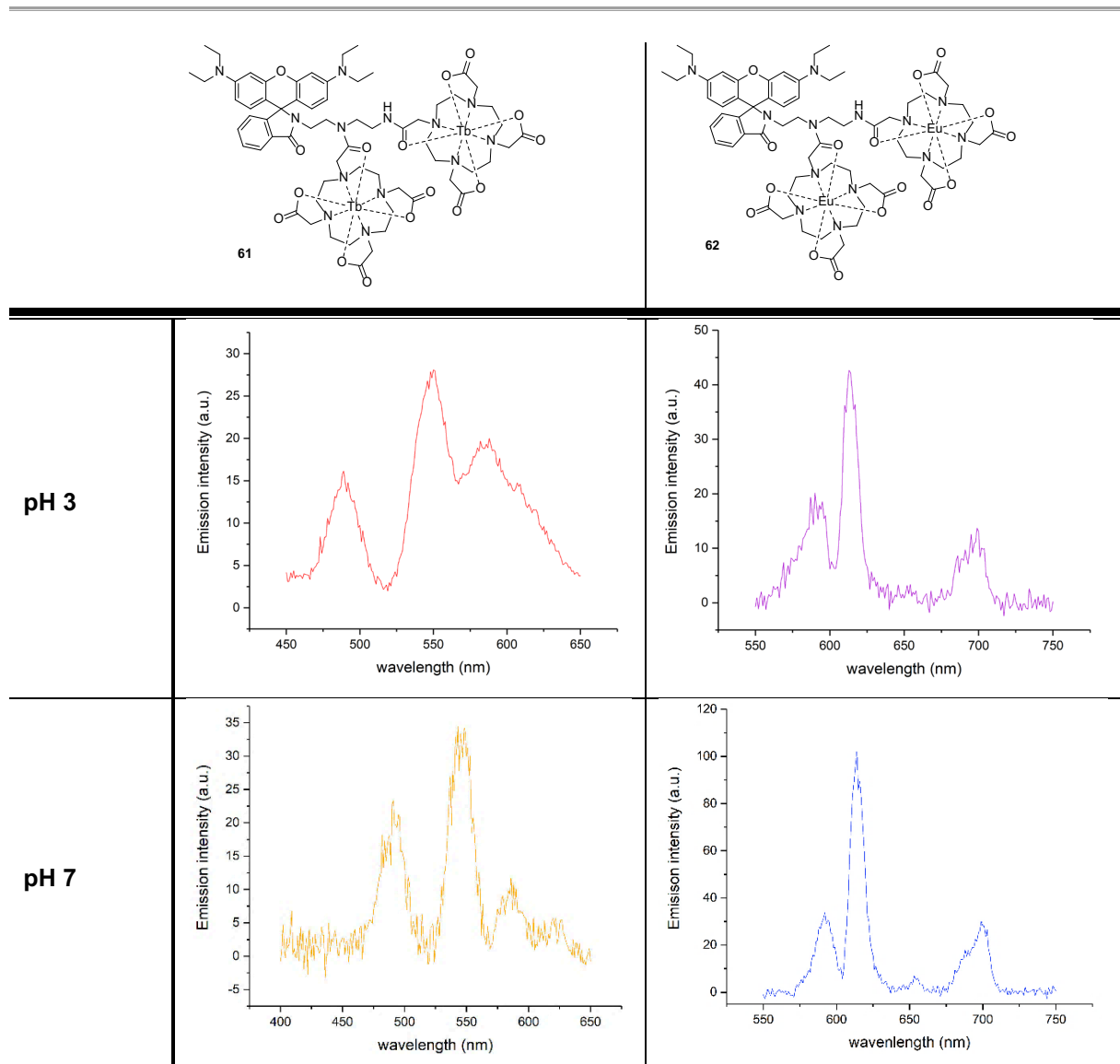


Table 6.4: Time-gated emission spectra of compounds **61** and **62** in 1:1 water/methanol (v/v) mixtures at pH 3 and pH 7 ($\lambda_{exc} = 350$ nm, 0.2 ms delay).

At neutral pH values, the detected long-lived emission originates solely from the lanthanide donor system and no notable donor-excited rhodamine emission was observed for both complexes. At pH 3, a slight increase in intensity at 580 nm appears within the spectrum of **62**, which is indicative of energy transfer to the rhodamine unit. Within the time-gated emission spectrum of the terbium analogue (**61**) obtained at pH 3, both lanthanide donor emission and rhodamine acceptor long-lived components are evident. Compared to the donor-only emission spectrum recorded at pH 7, the emission intensity of **61** at 580 nm is greatly enhanced at pH 3. Further, when compared to **58** (with a single bound Tb(III) ion), the second intramolecular donor lumiphore dramatically intensifies rhodamine-based donor-excited emission.

Quantification of these processes was achieved as outlined above, using equation 6.3. For the europium analogue (**62**), I_{490} was substituted with I_{612} in eq 6.3. The calculated Δ ratio of 0.904 for **61** is

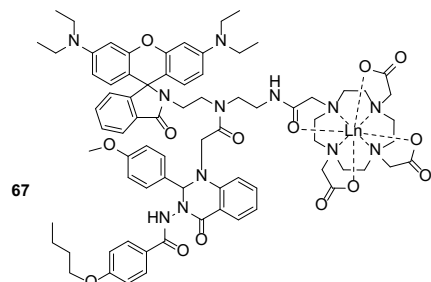
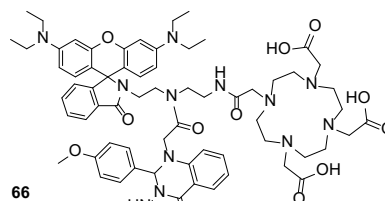
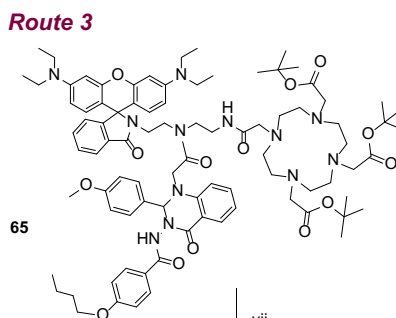
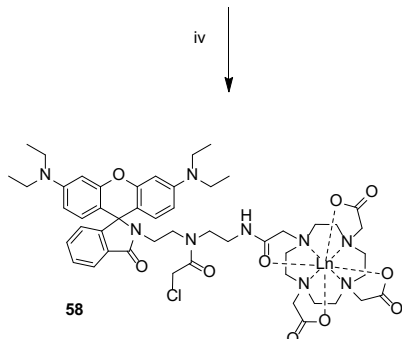
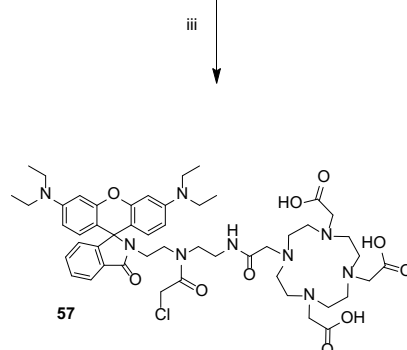
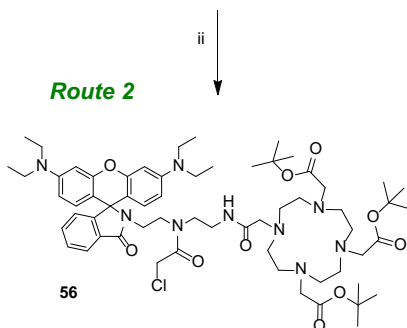
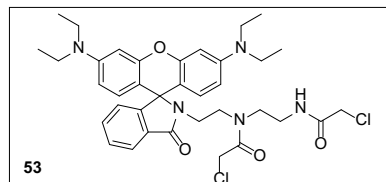
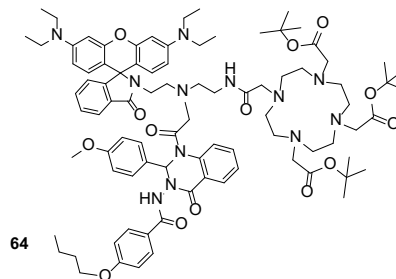
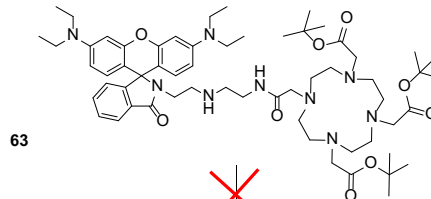
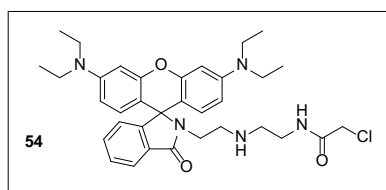
approximately double the value obtained for the mono-metallic complex **58**. The magnitude of LRET noted for compound **62** is much smaller ($\Delta\text{ratio} = 0.172$), which is likely due to the poor overlap between donor emission and acceptor absorption.

Taken together, the homo-bimetallic systems have proven useful to demonstrate that an enhancement in LRET magnitude is indeed observed with increased emission of the donor fluorophore. A similar increase is expected in the terbium-based tri-functionalised architecture **67**, as the emissive terbium states should be populated more efficiently using the Quin C1 fragment as an antenna chromophore, compared to the poorly matched rhodamine triplet states or direct lanthanide excitation. Further, although not detectable within the mono-metallic europium complex (Eu.**58**), the bimetallic analogue **62** enabled the weak energy transfer to the rhodamine fluorophore to be observed and quantified, proving that it does occur. Coupling with more suitable organic chromophores, that have absorption maxima around 600 nm, would enable an efficient europium LRET system to be constructed.

6.3.4 FPR2/ALX-targeted complexes for LRET

With these results in hand, construction of the trifunctional complex was attempted. The synthetic methodology is outlined in Scheme 6.3.

Initially, compound **54** was reacted with *tert*-butyl protected DO3A to form the macrocyclic compound **63**. Attempts at subsequent nucleophilic substitution using the hindered secondary amine site to react with Quin C1-chloro (**7**) proved unsuccessful (route 1, Scheme 6.3). Instead, the synthetic strategy was revised towards using the pre-chlorinated mono-macrocyclic species (**56**), which was postulated to be less hindered and provide a more approachable site for nucleophilic substitution. Both preparation pathways shown in routes 2 and 3 were simultaneously attempted and resulted in formation of the desired lanthanide constructs. Firstly, the ligand framework was fully built up before deprotection of the macrocyclic carboxylic acids and subsequent lanthanide complexation in the last step (route 3). Secondly, deprotection and lanthanide complexation was carried out before nucleophilic substitution of the chlorinated site by the targeting unit (route 2). Following the synthetic pathway detailed in route 3, Quin C1 (**6**) was attached to compound **56** in a nucleophilic substitution reaction. Although not a very efficient reaction, **65** was isolated after silica column chromatography and preparative thin layer chromatography, both eluting in dichloromethane with a methanol gradient (2 % - 50 %). Formation of the adduct was verified by HRMS. Assignment by ^1H -NMR spectroscopy was not possible due to the broad and overlapping nature of the signals detected, but relative integrations of the aromatic region with the characteristic 2H triplet at 4.06 ppm (belonging to the 2,3-dihydroquinazolinone unit), matched the predicted trifunctional compound. Subsequent deprotection afforded the carboxylic acid macrocycle (**66**) which was complexed with a slight excess of the respective terbium, europium and gadolinium trichloride hexahydrate species in aqueous conditions at pH 5.5.

Route 1

Scheme 6.3: Overview of synthetic pathway towards preparation of tri-functionalised molecules Ln.**67**. Reagents: i) *tert*-butyl DO3A, triethylamine, acetonitrile, 16 % yield, ii) to iv) discussed previously Scheme 1 and 2, vi) **6**, *N,N*-diisopropylethylamine, acetonitrile, 54 % yield, vii) concentrated hydrochloric acid, 60 % yield, viii) Ln(III) trichloride hexahydrate, water pH 5.5 (Tb.**67**: 51 % and Gd.**67**: 40 %).

The lanthanide compounds were isolated after purification by dialysis and were characterised by their typical isotope patterns as $[M+H_2O]^+$ ions in their respective HRMS spectra (at 1567.6248 a.m.u. and 1571.6178 a.m.u. for Eu.**67** and Gd.**67** respectively) or by MALDI as an $[M+MeOH+H]^+$ ion at 1588.9 a.m.u. for Tb.**67**.

6.3.4.1 Photophysical properties

Steady state photophysical properties of Tb.**67** were analysed in 1:1 methanol/water solutions at neutral and acidic pH values. The emission spectrum of Tb.**67** upon excitation at 350 nm at pH 3 is shown in Figure 6.9 (in red), along with excitation spectra at $\lambda_{em} = 450$ nm (in blue) and $\lambda_{em} = 580$ nm (in yellow below 500 nm and in green above 500 nm using smaller slit widths to observe the much more intense, main excitation peak at 560 nm). It was noted that at pH 3 the characteristic absorption, excitation and emission bands of both the rhodamine unit and the dihydro quinazolinone-derivative were apparent. As the pH was increased, the rhodamine spirocyclic equilibrium was shifted to the ring-closed form and at pH 7, no rhodamine-based absorption, excitation or emission was observed. The bands associated with the targeting group remained unchanged, as illustrated by the emission spectrum depicted inset in Figure 6.9.

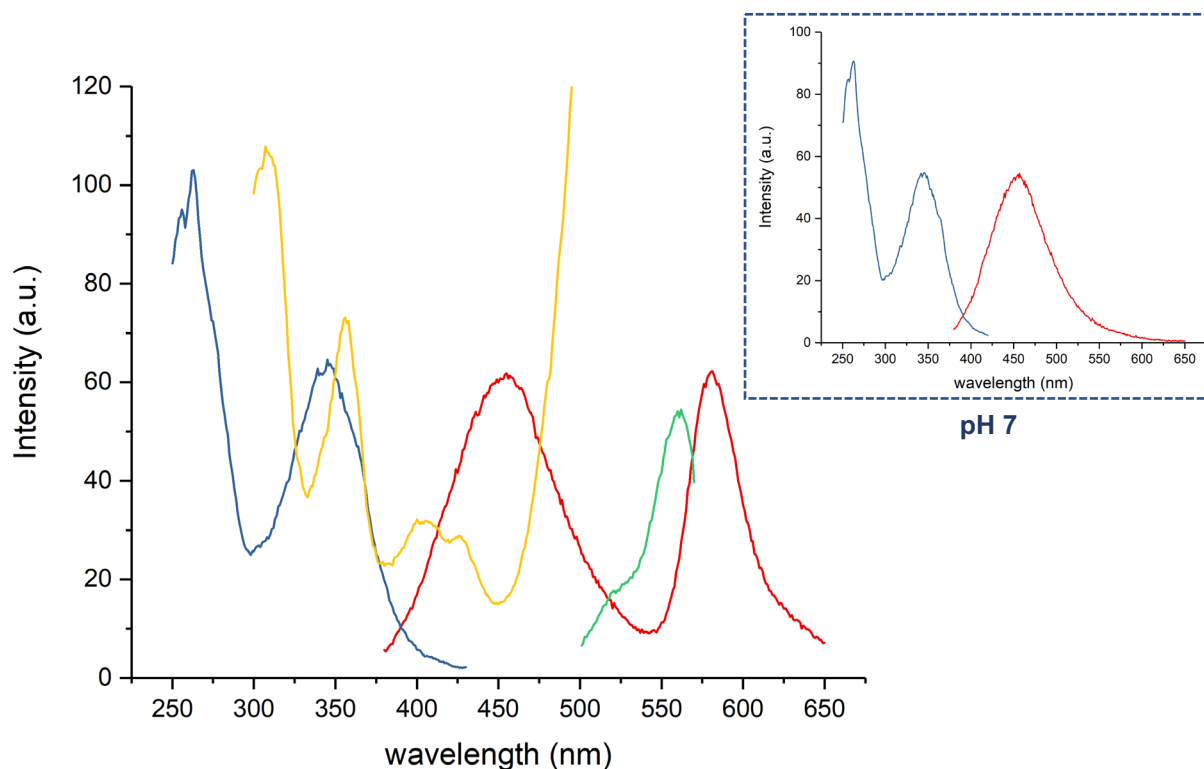


Figure 6.9: Steady state emission spectra (in red, $\lambda_{exc} = 350$ nm) and excitation spectra ($\lambda_{em} = 450$ in blue and $\lambda_{em} = 580$ nm in yellow (5 nm slit width) and green (2.5 nm slit width)) of compound Tb.**67** in 1:1 water/methanol (v/v) mixtures at pH 3. Inset above, steady state emission (in red, $\lambda_{exc} = 350$ nm) and excitation ($\lambda_{em} = 450$ in blue) spectra at pH 7.

When running these measurements, it was noted that a markedly slow ring-opening response upon addition of acid was observed for compound Tb.67, where appearance of the characteristic pink solution took minutes to almost half an hour. For comparison, previously described compounds containing a cyclisable rhodamine unit changed colour in a matter of seconds after addition of acid. In contrast, once the compound had been ring-opened, neutralisation using base resulted in an instantly observable loss of colour.

Kinetics governing the rhodamine ring-opening reaction have been previously investigated and the rate was found to mainly depend on the electronic rearrangement required for the protonated spiro lactam carbonyl, which is stabilised by charge delocalisation, to ring-open and afford the conjugated ring-opened rhodamine species (Figure 6.10).^{32,33}

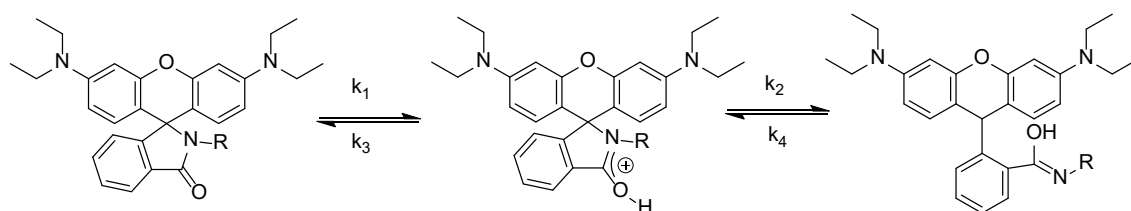


Figure 6.10: Molecular structures and rate constants associated with the steps leading from a spirocyclic rhodamine species (left) to a ring-opened form (right). The protonated intermediate with a delocalised charge is represented in the middle.

Specifically, ring-opening kinetics were reported to depend on electronic factors, but also show a possible dependence on the immediate sterics of the surrounding groups. For instance, Czaplyski *et al* showed that increasing the electron withdrawing nature of the ring amine substituent increased the rate of ring-opening.³³ Similarly, an increase in steric constraints around the spirocycle was found to correlate with both pK_A and fast ring-opening kinetics.^{33,34} These studies are consistent with the speculation that the slow ring-opening kinetics observed for Tb.67 may be a result of a high degree of intramolecular charge delocalisation stabilising the protonated intermediate. The highly stabilised intermediate results in a slow rearrangement step to the ring-opened analogue, which has previously been put forward as the rate-determining factor.^{33,34}

6.3.4.2 Time-gated luminescence measurements

Time-gated emission spectra at pH 3 and pH 7 were run with a 0.2 ms delay to ensure that short-lived signals from the acceptor fluorophore had decayed. Both emission spectra show the expected terbium-centred emission. At pH 3, the lanthanide emission was found to be superimposed on the donor-excited, long-lived rhodamine luminescence (Figure 6.11).

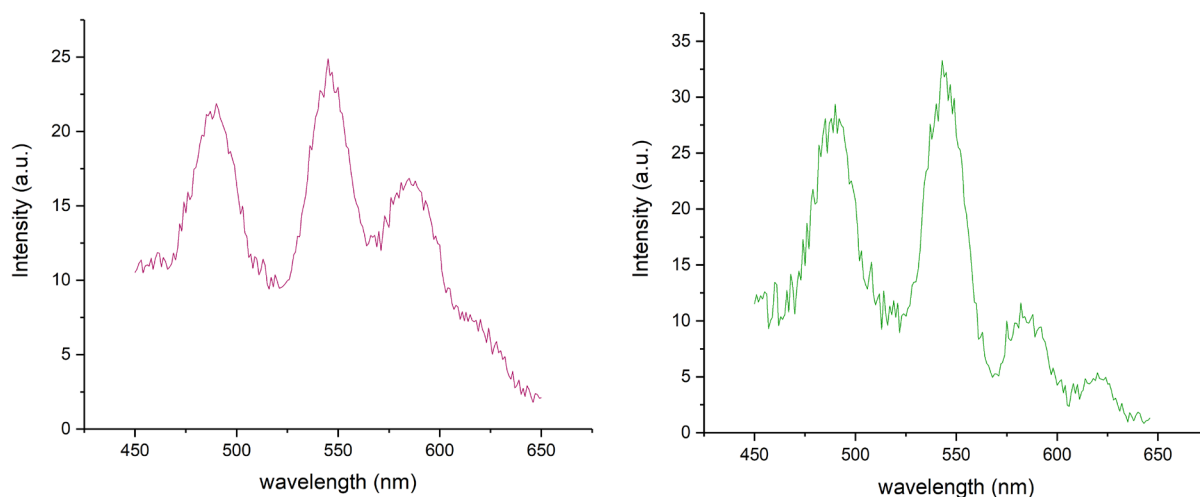


Figure 6.11: Time-gated emission spectra ($\lambda_{exc} = 350$ nm, delay = 0.2 ms) of Tb.67 in 1:1 methanol/water solutions at pH 3 (left) and pH 7 (right).

The magnitude of occurring luminescence resonance energy transfer was quantified by using equation 6.3, and Δ ratio was determined as 0.376. In line with expectations, the value is substantially higher than the value estimated for the compound with the same linker length (**58**, Δ ratio = 0.289) without the targeting group. This was attributed to the fact that the 2,3-dihydroquinazolinone derivative acts as a more efficient antenna unit to sensitise donor emission than the rhodamine or linker moiety. Comparison with **61** demonstrated that the values obtained for Δ ratio are still significantly below those recorded with two lanthanides, suggesting that the amount of donor emission, even using a more efficient excitation route, is still lower than that of two inefficiently sensitised donors.

6.3.4.3 Lifetime measurements

Luminescence lifetimes of Tb.67 were recorded in 1:1 methanol/water solutions (v/v) at 545 nm, both at pH 3 and pH 7. At the chosen wavelength, luminescence decay curves obtained could not be reliably fitted to a single exponential decay, revealing that a second, long-lived component is likely to significantly contribute to the observed luminescence. A bi-exponential fit was achieved using the in-built OriginPro 8 curve fitting function (Figure 6.12). By this method, the lifetime components were determined as $\tau_1 = 100$ μ s and $\tau_2 = 1544$ μ s at pH 7 (Figure 6.12, $\chi^2 = 0.9998$). The two components were tentatively assigned to terbium-based luminescence (τ_2) and donor-excited acceptor emission (τ_1). At pH 7, the contribution from τ_1 is small, as the rhodamine is predominantly in the cyclic form that does not absorb at relevant donor emission. The short-lived, acceptor-only signals (expected within the 10 ns range) cannot be detected in the presence of the 200 μ s time-gate. At pH 3, the value recorded for τ_1 increased slightly to 107 μ s, while at the same time a substantial decrease in donor-based lifetimes (to $\tau_2 = 721$ μ s) was noted. Additionally, the contribution from τ_1 to the overall decay approximately doubled compared

to what was recorded at pH 7, which is reflected by changes in the constant A_1 in equation 6.4 used to fit the data to a biexponential decay ($\chi^2 = 0.9997$). These results are consistent with an increased luminescence resonance energy transfer occurring, where the donor-only lifetime is expected to decrease, while donor-excited acceptor lifetimes introduce a second long-lived decay component.

$$y = y_0 + A_1 \exp\left(\frac{x-x_0}{\tau_1}\right) + A_2 \exp\left(\frac{x-x_0}{\tau_2}\right) \quad (\text{eq 6.4})$$

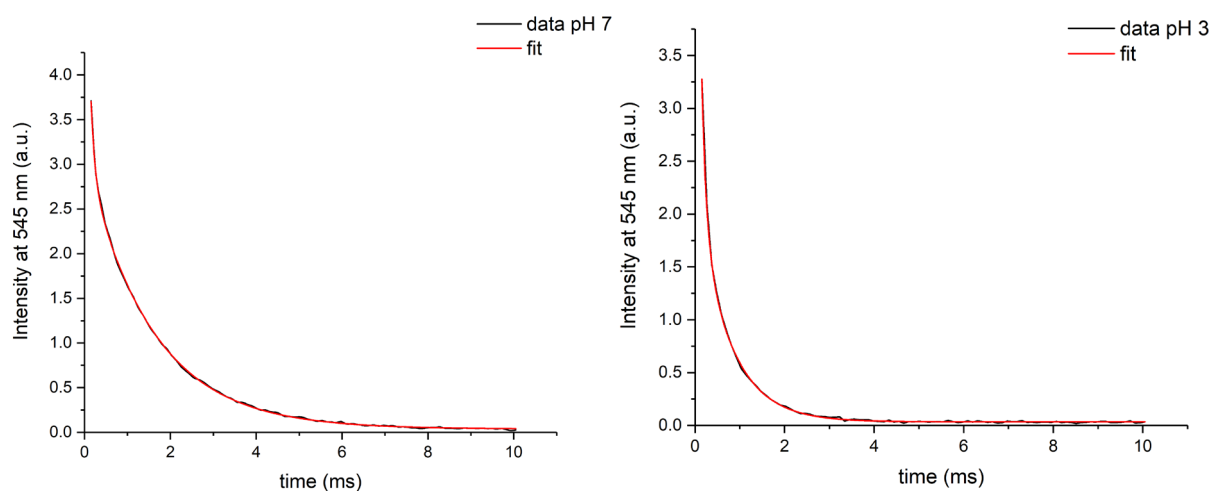


Figure 6.12: Time-gated emission lifetime decay components of Tb.67 at 545 nm ($\lambda_{\text{exc}} = 350$ nm, 0.1 ms delay) in 1:1 water/methanol solutions. Data and biexponential fit obtained at pH 7 (left) and pH 3 (right) are shown.

The efficiency of the LRET energy transfer can be calculated by rearranging equation 6.1 or can be derived from experimentally detected lifetimes (equation 6.5):

$$E = 1 - \left(\frac{\tau_{DA}}{\tau_D}\right) \quad (\text{eq 6.5})$$

Where τ_{DA} is the donor lifetime in presence of the acceptor and τ_D is the donor lifetime.^{35,5,4} By inserting the terbium-based lifetime (τ_2) at pH 7 as τ_D and the terbium-based lifetime determined at pH 3 as τ_{DA} into equation 6.5, the LRET efficiency was calculated as $E = 0.53$.

6.3.4.4 Relaxivity measurements

The relaxometric properties of Gd.**67** were investigated using inversion recovery experiments conducted by Peter Haycock at Imperial College London using the same method that was described in Chapter 4. First, sample concentrations were calculated from the shift difference between the H₂O and D₂O proton NMR peak in ppm of the compounds using the Evans method for three different samples. The r_1 value at 294 K using a 400 MHz spectrometer was determined via a graphical procedure outlined in section 4.3.2 as $r_1 = 0.6 \text{ mM}^{-1}\text{s}^{-1}$. In accord with typical octadentate ligand systems, an r_1 value of around $\sim 4 \text{ mM}^{-1}\text{s}^{-1}$ would be expected. Although not conclusively determined, a likely explanation for the large decrease in r_1 is that the increased steric demands around the ninth metal coordination site could lead to a q -value that is on average < 1 . Another plausible option is that the aforementioned steric bulk substantially affects the water exchange rates and increases proton residence times and thus reduces r_1 .

6.4 Conclusions

Compounds exhibiting lanthanide-based LRET properties were prepared, characterised and examined for their luminescent properties. Compounds with different linker lengths between the donor/acceptor lumiphore pair were prepared and the magnitude of energy transfer occurring was quantified relative to each other. A read-out of donor-only signals compared to donor-sensitised acceptor signals was facilitated by using systems where the acceptor fluorophore can be switched “on” or “off” by tuning the environmental pH values. A strong distance dependence of LRET efficiency was established and using two donor fragments within the same molecule was found to enhance the long-lived donor-excited emission. Using the insights gained from these studies, a compound with an “intermediate” linker length, which simultaneously enables coupling of the FPR targeting group Quin C1, while still holding the energy matched lumiphores in sufficient proximity, was designed. In principle, a construct of this nature should permit better visualisation of the receptors by making use of pH-dependent, time-gated techniques. An increased long-lived emission intensity resulting from the LRET-shortened lifetimes (and related higher photon flux) should further ameliorate the compound’s brightness. Unfortunately, time constraints on the project did not permit imaging of the compound in cells.

6.5 References

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Chapter 7

Conclusions and Future Work

This PhD work has focussed on preparing luminescent compounds for FPRs in inflammation. In particular, the role of FPR2/ALX is less well investigated than that of FPR1, leading us to focus on devising probes that could selectively visualise this receptor.

7.1 FPR2/ALX-targeted fluorescent compounds

Two structurally related FPR2/ALX ligands, Quin C1 and Quin C7 were prepared, as well as a TIPS-protected version of Quin C7 (Figure 7.1).^{1,2} For design of a targeted agent we found that the small-molecule FPR2/ALX ligand Quin C1 was both synthetically easier to manipulate as well as more suitable from a biological point of view than the antagonist counterpart Quin C7, which inhibited neutrophil function at a wider concentration range.

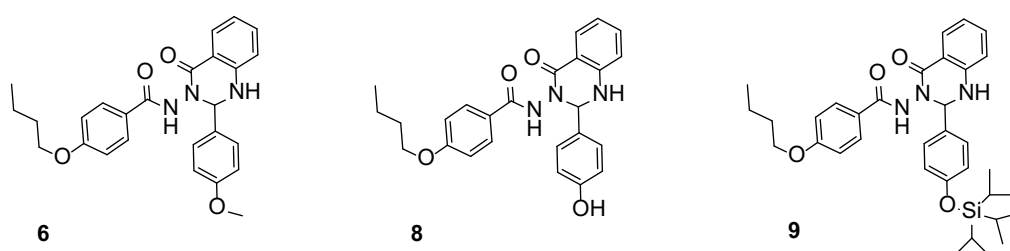


Figure 7.1: Structures of FPR2/ALX ligands Quin C1 (**6**), Quin C7 (**8**) and TIPS-Quin C7 (**9**) from Chapter 2.

Combination of Quin C1 with a conformationally locked, “always on” rhodamine-derivative, as well as a pH-responsive rhodamine dye, led to formation and characterisation of two fluorescent probes (Figure 7.2). Photophysical properties of both compounds were found to be appropriate for bioimaging applications. A pK_{cycl} value of 3.97 was determined for **13**, indicating the potential to selectively indicate acidic cellular conditions.

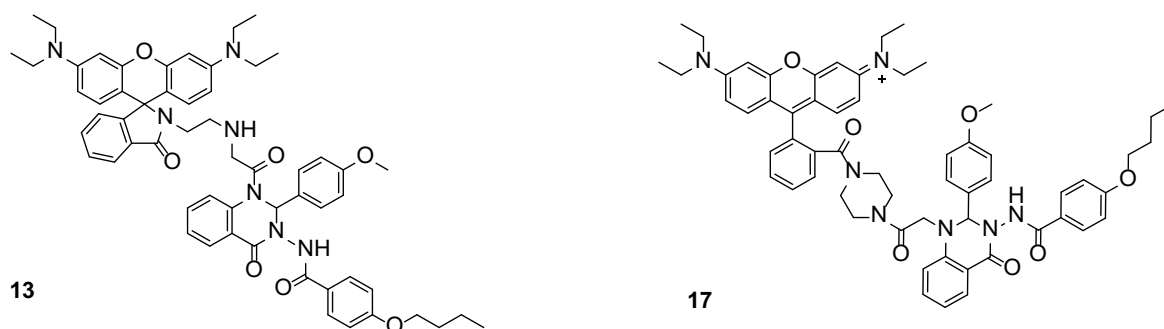


Figure 7.2: Quin C1-derived fluorescent probes with a switchable rhodamine unit (**13**) and a conformationally locked, ring-opened analogue (**17**) described in Chapter 3.

7.1.1 Biological validation of compounds **17** and **13**

Both **17** and **13** were tested *in vitro*, and neutrophil functional assays confirmed that the compounds did not alter cellular responses at concentrations relevant for biological imaging. Pharmacological blocking using a pan-FPR antagonist (Boc2) resulted in a significant attenuation of fluorescence compared to unblocked controls, demonstrating that neutrophil binding occurs via the FPR family. Selective blocking using an FPR2/ALX antagonist (WRW4) or an FPR1 antagonist (CsH) both resulted in a visible decrease of fluorescence intensity ratios, although results were not found to be statistically significant at $n = 3$. The data allowed binding to FPRs to be conclusively established, while selectivity for FPR2/ALX over FPR1 was observed as a trend within the results but could not be proven at this stage (no statistical significance). Apart from increasing n numbers for the experiment, another option to determine specific receptor subtype binding would be to assess binding properties in cell-lines stably expressing either just FPR1 or just FPR2/ALX (such as human embryonic 293 kidney cells).³

When tested in a model of TNF α -induced acute inflammation, **17** preferentially tagged neutrophils while significantly less labelling was observed in the saline control group. Similarly, in a separate group that had been treated with the anti-inflammatory compound BMS-470539 after administration of TNF α , results were comparable with the saline control. This study showed that the probe can selectively bind to neutrophils in an inflammatory context, whereas little uptake was observed under normal physiological conditions or when inflammation had been attenuated by administration of an antagonist for TNF α -induced inflammation. In all three groups, neutrophils that had emigrated outside the vessel (i.e. had become activated by an endogenous stimulus), were labelled. Taken together, this suggests that the probe allows neutrophils to be specifically tagged when they are in an activated state, and hence can be applied for tracking neutrophils and identifying inflammation *in vivo*.

Although the compound has demonstrated potential for preclinical biomedical studies, intravital microscopy using a visible light emissive fluorophore is limited to near-surface applications. Within the optical NIR window, between about 650 – 900 nm, biological autofluorescence is minimal. In addition, light of these wavelengths has a deeper tissue penetration and thus presents better opportunities for clinically relevant imaging.⁴ Nevertheless, NIR fluorophores tend to be less photostable which can compromise their utility. In recent years, an increased number of robust NIR-fluorophores have been reported.⁵ Amongst these, Nagano and co-workers reported the synthesis of stable rhodamine-derivatives where the oxygen has been replaced by a group 14 atom and thus the excitation and emission wavelength have been shifted into the far-red, NIR range (Figure 7.3).⁶ Grimm *et al* further reported how modifications of the rhodamine scaffold, by incorporating four-membered azetidine rings, allowed absorption and emission wavelengths to be systematically tuned towards lower energies.⁷ Taking these strategies into account, a possible future direction would be to replace the rhodamine B-derived fluorophore of **17** with a Si-rhodamine, an NIR-fluorescent analogue which would be more applicable for *in vivo* FPR targeting (Figure 7.3).

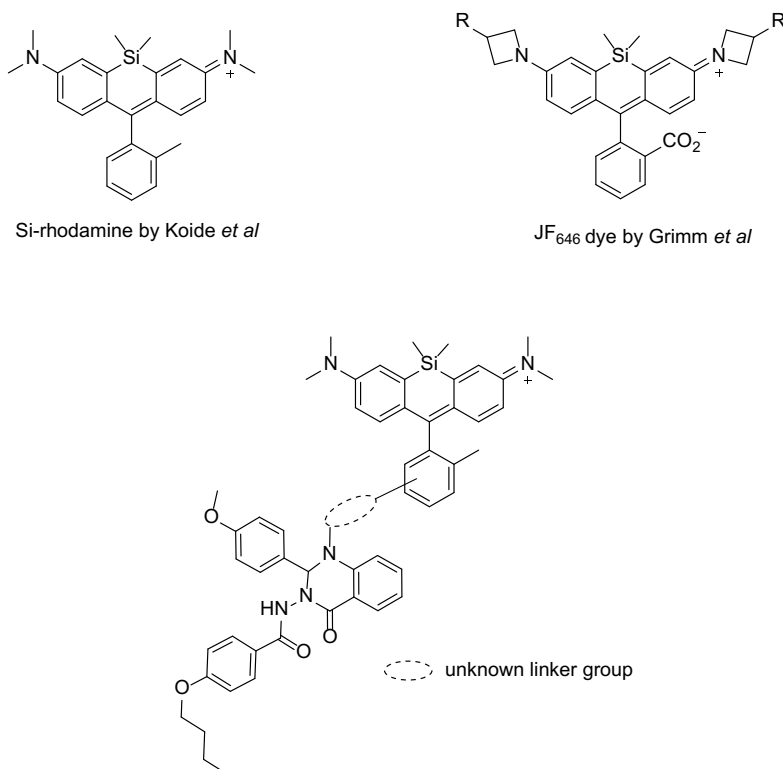


Figure 7.3: Structures of red-shifted rhodamine derivatives reported by Koide *et al* and Grimm *et al* (above) and molecular structure of potential candidate for far red/NIR FPR2/ALX targeting agents (below).^{6,7}

7.1.2 pH sensing using compound 13

Inflammatory conditions are accompanied by tissue acidosis and neutrophil activation is associated with a change in environmental pH values.^{8,9} For these reasons, a pH-responsive probe, **13**, was constructed. Photophysical properties of the compound were found to be compatible with cellular imaging applications in the acidic window ($pK_{cycl} = 3.97$). *In vitro* tests demonstrated that in an unactivated state, neutrophils that had been treated with the compound remained non-fluorescent, whereas prior stimulation of the cells resulted in “turn-on” rhodamine emission. These encouraging results indicate that the compound may allow neutrophils to be visualised *in vivo* by intravital microscopy specifically as they enter an activated state (fluorescence “turn on” response) and/or as the environmental pH returns to homeostasis (“turn off” response). Unfortunately, this was not established during the timeframe of the project, although the potential for *in vivo* pH sensing opportunities was demonstrated. Another future strategy, similar to what was mentioned in Figure 7.3, would be to extend the emission of the pH-responsive compound towards the NIR region by exchanging the fluorophore as represented in Figure 7.4.

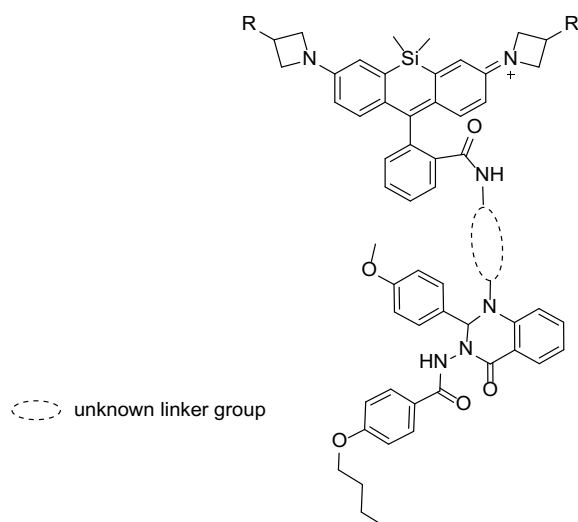


Figure 7.4: Potential structure of red-shifted, pH-sensitive rhodamine derivative based on JF₆₄₅ reported by Grimm et al for NIR FPR2/ALX targeting agents.⁷

7.2 Metal-based luminescence for targeting FPR2/ALX

Next, metal-based luminescence was investigated for time-resolved imaging of neutrophil samples. Macrocyclic, octadentate, DOTA-derived ligand frameworks were prepared to encapsulate the lanthanide ion and were appended to the targeting units via an ethylenediamine linker (Figure 7.5).

The terbium systems were found to be emissive upon application of a time-gate, whereas only very weak europium sensitisation was observed in these constructs. In the gadolinium analogues, comparatively high relaxivity values for an octadentate complex were measured ($r_1 = 6.9 \text{ mM}^{-1}\text{s}^{-1}$ compared to $r_1 \sim 4 \text{ mM}^{-1}\text{s}^{-1}$ for commercially used contrast agents), suggesting potential for future targeted MRI applications *in vivo*. Cellular imaging using a time-resolved confocal fluorescence microscope of activated and unactivated neutrophils pre-treated with Tb.**30** resulted in luminescence lifetime maps that contained long-lived components when the cells had been activated. Patterns representative of two different activated receptor distributions on the cell surface, one with a “speckled” fluorescence distribution corresponding to previously reported images of pseudopodia formation, and one with a ring-shaped structure around the circumference, were observed. However, the concentration of Tb.**30** required for visualisation was very high (1 mM), thus limiting the applicability of the compound.

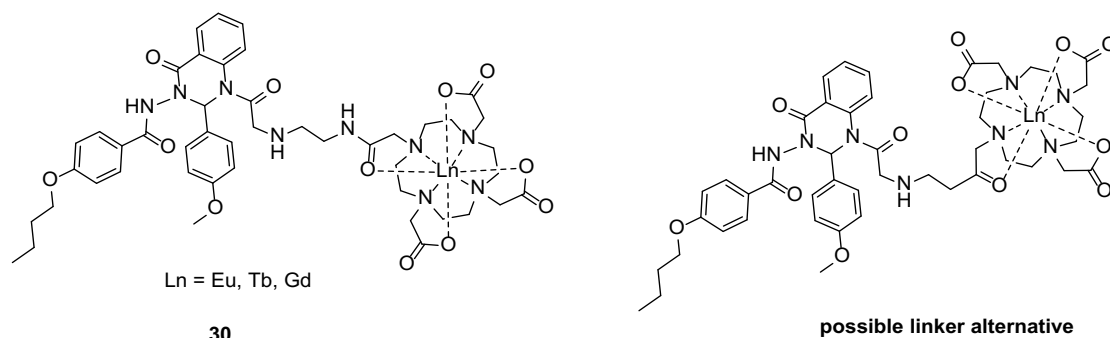


Figure 7.5: Structure of **Ln.30** as discussed in Chapter 4, and a possible alternative molecule to improve metal-based luminescence by excluding the proximal NH quenching group and shortening the antenna-lanthanide distance.

In order to improve lanthanide luminescence properties, design of the ligand system could be tweaked. One option would be to minimise non-radiative de-activation of the lanthanide excited state by limiting the close approach of solvent OH oscillators while at the same time optimising sensitisation efficiency. A nonadentate coordination environment, with no free solvent binding sites directly around the ion, for instance, may substantially improve luminescence. Similarly, the proximal NH oscillator introduced by the linker functionality may be causing substantial excited state deactivation. By changing the linker group, as shown in Figure 7.5, the distance between the antenna and the metal ion is decreased, in addition to circumventing the close approach of NH. Direct combination of Quin C1 acetyl chloride (**7**) with a tBu-DO3A macrocycle would give the shortest spacer between sensitiser and lanthanide, but the reaction was found to be infeasible, likely as a result of unfavourable steric interactions.

Another option that was explored in this project was introduction of a rhenium tricarbonyl species to tune metal-based luminescent properties for time-resolved microscopy applications (Figure 7.6). Re(I) complexes often exhibit long-lived emission in the microsecond to 100s of microsecond range originating from an MLCT excited state that can be populated via formally allowed excitation pathways. This also implies, that extinction coefficients are typically higher than those of lanthanide complexes and their uses in microscopy have been well established.¹⁰ Preparation and characterisation of the designed compound **47** was described, and proof of concept time-resolved microscopy images of the compound in murine neutrophils were obtained at a tenth of the concentration used for Tb.**30**.

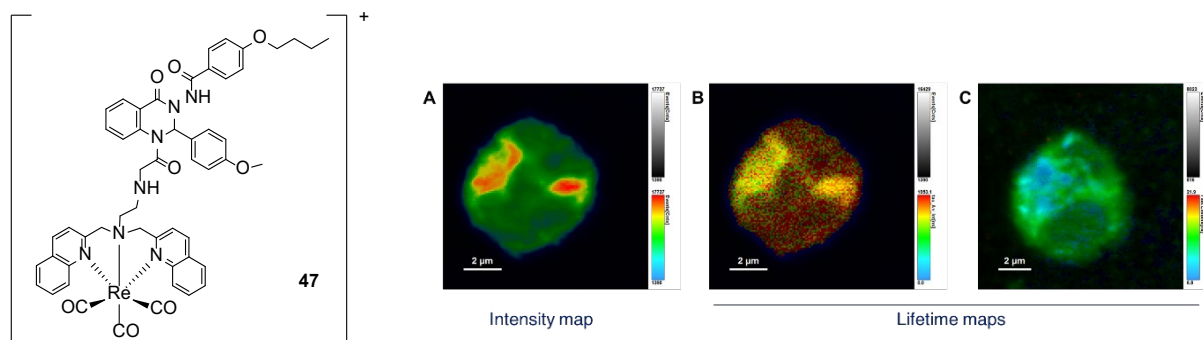


Figure 7.6: Structure of **47** and time-resolved microscopy images obtained using the rhenium tricarbonyl compound in stimulated murine neutrophils in Chapter 5.

Furthermore, due to their similar coordination chemistry, Re(I) is often considered as a mimic for the SPECT-active radioisotope $^{99\text{m}}\text{Tc}$.¹¹ Thus, complexation of $^{99\text{m}}\text{Tc}$ and the ligand framework could allow the FPR2/ALX probe to be translated into nuclear imaging applications and provides an avenue for future investigation.

7.3 Investigation into lanthanide-based luminescence resonance energy transfer and dual-modal probes

In the last chapter, design of compounds incorporating a spirocyclic rhodamine derivative combined with both the Quin C1 targeting group and a lanthanide complex is described.

Notably, the terbium complex exhibited pH-dependent energy transfer properties. When the rhodamine unit was in a spirocyclic state, long-lived terbium emission in the range of 1.5 ms was recorded. At low pH, when the rhodamine unit was in its fluorescent state, a luminescence resonance energy transfer between the terbium and the rhodamine moiety occurred, resulting in long-lived donor-excited rhodamine emission as well as a reduction in the lanthanide donor lifetime. The energy transfer efficiency was determined as $E = 0.53$. Combination of a lanthanide chelate with a non-attached, spectrally matched dye was recently reported to substantially increase the compound's brightness via alternative emission pathways, while still maintaining lifetimes in the microsecond range that allow for time-resolved microscopy.¹² It would therefore be interesting to visualise compound **67**, which combines both a rhodamine fluorophore and a terbium complex within the same molecule, in unstimulated neutrophils at physiological pH (i.e. where no LRET can occur) and compare results to stimulated conditions, when the rhodamine is in its fluorescent form (Figure 7.7).

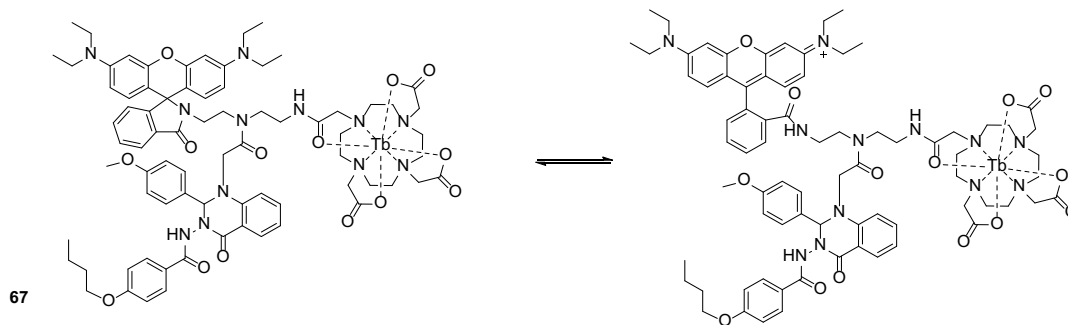


Figure 7.7: Molecular structure of Tb.67 described in Chapter 6, and the associated rhodamine spirocyclisation equilibrium.

By complexing gadolinium in the same ligand system, it is possible to prepare a compound that can provide both MRI contrast enhancement as well as fluorescence signals. However, the relaxivity values obtained were unusually low ($r_1 = 0.6 \text{ mM}^{-1}\text{s}^{-1}$ at 400 MHz, compared to $r_1 \sim 4 \text{ mM}^{-1}\text{s}^{-1}$ for typical octadentate contrast agents). This could be a result of the bulky ligand system shielding the metal ion from solvent approaches or a reduction of average hydration number to below 1 due to unwanted complexation and renders the compound unsuitable for MRI applications.

In order to form a more applicable dual-modal probe, a new design strategy was devised incorporating a cyanine dye. In this construct, the encapsulating, metal-binding unit is anchored via peptide coupling to a carboxylic acid terminal alkyl chain and therefore should be more accessible for solvent water exchange. In addition, the excitation and emission wavelengths of the Cy5 dye are red-shifted compared to the rhodamine unit and accordingly provide a better spectral match for LRET applications when coupled with a Eu(III) chelate, that could give rise to interesting applications. The envisaged molecule is depicted in Figure 7.8 but has not been previously mentioned within this thesis.

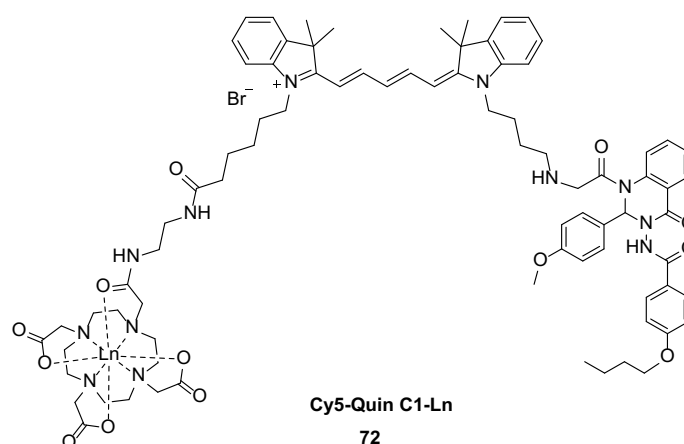
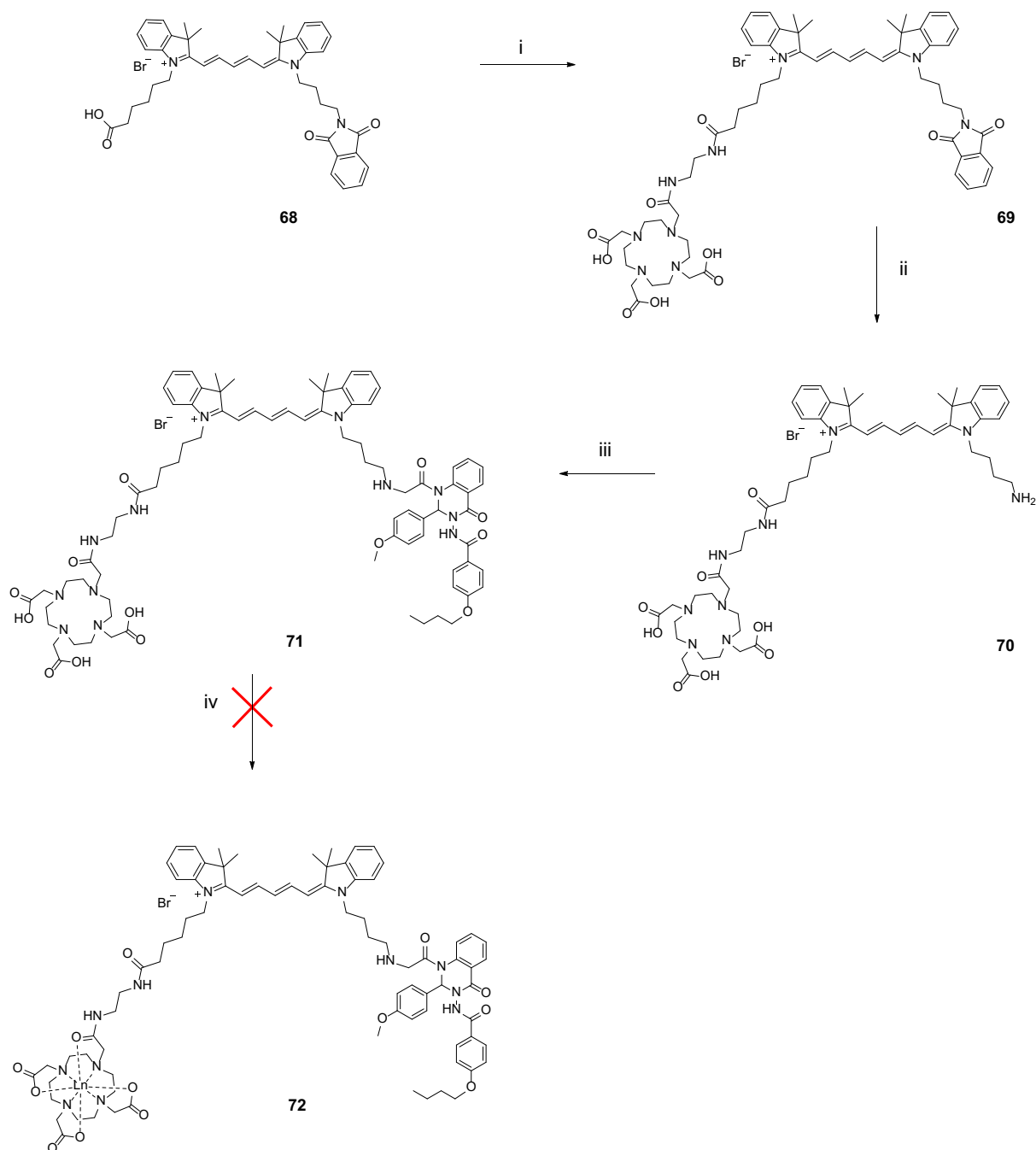


Figure 7.8: Molecular structure of potential dual-modal construct containing Cy5, Quin C1 and a Ln DOTA unit.



Scheme 7.1: Route towards preparation of a dual-modal, FPR2/ALX-targeted construct, **72**. Reagents: i) HBTU, *N,N*-diisopropylethylamine, acetonitrile, borate buffer, compound **23**, carried forward in presence of impurities, ii) methylamine (2M), methanol, 13 % yield, iii) **7**, potassium carbonate, acetonitrile, 69 % yield, iv) Ln(III) chloride hexahydrate, water, pH 5.5.

To prepare the target compound **72**, mono-protected Cy5 (**68**, Scheme 7.1) was prepared from 2,3,3-trimethyl-1-(5-carboxypentyl)-3H-indolium bromide, malonaldehyde bis(phenylimine) monohydrochloride and 2,3,3-trimethyl-1-[N-(phtalimidobutyl)]-3H-indolium bromide and isolated in poor yields (9 %) after repeated column chromatography. Next, the previously prepared, amine terminal, DOTA-derived ligand framework was attached to the dye carboxylic acid via HBTU peptide coupling to

yield **69**. As cyanine dyes are known to degrade under acidic conditions, the DOTA-derived unit was attached in its deprotected form. The phthalimide functionality was cleaved using a methylamine solution to unmask the terminal amine and afforded **70** after separation by repeated column chromatography. Finally, reaction with **7** under basic conditions resulted in formation of **71**. However, complexation with a lanthanide ion in mildly acidic conditions led to degradation of the cyanine dye and preparation of **72** via this route was found to be unsuccessful. To circumvent these issues, synthesis via pre-complexation of the lanthanide compound, followed by coupling with the dye and then introduction of the targeting unit could be attempted as a future direction.

7.4 Final remarks

The work conducted during the course of this thesis has identified the Quin C1 unit as a useful targeting group for the design of imaging agents, which, to the best of our knowledge, has not previously been used in this context. Preparation and characterisation of several Quin C1-derived, luminescent compounds that can act as convenient markers of FPRs was established. The compounds' selectivity for FPRs and utility for visualising activated neutrophils in an inflammatory context was demonstrated *in vitro* and *in vivo* and metal-based constructs enable time-resolved confocal microscopy images of neutrophils to be acquired. A lanthanide-appended dye-targeting group construct proved less successful as a dual-modal imaging agent (when Gd(III) was complexed), as a result of low r_1 values, but displayed interesting pH-sensitive energy transfer properties when Tb(III) was complexed, with an LRET efficiency of $E = 0.53$ at low pH and no energy transfer occurring under neutral and basic conditions.

Several points remain to be addressed in future design strategies. In order to move these ideas forward towards clinical applications, the poor depth penetration of compounds for optical imaging needs to be improved. In addition, it remains to be established whether compound **67** can be used in a cellular context to enhance photophysical properties of the lanthanide component.

7.5 References

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Chapter 8

Experimental Procedures

8.1 General experimental methods

All commercially available reagents were bought from Sigma Aldrich or Fisher Scientific and used without further purification. Deuterated solvents were bought from Goss Scientific. Thin-layer chromatography was conducted using pre-coated Merck Kieselgel 60, F254 plates or Aluminium Oxide 60 plates with. Column chromatography was performed using silica gel, activated alumina gel of different Brockman grades or a C-18 support and laboratory grade solvents, under mild pressure or using a Biotage automated column with Biotage SNAP KP-Sil cartridges of adequate sizes (10 g, 25 g or 50 g). Size exclusion chromatography was performed using Sephadex G-10. Dialysis was performed using a Float-A-Lyser system with a 1000 Da molecular weight cut-off cellulose membrane against distilled water.

8.2 General spectroscopic methods

^1H -NMR and ^{13}C -NMR spectra were recorded on a Bruker AMX-400 spectrometer at room temperature unless stated otherwise and assigned where possible using 2D correlation spectroscopy. NOESY and other specialist NMR experiments were conducted by Peter Haycock of the NMR Service Facility of Imperial College London, Department of Chemistry. Spectra were analysed using MestReNova software and internally referenced to residual solvent peaks.¹ Chemical shifts are quoted in parts per million (ppm), coupling constants are quoted in Hz and multiplicities are abbreviated as: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, dt = doublet of triplets, qu = quartet or m = multiplet, where substantial overlap of resonances or complex coupling of signals did not allow for precise assignment. Broadened signals were marked as br.

Electrospray Time-of-Flight and high-resolution mass spectra were obtained using a Waters LCT Premier by Lisa Haigh of the mass spectrometry facilities of Imperial College London, Department of Chemistry. MALDI-ToF spectra were obtained on a Micromass MALDI Micro MX spectrometer. CI spectra were recorded using ammonia as a reagent gas on a Micromass Platform II.

Infrared spectra were collected in the solid state using a Perkin Elmer Spectrum 100 FTIR Spectrometer.

Crystallographic data were collected and analysed by Dr Andrew White of the Department of Chemistry at Imperial College London on Agilent Xcalibur 3E diffractometer (Mo radiation source). Structures were refined by Dr Andrew White using Olex 2, SHELXTL and SHELX-2013 systems.^{2,3} Relevant data collected for the structure of **6** is collated in section 8.9

8.2.1 Photophysical measurements

UV-vis absorption spectra were recorded on a Perkin Elmer 650 spectrometer in quartz cuvettes. Concentrations were adapted to ensure a sensible absorbance range. Organic fluorophores were typically recorded at 10 μ M and lanthanide complexes at 0.1 mM.

Fluorescence excitation and emission spectra were obtained using a Varian Cary Eclipse spectrophotometer with settings specified on a case by case basis. For pH measurements a Jenway model 3510 pH/mV/temperature meter was used that had been calibrated against pH 10.00, pH 7.00 and pH 4.00 buffers.

Lanthanide-based lifetimes were measured using a Varian Cary Eclipse spectrophotometer in the phosphorescence mode, using 1 mM solutions in water, deuterium oxide or methanol/water mixtures as indicated on a case by case basis. The integrated intensity was recorded after excitation of the samples at 350 nm. The gate time was fixed at 0.1 or 0.2 ms with both excitation and emission slits set at 20 nm. The resulting decay curves were fitted to a single exponential decay in Microsoft Excel. Hydration number (q-values) were calculated according to the following equation.⁴

$$q = A(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - B)$$

with A = 5 ms and B = 0.06 ms⁻¹ for terbium.⁵

Short-lived organic component luminescence lifetimes were assessed in methanol solutions on a time resolved PicoQuant FluoTime 300 with a 375 nm picosecond pulsed laser using time correlated single photon counting (TCSPC). Fitting of the luminescence decay was conducted in FluoFit software.

8.2.2 Relaxometric measurements

Gadolinium(III) complexes were dissolved in water at 3 different molar concentrations. The three solutions and a water standard were added into a glass capillary of 1.7 mm diameter, sealed and inserted into an NMR tube containing D₂O. Concentrations of Gd(III) present were assessed by measuring the difference in solvent shifts according to the magnetic susceptibility measurements conducted using the Evans method.⁶ *T*₁ measurements were carried out by Peter Haycock using a Bruker Advance 400 spectrometer and inversion recovery measurements. Relaxivity values were calculated from the samples using a graphical procedure as described in the literature.⁷

8.3 Cellular studies

8.3.1 Neutrophil isolation protocol

Blood samples were collected by healthcare professionals from healthy volunteers after obtaining informed consent (50 mL). The study was approved by the institutional review board of the LSUHSC-S (STUDY00000261) and conducted in accordance with the Declaration of Helsinki. After discarding the

first 3 mL (to avoid thrombin contamination), 45 mL of the sample were mixed with anticoagulant citrate dextrose (ACD) solution (5 mL). The samples were centrifuged (800 rpm for 20 min at room temperature) and the upper layer of plasma was removed before adding 1 x PBS (10 mL). Subsequently, 6 % dextran (v/w, 1.8 g in 30 mL 1x PBS) solution (8 mL) was added, the sample was gently inverted several times and left to sediment for 15 minutes. The leukocyte layer was collected and separated over a histopaque 1077 gradient (10 mL per tube). After centrifugation (1500 rpm for 30 min at room temperature), the supernatant was removed. Cold water (9 mL) was added in order to lyse residual red blood cell, and subsequently mixed with 10 x PBS (1 mL). The volume was made up to 40 mL using 1 x PBS and centrifuged (1000 rpm for 10 min at room temperature). The supernatant was removed, and the cells were resuspended in 1 x PBS (5 mL) and the procedure was repeated. Finally, neutrophils were resuspended in DMEM with 3 % FCS (10 mL). The cells were counted using a Neubauer hemocytometer (diluted 1:1 with trypan blue) and the resulting cell count was diluted respectively for the number of cells required per assay performed.

8.3.2 *In vitro* methods

All *in vitro* experiments were run at LSUHSC Shreveport.

8.3.2.1 Chemotaxis assay

Chemotaxis assay were run using 3 μ m pore size ChemoTx® System 96 well plates (Neuro Probe, USA). LTB₄ (10^{-6} M, 29 μ L, Sigma Aldrich) as a chemotactic stimulus or PBS as a control (29 μ L) were added to the bottom of the wells. Freshly isolated neutrophils at a concentration of 4×10^6 cells/mL in DMEM and 3 % fetal calf serum (FCS) were treated with vehicle (PBS) or varying concentrations of the compound for 10 minutes prior to addition to the top of the well plate membranes (25 μ L). The plates were incubated for 3 hours at 37 °C with 5 % CO₂ before removal of the membrane. Migrated cells were manually counted using a Neubauer hemocytometer after dilution in a 1:1 ratio with trypan blue.

8.3.2.2 Transmigration assay

To assess cellular transmigration, 24-well plate inserts were treated with fibronectin for 30 minutes, before a monolayer of human umbilical vein endothelial cells (HUVECs, 32,000 cell/200 μ L) were grown for 72 hours. HUVEC media was removed and neutrophils (10^6 cells/mL in DMEM and 3 % FCS) that had been pre-treated with vehicle or varying compound concentrations for 10 minutes, were added to the inserts (500 μ L). PBS (vehicle, 500 μ L) or LTB₄ (10^{-6} M, 500 μ L) were added into a fresh 24-well plate set up and the inserts were transferred before incubation (3 hours at 37 °C with 5 % CO₂). Migrated cells were manually counted as described above.

8.3.2.3 Myeloperoxidase assay

Myeloperoxidase (MPO) release was quantified on cells that had been pre-activated by the transmigration assay. In order to achieve this, the neutrophil supernatant (20 μ L) was collected from the

bottom of transmigration plates and mixed in a 4:1 ratio with a 9:1:0.1 mixture of solutions A, B and C respectively that had been previously prepared. Solution A was made up of 5.4 g KH_2PO_4 , 1.875 g EDTA and 5 mL Triton X-100. Solution B was a solution of 3,3',5,5'-tetramethylbenzidine (TMB, 10 mM) in acetone (2 mL) and solution C was an aqueous solution of hydrogen peroxide (30 %, 25 μL) in water (10 mL). The cellular mixture was incubated at room temperature for approximately 5 minutes until a colour change occurred and the reaction was terminated by addition H_2SO_4 (1 M, 25 μL). Absorbance was read at 450 nm relative to a standard.

8.4 Immunofluorescence microscopy

Pharmacological blocking studies were carried out by seeding neutrophils (2×10^5 cells per well in 200 μL) on poly-L-lysine coated coverslips (Discovery Labware, Bedford, MA, USA) in a 24 well plate. After letting them rest for 30 minutes, they were treated with vehicle, Boc-2 (Pan FPR antagonist, 10 μM), Cyclosporin H (FPR1 antagonist, 10 μM) or WRW4 (FPR2 antagonist, 10 μM) and incubated for 15 minutes at 37 °C and 5 % CO_2 . Cells were subsequently stained with **17** (1 μM) and left for an hour before fixing using 10 % formalin. The nuclear stain 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) was added and cells were incubated for 10 minutes before washing. The coverslips were treated with mounting media (Fluoromont-G, Southern Biotech, Birmingham, Alabama, USA) and mounted on glass slides, where they were dried and attached using nail polish. Visualisation was performed using a Nikon Eclipse Ti inverted epifluorescence microscope (Minato-ku, Tokyo, Japan).

To assess compound **13** in a cellular context, neutrophils (2×10^5 cells per well in 200 μL) were stimulated with phorbol 12-myristate 13-acetate (PMA, 100 nM) or treated with vehicle (PBS) for one hour (37 °C, 5 % CO_2) and seeded on a poly-L-lysine coated coverslips (Discovery Labware, Bedford, MA, USA) in a 24 well plate, before fixing and mounting steps as described above.

Image quantification was accomplished by comparing relative fluorescence emission intensities for each sample (using an average from several fields of view) as a ratio between signals observed in the in-built Texas red channel and those observed in the in-built DAPI channel on NIS Elements Advanced Research (Nikon Instruments Inc.) software.

8.5 Time-resolved microscopy

Neutrophils were pre-treated with $\text{TNF}\alpha$ (50 ng/mL, 30 minutes) or with PBS (control) and incubated with varying concentrations of compound or vehicle for 45 minutes (37 °C, 5 % CO_2). Cells were seeded on poly-L-lysine sheets (300,000 cells per sheet) and fixed using paraformaldehyde (20 minutes) followed by direct transfer onto Fluoromont G coated glass slides. The sheets were firmly adhered to the slides using a nail base coat. Visualisation was performed on a PicoQuant Microtime 200 time-resolved confocal fluorescence microscope and terbium-centred emission was recorded using pulsed 375 nm laser excitation.

8.6 *In vivo* protocols

All *In vivo* work was performed by Dr Elena Senchenkova at LSUSHC Shreveport in accordance with the Animal Care and Use Committee of LSUHSC-S.

Male C57bl/6 mice were anaesthetised with ketamine hydrochloride (150 mg/kg body weight, i.p. and xylazine (7.5 mg/kg body weight, i.p.) and the jugular vein was cannulated for drugs/antibody administration. Next, mice were injected intrascrotally with either saline (control group) or recombinant mouse TNF α (250 ng in 200 μ L saline) to induce an acute inflammatory response. A separate group was treated with recombinant mouse TNF α (250 ng in 200 μ L saline) and a subsequent injection of BMS-470539 (20 mg/kg, i.p.) one hour after the initial dosage to attenuate inflammation. One hour prior to neutrophil observation, **17** (15 μ g in 50 μ L of saline) was injected for recirculation. After two hours from the initial injection, the cremaster muscle was prepared for visualisation by intravital microscopy according to surgical procedures previously described.⁸ 5 minutes before visualisation, Alexa Fluor 488 conjugated anti-mouse Ly-6G (Gr-1) clone RB6-8C5 (2 μ g/mouse) was injected to enable identification of neutrophils. The animal body temperature was maintained at 35 - 37°C and after a 20 minute stabilisation period, the microcirculation was visualised using a spinning disc intravital microscope (Olympus BX51WI upright microscope with a 40XW (LUMPLFLN) (Olympus, Center Valley, PA, USA) objective equipped with a 3i LaserStack laser launch (3i, Denver, CO, USA), Yokogawa CSU-X1-A1N-E spinning disk confocal unit (Yokogawa Electric Corporation, Musashino, Tokyo, Japan) and electron multiplier CCD camera (C9100-13; Hamamatsu, Bridgewater, NJ, USA)). Within the recorded venular segments (5 - 11 per run) with a diameter of 20 - 50 μ m, the number of firmly adherent neutrophils (stationary for $\geq$ 2 seconds) and the number of emigrated neutrophils were quantified. Emigrated cells were defined as neutrophils up to 50 μ m either side of the vessel. Data was measured per vessel segment and averaged for each animal. Analysis was conducted offline using Slidebook software (3I, Denver, CO, USA).

8.7 Statistical analysis

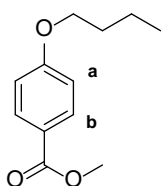
Statistical analysis was performed using Graph Pad Prism8 software (San Diego, USA). Data was assessed using Student's *t*-test (for two groups) or ANOVA with Bonferroni post-tests (for more than two groups) and represented as mean values $\pm$ standard error of the mean (SEM) and differences were considered statistically significant at a value of $p < 0.05$. Each experiment was run in duplicate or triplicate per compound and per concentration on a minimum of $n = 3$ independent donors per compound.

8.8 Synthetic procedures

Tert-butyl DO3A,⁹ Rhodamine B-piperazine,¹⁰ Rhodamine B ethylene diamine¹¹ and DOTAGA¹² are well-known in the literature and were prepared as outlined in reported protocols. Compound **50** was prepared as published by Rivas *et al.*¹³

8.8.1 Synthesis of compounds in Chapter 2

Methyl 4-butoxybenzoate (**2**)¹⁴

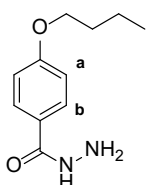


Potassium carbonate (6.83 g, 50 mmol) and methyl 4-hydroxybenzoate (5.000 g, 33 mmol) were dissolved in methanol (60 mL) and stirred under nitrogen at 0 °C for 20 minutes. 1-Bromobutane (4.970 g, 37 mmol) was added dropwise before the mixture was heated at reflux for 23 hours. The solvents were removed *in vacuo*, the reaction mixture was dissolved in dichloromethane (50 mL), washed with water (3 x 50 mL), brine (2 x 50 mL), dried over magnesium sulphate and concentrated. Purification was achieved by silica column chromatography in dichloromethane/petroleum ether (3:1) and yielded a clear oil (4.090 g, 60 %).

¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 0.99 (3H, t, ³*J*_{HH} = 7.4 Hz), 1.51 (2H, m, ³*J*_{HH} = 7.6 Hz), 1.80 (2H, m, ³*J*_{HH} = 6.5 Hz), 3.89 (3H, s), 4.02 (2H, t, ³*J*_{HH} = 6.5 Hz), 6.91 (2H, d, ³*J*_{HH} = 8.9 Hz, H_a), 7.98 (2H, d, ³*J*_{HH} = 8.9 Hz, H_b).

CI⁺: *m/z* 209 {M+H}⁺ (~25% relative intensity), 226 {M+NH₄}⁺ (100 % relative intensity)

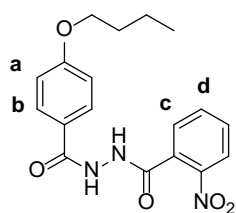
4-Butoxybenzohydrazide (**3**)¹⁴



2 (2.700 g, 13 mmol) and hydrazine monohydrate (2.600 g, 52 mmol) were dissolved in ethanol (50 mL) and heated at 65°C under a nitrogen atmosphere for 24 hours, before further 4 equivalents of hydrazine monohydrate were added. The reaction was heated for further 30 hours. Subsequently, the reaction mixture was concentrated *in vacuo* and the resulting oil was purified by activated alumina column chromatography (Brockman grade V), eluting initially with dichloromethane. Once the starting material had eluted, a methanol gradient (up to 20 %) was added to afford a crystalline white solid (1.900 g, 70 %).

¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 0.99 (3H, t, ³*J*_{HH} = 7.4 Hz), 1.51 (2H, m, ³*J*_{HH} = 7.6 Hz), 1.79 (2H, m, ³*J*_{HH} = 6.5 Hz), 4.01 (2H, t, ³*J*_{HH} = 6.5 Hz), 4.07 (2H, br, -NH₂), 6.93 (2H, d, ³*J*_{HH} = 8.9 Hz, H_a), 7.70 (2H, d, ³*J*_{HH} = 8.9 Hz, H_b).

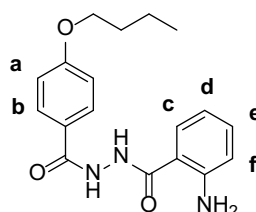
ES⁺: *m/z* 209 {M+H}⁺ (100 % relative intensity)

2-(4-Butoxybenzoyl)hydrazide 2-nitro-benzoic acid (4)

4-Butoxybenzohydrazine (1.800 g, 9 mmol) and potassium carbonate (1.200 g, 9 mmol) were dissolved in dichloromethane at 0 °C under a nitrogen atmosphere. 2-Nitro-benzoyl chloride (1.0 mL, 7.8 mmol) was added slowly to the solution, which was gradually allowed to warm to room temperature. After stirring for further 15 hours, the reaction mixture was washed with water (2 x 50 mL) and brine (2 x 50 mL), the organic fractions were combined and dried over magnesium sulphate before the remaining solvents were removed *in vacuo* to yield a pale yellow solid (1.38 g, 43 %).

$^1\text{H-NMR}$ (400 MHz, MeOD) δ_{H} (ppm): 1.01 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.53 (2H, m, $^3J_{\text{HH}} = 7.4$ Hz), 1.80 (2H, m, $^3J_{\text{HH}} = 6.4$ Hz), 4.07 (2H, t, $^3J_{\text{HH}} = 6.4$ Hz), 7.01 (2H, d, $^3J_{\text{HH}} = 8.9$, H_{a}), 7.72-8.16 (6H, br, $\text{H}_{\text{b-f}}$).

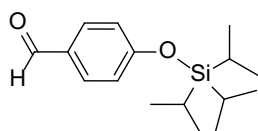
ES+: m/z 358 $\{\text{M}+\text{H}\}^+$ (100 % relative intensity)

2-(4-Butoxybenzoyl)hydrazide 2-amino-benzoic acid (5)

4 (1.380 g, 3.8 mmol) was dissolved in a mixture of acetic acid and dichloromethane (1:1, 80 mL) at 0°C. Excess zinc dust (2.500 g, 38.4 mmol) was added to the stirring mixture which was subsequently gradually allowed to warm to room temperature. After 16 hours, the mixture was filtered through celite and washed with dichloromethane (10 x 20 mL). The solvents were removed *in vacuo*, before the reactants were dissolved in dichloromethane, washed with saturated sodium bicarbonate (3 x 50 mL) and dried over magnesium sulphate to yield an off-white solid (0.870 g, 70 %).

$^1\text{H-NMR}$ (400 MHz, MeOD) δ_{H} (ppm): 1.01 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.54 (2H, m, $^3J_{\text{HH}} = 7.6$ Hz), 1.79 (2H, m, $^3J_{\text{HH}} = 6.4$ Hz), 4.07 (2H, t, $^3J_{\text{HH}} = 6.4$ Hz), 6.75 (2H, m, H_{Ar}), 7.02 (2H, m, H_{a}), 7.32 (1H, m, H_{Ar}), 7.73 (1H, m, H_{Ar}), 7.90 (2H, m, H_{b}).

ES+: m/z 368 $\{\text{M}+\text{MeCN}\}^+$ (100 % relative intensity)

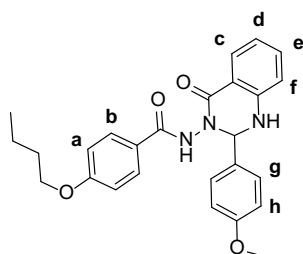
4-(Triisopropylsilyloxy) benzaldehyde¹⁵

Triethylamine (0.200 g, 2.0 mmol) and 4-dimethylaminopyridine (0.200 g, 1.6 mmol) were added to a stirring solution of 4-hydroxy benzaldehyde (2.000 g, 16.4 mmol) in dichloromethane (60 mL) at 0 °C. After 10 minutes, 4-triisopropylsilyl chloride (3.5 g, 18 mmol) was added and the reaction mixture was warmed to room temperature slowly. After stirring for 16 hours the reaction mixture was washed with water, the aqueous layer was extracted with dichloromethane twice and the organic fractions were combined. The crude product was dried over sodium sulphate and concentrated *in vacuo*. Isolation of the product was achieved by silica column chromatography eluting with petroleum ether/ethyl acetate (20:1) to yield a pale yellow oil (1.350 g, 30 %).

$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ_{H} (ppm): 1.11 (18H, m), 1.23-1.34 (3H, m), 6.98 (2H, d, $^3J_{\text{HH}} = 8.5$ Hz), 7.78 (2H, d, $^3J_{\text{HH}} = 8.7$ Hz), 9.88 (1H, s).

ES+: m/z 279 $\{M+H\}^+$ (~35 % relative intensity)

Quin C1 (6)¹⁶



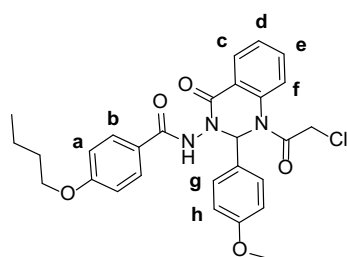
2-(4-Butoxybenzoyl)hydrazide 2-amino-benzoic acid (0.536 g, 1.6 mmol) and 4-methoxy benzaldehyde (0.268 g, 2.0 mmol) were dissolved in ethanol (20 mL) and heated at reflux for an hour before addition of citric acid (0.378 g, 2.0 mmol). The reagents were further refluxed for 48 hours. Residual solvent was removed *in vacuo* and the reaction mixture was dissolved in dichloromethane and filtered. The filtrate was purified by silica column chromatography eluting with a dichloromethane and acetone gradient of 0 % - 50 % to yield a white solid (0.590 g, 83 %).

$^1\text{H-NMR}$ (400 MHz, d_6 -acetone) δ_{H} (ppm): 0.95 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.47 (2H, m, $^3J_{\text{HH}} = 7.6$ Hz), 1.74 (2H, m, $^3J_{\text{HH}} = 6.5$ Hz), 3.78 (3H, s), 4.02 (2H, t, $^3J_{\text{HH}} = 6.5$ Hz), 6.30 (2H, br, NH and NC(H)N), 6.83 (1H, td, $^3J_{\text{ortho}} = 7.1$ Hz, $^4J_{\text{meta}} = 1.0$ Hz, H_d), 6.90 (5H, m, $\text{H}_{a,h,f}$), 7.35 (1H, td, $^3J_{\text{ortho}} = 8.0$ Hz, $^4J_{\text{meta}} = 1.6$ Hz, H_e), 7.49 (2H, d, $^3J_{\text{ortho}} = 8.6$, H_g), 7.66 (2H, m, H_b), 7.84 (1H, dd, $^3J_{\text{ortho}} = 7.8$ Hz, $^4J_{\text{meta}} = 1.5$ Hz, H_c), 9.38 (1H, br). Assignment aided by NOESY spectroscopy.

$^{13}\text{C-NMR}$ (400 MHz, d_6 -acetone) δ_{H} (ppm): 14.1, 19.8, 31.9, 55.6, 68.5, 74.8, 114.4, 114.8, 115.5, 119.1, 129.3, 130.2, 130.4, 134.6, 149.7, 161.4, 162.9, 165.3, 166.3, 180.7.

HRMS: m/z calculated: 446.2080, found: 446.2089 for $\{M+H\}^+$ (~50 % relative intensity)

Quin C1-acetyl chloride (7)



Compound **6** (0.100 g, 0.22 mmol) and triethylamine (0.033 g, 0.33 mmol) were dissolved in dichloromethane (3 mL) under a nitrogen atmosphere. The stirring solution was immersed in an ice/acetone bath and chloroacetyl chloride (0.030 g, 0.26 mmol) in dichloromethane (2 mL) was added dropwise. The reaction mixture was gradually warmed to room temperature and stirred overnight, before it was washed with

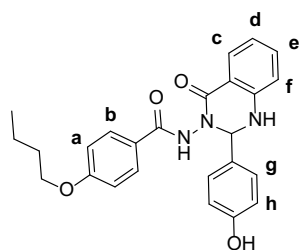
water (2 x 5 mL) and dried over magnesium sulphate. Purification was achieved by silica column chromatography eluting with a dichloromethane/acetone mixture (95:5) to afford an off-white solid (0.070 g, 60 %).

$^1\text{H-NMR}$ (400 MHz, d_6 -acetone) δ_{H} (ppm): 0.97 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.50 (2H, m, $^3J_{\text{HH}} = 7.6$ Hz), 1.78 (2H, m, $^3J_{\text{HH}} = 7.2$ Hz), 3.72 (3H, s), 4.08 (2H, t, $^3J_{\text{HH}} = 6.5$ Hz), 4.84 (2H, br), 6.81 (2H, dt, $^3J_{\text{ortho}} = 8.9$ Hz, $^4J_{\text{meta}} = 2.1$ Hz, H_h), 7.02 (2H, m, H_a), 7.13 (1H, br, s, NC(H)N), 7.39 (3H, m, $\text{H}_{g,e}$), 7.58 (2H, m, $\text{H}_{f,c}$), 7.96 (3H, m, $\text{H}_{b,d}$), 10.37 (1H, br). Exact assignment aided by NOESY spectroscopy.

^{13}C -NMR (400 MHz, d_6 -acetone) δ_{H} (ppm): 13.1, 18.8, 30.9, 54.5, 67.6, 113.7, 114.1, 124.0, 126.4, 127.7, 128.5, 129.6, 132.9, 159.8, 160.4, 162.5, 165.7.

HRMS: m/z calculated: 552.1790, found: 552.1793 for $\{\text{M}+\text{H}\}^+$ (~70 % relative intensity)

Quin C7 (8)



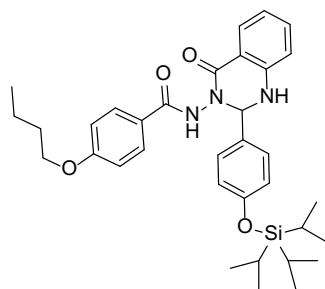
2-(4-Butoxybenzoyl)hydrazide 2-amino-benzoic acid (0.13 g, 0.4 mmol) and 4-hydroxy benzaldehyde (0.06 g, 0.5 mmol) were refluxed in ethanol (5 mL) for half an hour before addition of citric acid (0.08 g, 0.4 mmol). After three days, residual solvents were evaporated and the product was isolated by silica column chromatography eluting in dichloromethane and acetone (9:1) (0.080 g, 47 %).

^1H -NMR (500 MHz, d_6 -acetone) δ_{H} (ppm): 0.98 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.49 (2H, m, $^3J_{\text{HH}} = 7.7$ Hz), 1.75 (2H, m, $^3J_{\text{HH}} = 6.4$ Hz), 4.00 (2H, t, $^3J_{\text{HH}} = 6.2$ Hz), 6.25 (2H, s, NH and NC(H)N), 6.78-6.95 (6H, m, $\text{H}_{\text{h,d,f,a}}$), 7.34 (1H, td, $^3J_{\text{ortho}} = 7.2$ Hz, $^4J_{\text{meta}} = 1.6$ Hz, H_{e}), 7.39 (2H, m, H_{g}), 7.66 (2H, m, H_{b}), 7.82 (1H, m, H_{c}), 8.50 (1H, s), 9.31 (1H, br).

^{13}C -NMR (400 MHz, d_6 -acetone) δ_{H} (ppm): 14.1, 19.8, 31.9, 55.0, 68.5, 75.7, 114.8, 115.4, 115.8, 116.4, 119.0, 125.8, 129.3, 129.7, 130.1, 130.5, 134.5, 149.1, 159.1, 162.9, 164.6, 166.2.

HRMS: m/z calculated: 432.1923, found: 432.1915 for $\{\text{M}+\text{H}\}^+$ (100 % relative intensity)

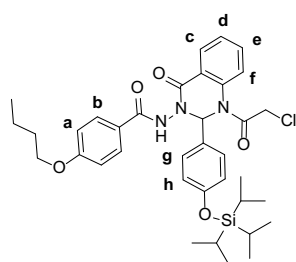
Quin C7-TIPS (9)



2-(4-Butoxybenzoyl)hydrazide 2-amino-benzoic acid (0.400 g, 1.2 mmol), citric acid (0.240 g, 1.2 mmol) and 4-(triisopropylsilyloxy) benzaldehyde (0.400 g, 1.5 mmol) were dissolved in ethanol (20 mL) and heated at reflux for 60 hours. Residual solvent was removed *in vacuo* to give a dark brown oil. The product was isolated by silica column chromatography eluting initially with dichloromethane and then acetone (5 %) was added to yield a pale yellow oil (0.100 g, 14 %).

^1H -NMR (400 MHz, CDCl_3) with impurities present δ_{H} (ppm): 1.00 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.20-1.37 (21H, m, br), 1.52 (2H, m), 1.80 (2H, m), 4.02 (2H, t, $^3J_{\text{HH}} = 6.6$ Hz), 6.84- 7.03 (4H, br), 7.53 (1H, m), 7.62-7.73 (3H, m), 7.83 (2H, m), 8.32 (1H, d, $^3J_{\text{HH}} = 7.8$).

HRMS: m/z calculated: 586.3101, found: 586.3124 for $\{\text{M}-\text{H}\}^+$ (100 % relative intensity)

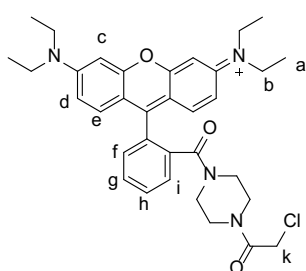
Quin C7-TIPS acetyl chloride (10)

8 (0.098 g, 0.17 mmol) and triethylamine (0.051 g, 0.50 mmol) were dissolved in dichloromethane (3 mL) under a nitrogen atmosphere. The stirring solution was immersed in an ice/acetone bath and chloroacetyl chloride (0.038 g, 0.33 mmol) in dichloromethane (2 mL) was added dropwise. The reaction mixture was gradually warmed to room temperature and stirred overnight. The reaction mixture was washed with water (10 mL) and dried over magnesium sulphate. Purification was achieved by silica column chromatography eluting with dichloromethane to afford a clear oil (0.015 g, 13 %).

$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ_{H} (ppm): 0.95 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.21 (18H, br), 1.23-137 (3H, m, br), 1.57 (2H, m), 1.80 (2H, m), 4.04 (2H, t, $^3J_{\text{HH}} = 6.6$ Hz), 4.53 and 4.80 (2H, ABq, $^2J_{\text{AB}} = 15.3$ Hz), 6.89 (2H, d, $^3J_{\text{HH}} = 9.1$ Hz, H_a), 7.04 (2H, d, $^3J_{\text{HH}} = 8.9$ Hz, H_h), 7.42 (2H, d, $^3J_{\text{HH}} = 9.0$ Hz, H_b), 7.56 (2H, d, $^3J_{\text{HH}} = 8.8$ Hz, H_g), 7.66 (1H, m, H_d or e), 7.76 (1H, m, H_c or f), 7.95 (1H, m, H_d or e), 8.27 (1H, m, H_c or f).

$^{13}\text{C-NMR}$ (400 MHz, d_6 -acetone) δ_{H} (ppm): 13.4, 14.0, 18.3, 19.8, 31.8, 44.6, 68.8, 115.1, 120.6, 121.6, 125.1, 126.5, 128.0, 128.6, 129.0, 131.3, 132.0, 136.6, 147.9, 155.2, 159.2, 164.3, 169.0.

HRMS: m/z calculated: 662.2817, found: 662.2806 for $\{\text{M-H}\}^+$ (100 % relative intensity)

8.8.2 Synthesis of compounds in Chapter 3**Rhodamine B piperazine amide acetyl chloride (16)**

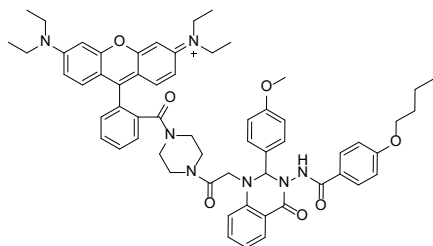
Rhodamine B piperazine amide (1.00 g, 2.0 mmol) was prepared according to literature procedures¹⁰ and dissolved in dichloromethane (30 mL) at 0°C. Triethylamine (1.113 mL, 8.0 mmol) was added to the mixture followed by dropwise addition of chloroacetyl chloride (0.175 mL, 2.2 mmol). The solution was stirred and slowly warmed to room temperature. After 3 hours water was added, and the aqueous phase was extracted with dichloromethane. Organic fractions were combined and dried over

magnesium sulphate prior to separation of the desired product by silica column chromatography eluting in dichloromethane with an acetone gradient, to yield a pink solid. (0.460 g, 39 %)

$^1\text{H-NMR}$ (400 MHz, d_6 -acetone), δ_{H} (ppm): 1.33 (12H, t, $^3J_{\text{HH}} = 7.9$ Hz, H_a), 3.24- 3.67 (8H, br), 3.77 (8H, qu, $^3J_{\text{HH}} = 7.1$ Hz, H_b), 4.23 (2H, br s, H_k), 6.95 (2H, br m, H_c), 7.17 (2H, br, H_d), 7.35 (2H, d, $^3J_{\text{HH}} = 9.5$ Hz, H_e), 7.54-7.60 (1H, m, H_f), 7.73-7.82 (3H, m, $\text{H}_{g,h,i}$).

$^{13}\text{C-NMR}$ (400 MHz, d_6 -acetone) δ_{H} (ppm): 12.8, 38.7, 42.0, 46.6, 96.9, 114.6, 115.1, 128.6, 130.7, 131.3, 132.1, 133.1, 156.7, 158.7.

MS m/z : 587 $\{\text{M}\}^+$ (100 % relative intensity)

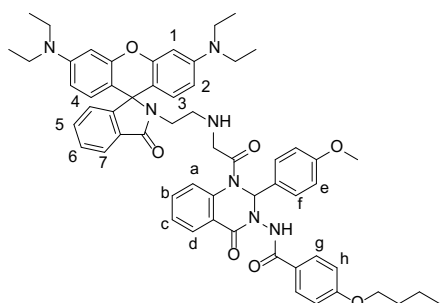
Rhodamine piperazine-Quin C1 (17)

To a solution of Rhodamine B piperazine amide acetyl chloride (0.012 g, 20 μ mol) in acetonitrile (5 mL), N,N-diisopropylethylamine (18 μ L) and **6** (0.010 g, 22 μ mol) were added and the mixture was stirred overnight at 65°C. Purification was achieved by repeated silica column chromatography, initially eluting in dichloromethane and acetone (10 %), with an added gradient of methanol (up to 30 %). Subsequently, the fractions containing the desired product were separated again using a dichloromethane/acetone (4:1) eluent. The product was obtained as a deep pink solid (0.006 g, 30 %).

$^1\text{H-NMR}$ (400 MHz, d_6 -acetone), δ_{H} (ppm): 0.99 (3H, m) 1.06-1.40 (12H, m), 1.51 (2H, m), 1.77 (2H, m), 3.12- 4.18 (23H, br), 6.10-8.61 (22H, br).

$^{13}\text{C-NMR}$ (500 MHz, d_6 -acetone) δ_{H} (ppm), spectrum faint: 11.7, 12.8, 14.4, 19.8, 23.3, 23.7, 32.6, 33.9, 35.2, 37.6, 41.2, 46.6, 64.5, 66.2, 66.5, 73.4, 97.0, 113.4-116.1, 129.3, 130.6, 132.0, 133.5, 135.6, 156.8, 157.1, 158.7, 168.0.

HRMS m/z : found: 996.5035 calculated: 996.5024 $\{M+H\}^+$ (100 % relative intensity)

Rhodamine ethylenediamine Quin C1 (13)

Rhodamine ethylenediamine (**12**), prepared according to literature procedures¹¹ (0.011 g, 22 μ mol), 4-butoxy-N-(1-(2-chloroacetyl)-2-(4-methoxyphenyl)-4-oxo-1,4-dihydroquinazolin-3(2H)-yl)benzamide (0.010 g, 18 μ mol) and potassium carbonate (0.015 g, 109 μ mol) were stirred in acetonitrile (2 mL) at 60°C for 17 hours. The product was isolated by silica column chromatography eluting in a dichloromethane and acetone mixture (9:1) as a pink solid (0.011g, 63 %).

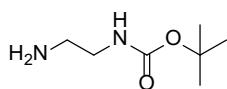
$^1\text{H-NMR}$ (400 MHz, d_6 -acetone), δ_{H} (ppm): 0.97 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz) 1.12 (12H, t, $^3J_{\text{HH}} = 7.1$ Hz), 1.50 (2H, m), 1.77 (2H, m), 2.50- 2.64 (2H), 3.26- 3.42 (10H, br), 3.72 (3H, s), 4.08 (2H, t, $^3J_{\text{HH}} = 6.5$ Hz), 6.32-6.54 (6H, m, $\text{H}_{1,2,3}$), 6.80 (2H, d, H_{e}), 6.98-7.06 (3H, m, $\text{H}_{\text{h},4}$), 7.30 (1H, m, br, H_{c}), 7.41 (2H, br m, $\text{H}_{\text{a,b}}$), 7.47- 7.56 (4H, m, $\text{H}_{5,6,\text{f}}$), 7.77-8.05 (4H, m, $\text{H}_{\text{g,d},7}$), 10.32 (1H, br).

$^{13}\text{C-NMR}$ (500 MHz, d_6 -acetone) δ_{H} (ppm): 12.9, 14.1, 19.8, 31.9, 40.4, 44.9, 48.2, 50.6, 51.6, 55.5, 68.6, 98.4, 109.1, 114.5, 115.0, 123.2, 124.5, 128.8, 129.5, 130.0, 130.6, 132.0, 132.2, 133.3, 149.7, 154.1, 154.7, 160.7, 161.4, 163.4, 168.3, 168.7.

HRMS m/z : found: 970.4863 calculated: 970.4867 $\{M+H\}^+$ (~30 % relative intensity)

8.8.3 Synthesis of compounds in Chapter 4

N-(*tert*-butoxycarbonyl)-1,2-diaminoethane (**20**)¹⁷

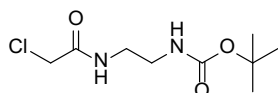


Ethylene diamine (10 mL, 150 mmol) was dissolved in ethanol (100 mL) and stirred at 0°C under a nitrogen atmosphere. Di-*tert*-butyl dicarbonate (3.280 g, 15 mmol) in ethanol (10 mL) was added dropwise and the reaction was slowly warmed to room temperature and left to stir overnight. The reaction mixture was evaporated to dryness under reduced pressure, re-dissolved in dichloromethane (100 mL) and washed with aqueous sodium hydroxide (2M, 100 mL). The organic layers were combined, dried over magnesium sulphate and concentrated *in vacuo* to yield an off-white oily solid (1.980 g, 83 %).

¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 1.45 (9H, s), 2.80 (2H, t, ³J_{HH} = 6.0 Hz), 3.17 (2H, br, qu), 4.84 (1H, br, s).

ES+: *m/z* 161 {M+H}⁺ (100 % relative intensity)

N-(*tert*-butoxycarbonyl)-N'-aminoacetylchloride (**21**)¹⁸



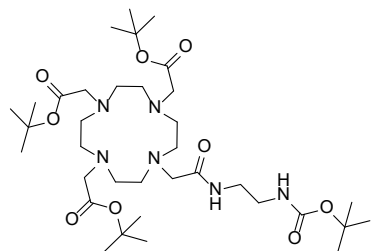
N-(*tert*-butoxycarbonyl)-1,2-diaminoethane (1.000 g, 6.3 mmol) and triethylamine (1.3 mL, 9.4 mmol) were dissolved in dichloromethane (20 mL) and stirred in an ice/acetone bath under nitrogen. Chloroacetyl chloride (0.85

g, 7.5 mmol) in dichloromethane (20 mL) was added dropwise over the course of an hour and the reaction was allowed to warm to room temperature. After 15 hours, the solvent was removed *in vacuo* and the crude product was washed with water, dried over magnesium sulphate and concentrated *in vacuo* to yield a light brown solid (1.040 g, 70 %).

¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 1.45 (9H, s), 3.33 (2H, m), 3.42 (2H, m), 4.04 (2H, s), 4.83 (1H, br, s), 7.18 (1H, br, s).

ES+: *m/z* 300 {M+MeCN+Na}⁺ (~65 % relative intensity)

1,4,7-Tris(*tert*-butoxycarbonylmethyl)-10-(N-(2-*tert*butoxycarbonylaminoethyl)acetamide)-1,4,7,10-tetraazacyclododecane (**22**)¹⁹

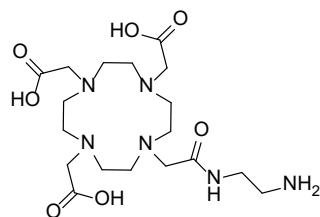


Tert-butyl DO3A (2.820 g, 5.5 mmol), sodium hydrogen carbonate (3.300 g, 38.5 mmol) and **21** (1.310 g, 5.5 mmol) were dissolved in acetonitrile (30 mL) and heated at reflux for 20 hours. The reaction mixture was subsequently cooled, inorganic salts were removed by filtration and the crude was concentrated *in vacuo*. Purification via silica column chromatography eluting in dichloromethane with a methanol gradient from 2 % to 20 % resulted in a dark brown oil (1.290 g, 33 %).

¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 1.37-1.49 (36H, br), 2.20-3.58 (not resolved, v br), 4.10 (2H, m), 6.08 (1H, br s), 8.77 (1H, br s).

ES+: m/z 358 $\{M+2H\}^{2+}$ (~45% relative intensity) and 716 $\{M+H\}^+$ (100 % relative intensity)

1,4,7-Tris(carbonylmethyl)-10-(aminoethyl-N-acetyl)-1,4,7,10-tetraazacyclododecane (**23**)¹⁹



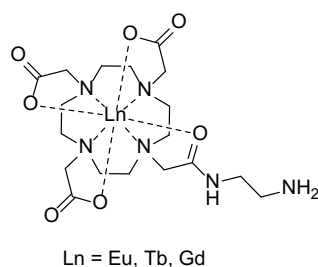
To a stirring solution of **22** (1.290 g, 1.8 mmol) in dichloromethane (10 mL), trifluoroacetic acid (5 mL) was added dropwise. The reaction was left to stir at room temperature for 24 hours before the solvents were removed under reduced pressure. The residue was consecutively washed and evaporated to dryness with dichloromethane (3 x 30 mL) and methanol (3 x 30 mL) and finally recrystallised from a methanol and diethyl ether mixture to yield an off-white solid (0.790 g, 98 %).

¹H-NMR (400 MHz, CDCl₃) δ_H (ppm): 2.93 - 4.04 (28H not resolved, br).

IR (solid) in cm⁻¹: 3099, 2359, 2341, 1674, 1458, 1361, 1200, 1127.

ES+: m/z 447 $\{M+H\}^+$ (100 % relative intensity)

General synthetic procedure for preparation of Ln.**29**



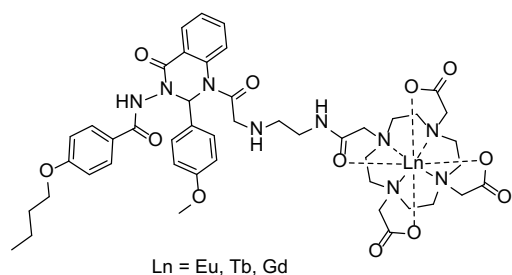
Compound **23** and the respective lanthanide salt were dissolved in water in a 1:1.1 molar ratio, the pH was adjusted to 5.5 and the solution was stirred overnight at room temperature. Subsequently, the pH of the mixture was adjusted to 10 using a sodium hydroxide solution (1 M) and stirred for 45 minutes. The suspension was centrifuged and filtered to remove inorganic solids. Next, the pH of the filtrate was readjusted to 7 using a hydrochloric acid solution (1 M) and a xylene orange test was

performed. Water was removed *in vacuo* and the residue was recrystallised from a methanol and diethyl ether mixture and repeatedly washed with diethyl ether.

Tb.**29**: (0.053 g, 59 %). IR (solid) in cm⁻¹: 3472, 2359, 1599, 1438, 1406, 1256, 1168, 1036. HRMS: m/z calculated: 603.1575 found: 603.1580 for $\{M+H\}^+$ (100 % relative intensity)

Eu.**29**: (0.210 g, 88 %). ¹H-NMR (400 MHz, D₂O) δ_H (ppm): -16.66, -16.20, -15.60, -14.97, 14.67, -14.19, -13.91, -12.60, -11.58, -10.91, -9.41, -8.47 to -6.95, -6.68, -5.53, -5.02, -4.25, -3.24, -2.31, -1.72, -1.03, -0.45, 0.38, 1.17, 1.48, 2.49, 2.81 – 4.51, 6.78, 10.53, 11.30, 11.77 – 12.85, 13.52, 30.74, 31.88, 33.70, 34.07. HRMS: m/z calculated: 595.1531 found: 595.1548 for $\{M+H\}^+$ (95 % relative intensity)

Gd.**29**: (0.050 g, 35 %). HRMS: m/z calculated: 602.1574, found: 602.1576 for $\{M+H\}^+$ (~70 % relative intensity)

General synthetic procedure for preparation of Ln.30

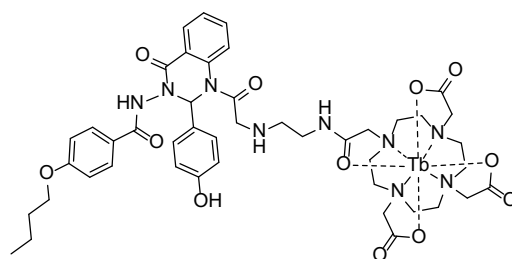
Potassium carbonate and Ln.**29** (in 5:1 molar ratios) were suspended in acetonitrile and stirred for half an hour before addition of **7** (1 equivalent). The mixture was heated at 60°C for two days. Inorganic salts were removed by filtration and residual solvents were evaporated to yield a white solid. The crude product was isolated by dialysis against distilled water using a 1000 Da molecular weight

cut-off cellulose membrane and/or by Sephadex G-10 size exclusion chromatography eluting with MilliQ water.

Tb.**30**: (0.019 g, 25 %). IR (solid) in cm⁻¹: 2361, 1607, 1251, 1173. HRMS: *m/z* calculated: 1089.4792, found: 1089.4794 for {M+H}⁺ (~40 % relative intensity)

Eu.**30**: ¹H-NMR (400 MHz, D₂O) δ_H (ppm): -17.17 to -15.97, -15.76, -13.40, -12.79 to -10.07, -9.37, -8.25 to -6.79, -6.59, -5.38, -4.85, -3.28, -2.59 to -1.77, -1.00, -0.45, 0.51, 0.96, 1.26, 1.48, 1.79, 1.91, 2.09, 2.69, 3.52-4.27, 5.89, 5.94, 5.99, 6.72-8.55, 10.92-14.33, 29.55-34.30. HRMS: *m/z* found: 1182.5885 for {M+H₂O+2 MeCN+H}⁺ (~10 % relative intensity)

Gd.**30**: (0.019 g, 30 %). HRMS *m/z*: calculated: 1087.3524, found: 1087.3661 for {M+H}⁺ (~10 % relative intensity)

Tb-DO3A-Quin C7 (31)

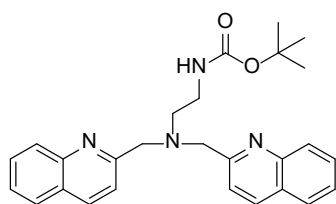
Potassium carbonate (0.014 g, 0.10 mmol), Tb.**29** (0.010 g, 0.02 mmol) and **10** (0.010 g, 0.02 mmol) were suspended in acetonitrile (3 mL) and heated at 50°C for 3 days. The crude product was dialysed against distilled water using a Float-A-Lyser G2 setup with a 500-1000 Da membrane to yield a yellow oil, which was dissolved in

tetrahydrofuran (1 mL). Tetra-*n*-butylammonium fluoride solution (1M in tetrahydrofuran, 11 μL) was added slowly and the mixture was immersed in an acetone/ice bath under a nitrogen atmosphere. The reaction was gradually warmed to room temperature and stirred for 5 hours. The solvents were removed *in vacuo* and the crude was purified by dialysis against a 1000 Da molecular weight cut-off cellulose membrane to yield an off-white solid.

HRMS *m/z*: 1073.6278 {M-H}⁺ (~50 % relative intensity)

8.8.4 Synthesis of compounds in Chapter 5

***Tert*-butyl (2-(bis(quinolin-2-ylmethyl)amino)ethyl)carbamate (42)**

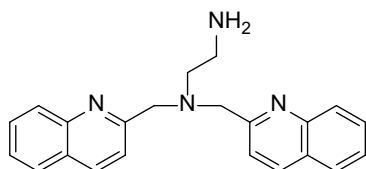


To a stirring solution of *N*-*tert*-butoxycarbonyl-ethylenediamine (0.146 g, 0.9 mmol) in dichloroethane (5 mL), 2-quinolinecarboxaldehyde (0.300 g, 1.9 mmol) in dichloroethane (5 mL) was added at ambient temperature. After two hours, the mixture was cooled to 0°C and sodium triacetoxyborohydride (0.439 g, 2.1 mmol) was added slowly and further reacted for two hours at room temperature. Water was added to the crude and the organic fractions were extracted with dichloromethane, washed with water and brine, combined and dried over magnesium sulphate. The mixture was purified by silica flash column chromatography eluting with a dichloromethane/methanol gradient (0 % - 60%) to yield a pale yellow oily solid (0.305 g, 77 %).

¹H-NMR (400 MHz, CDCl₃), δ_H (ppm): 1.51 (9H, s), 2.85 (2H, br), 3.33 (2H, m), 4.09 (4H, s), 6.65 (1H, br), 7.42-7.63 (4H, m), 7.69 (2H, m), 7.77 (2H, d, ³*J*_{HH} = 8.1 Hz), 8.06 (2H, d, ³*J*_{HH} = 8.4 Hz), 8.18 (2H, d, ³*J*_{HH} = 8.4 Hz).

MS *m/z*: {M-H}⁺ 441 (100 % relative intensity)

***N*1,*N*1-bis(quinolin-2-ylmethyl)ethane-1,2-diamine (43)**

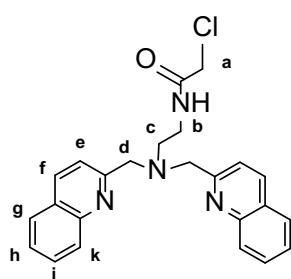


Tert-butyl (2-(bis(quinolin-2-ylmethyl)amino)ethyl)carbamate (0.05 g, 0.11 mmol) was dissolved in concentrated hydrochloric acid (0.7 mL) and methanol (0.5 mL) and stirred at room temperature for one hour. Subsequently 1 M aqueous sodium hydroxide solution was added until a pH value of 12 was reached. The product was extracted with dichloromethane (5 x 10 mL) to yield a pale yellow solid that was carried forward without further purification steps (0.025 g, 66 %).

¹H-NMR (400 MHz, CDCl₃), δ_H (ppm): 2.74 (2H, br), 2.85 (2H, m), 4.05 (4H, s), 7.50 (2H, m), 7.65-7.83 (6H, m), 8.05 (2H, m), 8.12 (2H, d, ³*J*_{HH} = 8.4 Hz).

HRMS *m/z*: calculated: 343.1923 found: 343.1923 for {M+H}⁺ (100 % relative intensity)

***N*-(2-(Bis(quinolin-2-ylmethyl)amino)ethyl)-2-chloroacetamide (44)**



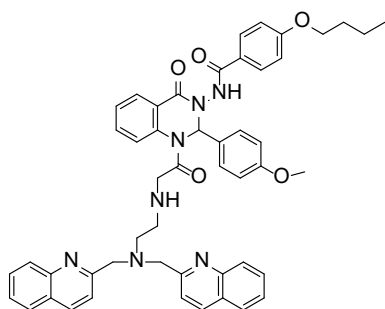
N,N-Bis(quinolin-2-ylmethyl)ethane-1,2-diamine (0.041 g, 0.12 mmol) and triethylamine (0.024 g, 0.24 mmol) were dissolved in dichloromethane (5 mL) and stirred at room temperature for half an hour. The reaction was cooled to 0°C and chloroacetyl chloride (0.014 g, 0.12 mmol) in dichloromethane (2 mL) was added slowly. After 3 hours stirring at room temperature, the mixture was washed with water and dried over magnesium sulphate. The resulting crude oil was purified by silica column

chromatography eluting in dichloromethane with an acetone gradient to yield a pale brown solid (0.012 g, 24 %).

$^1\text{H-NMR}$ (400 MHz, CDCl_3), δ_{H} (ppm): 2.88 (2H, t, $^3J_{\text{HH}} = 5.9$ Hz, H_{c}), 3.44 (2H, m, H_{b}), 4.02 (2H, s, H_{a}), 4.08 (4H, s, H_{d}), 7.53 (2H, m, $\text{H}_{\text{h or i}}$), 7.60 (2H, d, $^3J_{\text{HH}} = 8.4$ Hz, $\text{H}_{\text{e or f}}$), 7.71 (2H, m, $\text{H}_{\text{h or i}}$), 7.79 (2H, m, $\text{H}_{\text{g or k}}$), 8.05– 8.13 (4H, m).

MS m/z : $\{\text{M}+\text{H}\}^+$ 419 (100 % relative intensity)

Bisquinoline-C1 (46)



N1,N1-bis(quinolin-2-ylmethyl)ethane-1,2-diamine (0.013 g, 0.039 mmol) and potassium carbonate (0.027 g, 0.196 mmol) were suspended in acetonitrile (2 mL) and stirred at 60°C. After half an hour, **7** (0.017 g, 0.033 mmol) in acetonitrile (1 mL) was added and the reaction was heated at 60°C overnight. Residual solvents were removed, the mixture was taken up in dichloromethane, washed with water and dried over magnesium sulphate. Subsequently, purification was achieved by flash column chromatography eluting in a dichloromethane/acetone (9:1) mixture. The product was obtained as a yellow oil (0.007 g, 26 %).

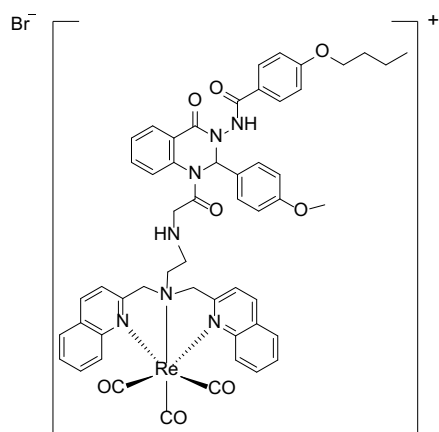
$^1\text{H-NMR}$ (400 MHz, d_6 -acetone), δ_{H} (ppm): 0.96 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.49 (2H, m), 1.77 (2H, m), 3.43 (4H, s), 3.83 (3H, s), 3.91 (2H, br), 4.07 (2H, t, $^3J_{\text{HH}} = 6.5$ Hz), 6.95– 8.58 (24H, m).

$^{13}\text{C-NMR}$ (400 MHz, d_6 -acetone) δ_{H} (ppm): signals weak 14.0, 19.8, 31.9, 54.5, 55.0, 55.7, 68.2, 68.6, 75.7, 76.0, 114.0, 115.2, 119.5, 124.7, 127.6, 128.7, 130.2, 130.4, 131.5, 135.6, 137.4.

IR (solid) in cm^{-1} : 3248, 3065, 2957, 2931, 2873, 2855, 2838, 1691, 1680, 1603, 1576, 1513, 1470, 1441, 1387, 1352, 1305, 1250, 1176.

HRMS m/z : $\{\text{M}+\text{isopropyl}+\text{H}\}^+$ 887.1917 (~90 % relative intensity)

Re(I) bisquinoline-C1 (47)



Compound **46** (0.007 g, 0.008 mmol) in methanol (1 mL) was added to a solution of $[\text{NEt}_4]_2[\text{Re}(\text{CO})_3\text{Br}_3]$ (0.010 g, 0.013 mmol) in methanol (2 mL) and stirred overnight at 60°C. The resulting mixture was separated by flash column chromatography eluting in dichloromethane to yield a yellow solid (0.008 g, 89 %).

$^1\text{H-NMR}$ (400 MHz, d_6 -acetone), δ_{H} (ppm): 0.97 (3H, t, $^3J_{\text{HH}} = 7.4$ Hz), 1.50 (2H, m), 1.77 (2H, m), 3.43 (4H, s), 3.83 (3H, s), 3.92 (2H, br), 4.08 (2H, t, $^3J_{\text{HH}} = 6.4$ Hz), 6.93– 8.42 (24H, m).

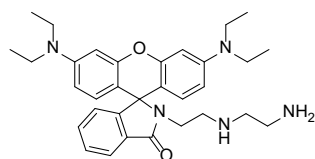
^{13}C -NMR (400 MHz, d_6 -acetone) δ_{H} (ppm): signals weak 18.9, 31.0, 53.6, 54.8, 64.4, 67.7, 102.9, 105.9, 113.1, 114.3, 118.6, 126.7, 127.8, 129.4, 129.5, 130.6, 134.7, 135.7, 136.5.

IR (solid) in cm^{-1} : 3243, 3066, 2971, 2933, 2873, 2855, 2846, 2021, 2007, 1881, 1718, 1667, 1612, 1564, 1518, 1455, 1363, 1304, 1235, 1166.

MS m/z : $\{\text{M}+\text{H}\}^+$ 1099 (~25 % relative intensity)

8.8.5 Synthesis of compounds in Chapter 6

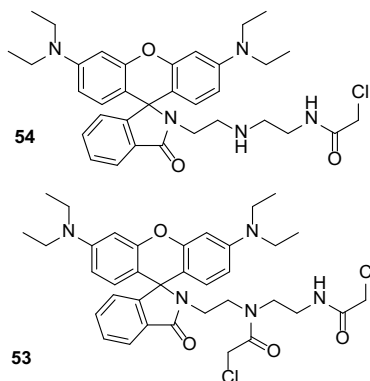
Rhodamine-B diethylene triamine (**52**)²⁰



Rhodamine B (1.000 g, 2.1 mmol) was dissolved in a stirring solution of ethanol (10 mL). Diethylene triamine (1.5 mL, 14.6 mmol) was added dropwise and the mixture was refluxed for 20 hours. After allowing the reaction mixture to cool to room temperature, water was added and the desired product was extracted with dichloromethane. Organic fractions were combined and dried over magnesium sulphate to yield a pale orange oil (0.840 g, 76 %).

^1H -NMR (400 MHz, CDCl_3) δ_{H} (ppm): 1.15 (12H, t, $^3J_{\text{HH}} = 7.0$ Hz), 1.31 (2H, br, s), 2.41 (2H, m), 2.58 (2H, t, $^3J_{\text{HH}} = 6.0$ Hz), 3.21-3.39 (10H, br), 6.21-6.47 (6H, m), 7.07 (1H, m), 7.42 (2H, m), 7.89 (1H, m). HRMS m/z : calculated: 528.3339 found: 528.3339 for $\{\text{M}+\text{H}\}^+$ (100 % relative intensity)

Rhodamine-B diethylene triamine acetyl chloride (**54** and **53**)



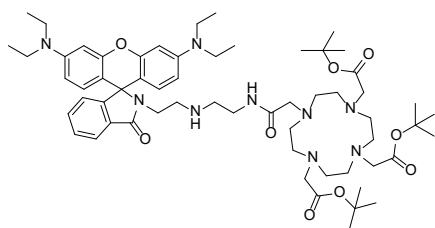
Rhodamine B diethylene triamine (0.150 g, 0.28 mmol) and triethylamine (60 μL , 0.56 mmol) were dissolved in dichloromethane (10 mL) and cooled to 0°C . chloroacetyl chloride (0.026 g, 0.23 mmol) in dichloromethane (2 mL) was added dropwise. After 3 hours stirring at room temperature, the mixture was washed with water and dried over magnesium sulphate. Both **53** and **54** were isolated from the crude mixture by flash column chromatography as deep pink/purple solids (**54**: 0.080 g, 58 % and **53**: 0.037 g, 24 %).

54: ^1H -NMR (400 MHz, CDCl_3) δ_{H} (ppm): 1.17 (12H, t, $^3J_{\text{HH}} = 7.1$ Hz), 2.86 (2H, br, m), 3.16 (2H, br, m), 3.34 (10H, br, overlaid signals q, $^3J_{\text{HH}} = 7.0$ Hz), 3.38 (2H, br, m), 4.17 (2H, s), 6.19-6.51 (6H, m), 7.10 (1H, m), 7.49 (2H, m), 7.87 (1H, m).

HRMS m/z : calculated: 604.3054, found: 604.3064 for $\{\text{M}+\text{H}\}^+$ (100 % relative intensity)

53: ^1H -NMR (400 MHz, CDCl_3) δ_{H} (ppm): 1.16 (12H, t, $^3J_{\text{HH}} = 7.1$ Hz), 3.03-3.41 (16H, br), 3.78 (2H, s), 3.90 (2H, s), 6.15-6.48 (6H, m), 7.12 (1H, m), 7.48 (2H, m), 7.91 (1H, m).

HRMS m/z : calculated: 680.2770, found: 680.2777 for $\{\text{M}+\text{H}\}^+$ (~80 % relative intensity)

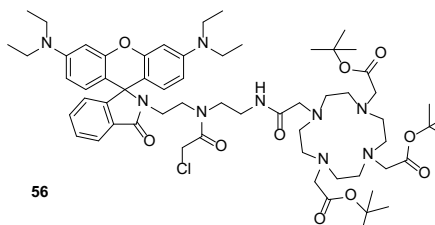
Rhodamine-B diethylene triamine *tert*-butyl DO3A

Tert-butyl DO3A (0.058 g, 0.11 mmol) and triethylamine (29 μ L, 0.19 mmol) were stirred in acetonitrile (15 mL) and heated to reflux for an hour. **54** (0.057 g, 0.09 mmol) was subsequently added to the mixture and the reaction was heated at reflux overnight. The desired product was isolated by repeated silica column chromatography as a pale pink solid (0.016 g, 16 %).

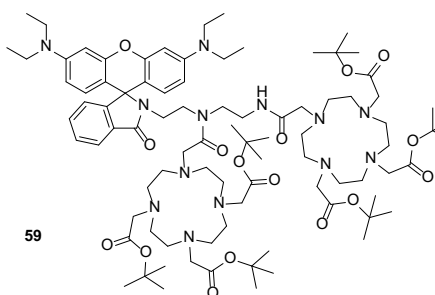
$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ_{H} (ppm): 1.16 (12H, t, $^3J_{\text{HH}} = 7.0$ Hz), 1.37 (2H, t, $^3J_{\text{HH}} = 7.3$ Hz), 1.41-2.03 (27H, m, br), 2.10 (2H, t, $^3J_{\text{HH}} = 7.1$ Hz), 2.57 (2H, t, $^3J_{\text{HH}} = 6.3$ Hz), 2.71- 3.43 (28 H, m, br), 5.69 (1H, br, s), 6.17-6.48 (6H, m), 7.09 (1H, m), 7.43 (2H, m), 7.88 (1H, m).

$^{13}\text{C-NMR}$ (400 MHz, CDCl_3) δ_{H} (ppm): 8.8, 12.7, 28.3, 37.3, 41.6, 44.5, 46.1, 47.6, 49.4, 51.5, 54.6, 57.1, 58.3, 65.0, 81.8, 97.8, 105.7, 108.2, 110.1, 122.8, 123.9, 128.1, 129.1, 132.5, 148.9, 153.5, 168.2, 169.5, 170.7

HRMS m/z : found: 1111.6785 for $\{\text{M}+30\}^+$ (~95 % relative intensity) and small peak assigned to $\{\text{M}+\text{H}\}^+$: calculated: 1082.7018, found: 1082.7035.

Rhodamine-B diethylene triamine acetyl chloride *tert*-butyl DO3A (56**) and Rhodamine-B diethylene triamine di- *tert*-butyl DO3A (**59**)****56**

Tert-butyl DO3A (0.108 g, 0.21 mmol) and potassium carbonate (0.122 g, 0.88 mmol) were suspended in acetonitrile (15 mL) and stirred for about an hour. Subsequently, **53** (0.200 g, 0.29 mmol) was added and the reaction mixture was heated at reflux overnight. Inorganic salts were filtered off and the crude mixture was purified by silica automated column chromatography eluting with a methanol/dichloromethane gradient (5 % - 50 %). **56** and **59** were both isolated as pale pink solids (**56**: 0.019 g, 79 % and **59**: 0.032 g, 9 %).

**59**

56: $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ_{H} (ppm): 1.05- 3.51 (81 H, broad, overlapping resonances), 3.99 (2H, s), 6.20-6.47 (6H, m), 7.07 (1H, m), 7.45 (2H, m), 7.86 (1H, m).

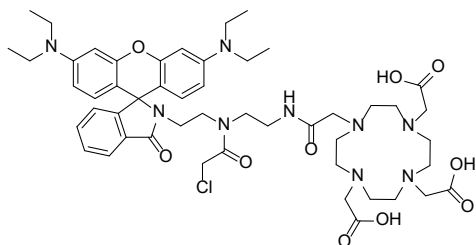
$^{13}\text{C-NMR}$ (400 MHz, CDCl_3) δ_{H} (ppm): 12.8, 28.0, 28.3, 42.8, 44.5, 45.1, 46.0, 47.6, 49.4, 51.5, 54.5, 55.9, 58.3, 65.0, 81.8, 97.9, 105.1, 108.5, 122.9, 124.1, 128.4, 128.9, 149.1, 153.2, 153.6, 166.6, 167.9, 169.8, 170.6, 172.8

HRMS m/z : calculated: 1158.6734, found: 1158.6766 for $\{\text{M}+\text{H}\}^+$ (~60 % relative intensity) and 1180.6617 $\{\text{M}+\text{Na}\}^+$ (100 % relative intensity)

59: $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ_{H} (ppm): 0.75- 3.96 (130H, br, overlapping resonances), 4.28 (2H, br, s), 6.15-6.59 (6H, m), 7.00 (1H, m), 7.40 (2H, m), 7.71 (1H, m).

HRMS m/z : calculated: 1637.0698, found: 1637.0835 for $\{M+H\}^+$ (100 % relative intensity)

Rhodamine-B diethylene triamine acetyl chloride DO3A (57)



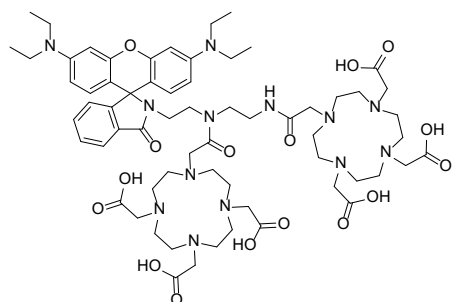
56 (0.200 g, 0.17 mmol) was dissolved in a minimum amount of methanol, before concentrated hydrochloric acid (4 mL) was added. The reaction mixture was stirred at ambient temperature for three hours, before an aqueous solution of sodium hydroxide (1 M) was carefully added until the pH value was adjusted to 10. The aqueous phase was subsequently

extracted with dichloromethane (3 x 20 mL) and dried over magnesium sulphate to yield a yellow oil (0.082 g, 48 %).

$^1\text{H-NMR}$ (400 MHz, CDCl_3), δ_{H} (ppm): 0.95 - 4.14 (54H, br, overlapping resonances), 6.14-6.54 (6H, m), 7.11 (1H, m), 7.49 (2H, m), 7.87 (1H, m).

HRMS m/z : calculated: 990.4856, found: 990.4832 for $\{M+H\}^+$ (~35 % relative intensity)

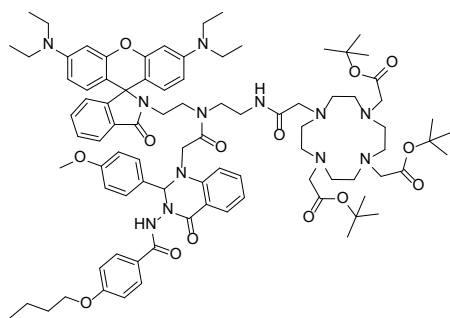
Rhodamine-B diethylene triamine di-DO3A (60)



Compound **59** (0.032 g, 0.02 mmol) was stirred at room temperature in concentrated hydrochloric acid (2 mL) for three hours. Next, an aqueous solution of sodium hydroxide (1 M) was carefully added until the pH value was adjusted to 10 and extracted using dichloromethane (3 x 5 mL). the organic fractions were combined and dried over magnesium sulphate to yield a dark yellow solid (0.012 g, 46 %).

$^1\text{H-NMR}$ (400 MHz, CDCl_3), δ_{H} (ppm): 0.55 - 4.46 (76H, br, overlapping resonances), 6.04-6.69 (6H, br m), 7.09 (1H, br), 7.45 (2H, br), 7.87 (1H, br).

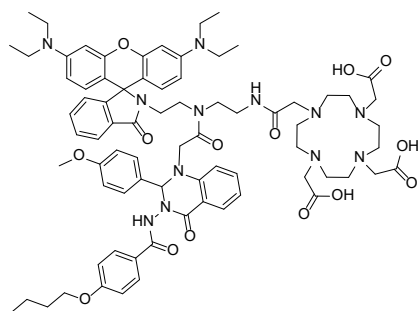
HRMS m/z : calculated: 1300.6942, found: 1300.6882 for $\{M+H\}^+$ (~60 % relative intensity) and 675.8379 for $\{M+2H\}^{2+}$ (100 % relative intensity)

Rhodamine-B diethylene triamine *tert*-butyl DO3A Quin C1 (65)

Compound **6** (0.033 g, 0.07 mmol) and *N,N*-diisopropylethylamine (30 μ L, 0.17 mmol) were stirred in acetonitrile (6 mL) for half an hour before addition of **56** (0.060 g, 0.05 mmol). The reaction mixture was heated at 65 $^{\circ}$ C for 48 hours. Inorganic salts were removed by filtration. The crude mixture was separated by silica column chromatography eluting in a methanol/ dichloromethane gradient (2 % - 50 %) to afford a pale pink solid (0.044 g, 54 %).

$^1\text{H-NMR}$ (400 MHz, d_6 -acetone), δ_{H} (ppm): 0.80- 3.94 (> 87H, br, partly obscured by solvents), 4.06 (2H, t, $^3J_{\text{HH}} = 6.4$ Hz), 6.04-7.99 (22H, br).

HRMS m/z : calculated: 1567.8969, found: 1567.8879 for $\{\text{M}+\text{H}\}^+$ (~50 % relative intensity)

Rhodamine-B diethylene triamine DO3A Quin C1 (66)

Compound **65** (0.010 g, 0.01 mmol) was stirred at room temperature in concentrated hydrochloric acid (1 mL) for two hours before dropwise addition of an aqueous sodium hydroxide (1 M) until the pH value was adjusted to 10. The aqueous phase was extracted using dichloromethane (3 x 5 mL), the organic fractions were combined and dried over magnesium sulphate to yield an orange solid. (0.005 g, 60 %)

$^1\text{H-NMR}$ (400 MHz, d_6 -acetone), δ_{H} (ppm): 0.72- 3.61 (> 61H, br, partly obscured by solvents), 3.91(3H, s), 4.05 (2H, t, $^3J_{\text{HH}} = 6.4$ Hz), 6.04-7.99 (22H, br).

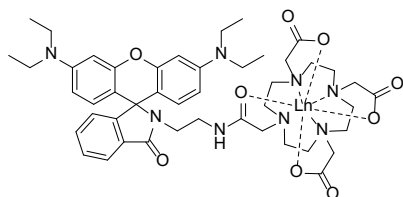
HRMS m/z : calculated: 1396.7570, found: 1396.8394 for $\{\text{M}+\text{H}\}^+$ (~30 % relative intensity)

General synthetic procedure for preparation of lanthanide complexes

The lanthanide trichloride hexahydrate salt (1.5 equivalents) was added to the macrocyclic ligand system (1 equivalent) in a small amount of water and stirred at pH ~5.5 at room temperature overnight. Subsequently, the aqueous pH was raised to 12 and the appearing lanthanide precipitate was removed by centrifugation followed by filtration. Next, the pH of the filtrate was adjusted to pH ~7, solvents were removed in vacuo and the compound was dissolved in water and purified by dialysis using a 500-1000 Dalton cut-off membrane.

Characterisation data for lanthanide complexes in Chapter 6

Compound Ln.51



Tb.**51** ES⁺: m/z 1027.4 {M+H}⁺ (~65 % relative intensity)

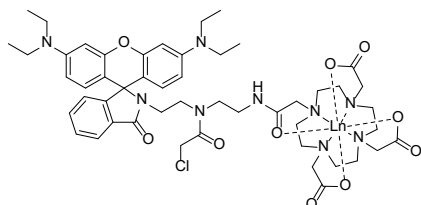
Eu.**51** ¹H-NMR (400 MHz, D₂O) δ_H (ppm): -17.09, -15.84, -15.28 to -14.09, -12.27, -11.60, -11.18, -8.70 to -6.50, -5.54, -4.81, -3.14, -2.09, -1.57 to 4.14, 6.34, 7.05-7.30, 7.37-7.76, 7.81, 8.05,

8.25, 8.46, 9.40, 11.04-12.84, 30.73, 31.39, 32.48, 33.61.

HRMS: calculated: 1021.3678; found: 1021.3675 for {M+H}⁺ (100 % relative intensity)

Gd.**51** HRMS: calculated: 1026.3724, found: 1026.3750 for {M+H}⁺ (~85 % relative intensity)

Compound Ln.58



Tb.**58** HRMS: calculated: 1146.3875, found: 1146.3888 for {M+H}⁺ (100 % relative intensity)

Eu.**58** ¹H-NMR (400 MHz, D₂O) δ_H (ppm): broad, structureless resonances -17.98 to 15.13 and 29.38 to 36.23.

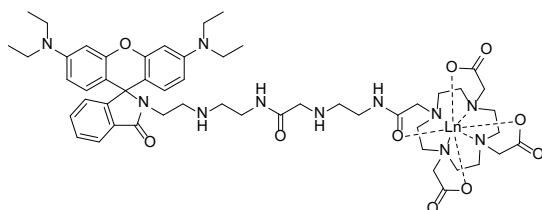
HRMS: calculated: 1138.3820, found: 1138.3866 for {M+H}⁺

(100 % relative intensity)

Gd.**58** HRMS: calculated: 1145.3862, found: 1145.3895 for {M+H}⁺ (~35 % relative intensity) and 573.1929 for {M+2H}²⁺ (100 % relative intensity)

Yields ~90 % for all three inexact due to hygroscopic nature.

Compound Ln.55

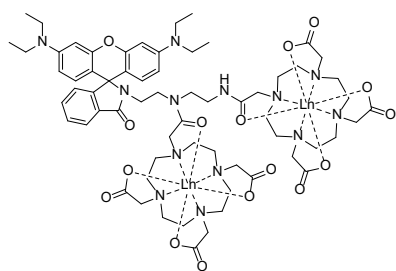


Tb.**55** (0.008 g, 17 %). HRMS: calculated: 1171.6428, found: 1171.6399 for {M+H}⁺ (40 % relative intensity)

Eu.**55** (0.010 g, 43 %) ¹H-NMR (400 MHz, D₂O) δ_H (ppm): -16.45, -15.84 to -14.38, -12.86, -12.54, -12.18,

-11.02, -10.59, -10.22, -8.28, -7.83, -7.48, -7.01, -6.73, -5.73, -5.20, -3.81, -3.07, -1.97, -1.75, -1.02, -0.39, -0.09, 0.81-8.22, 10.78-14.77, 31.42, 31.89, 32.57, 32.94, 33.60, 33.75, 34.45.

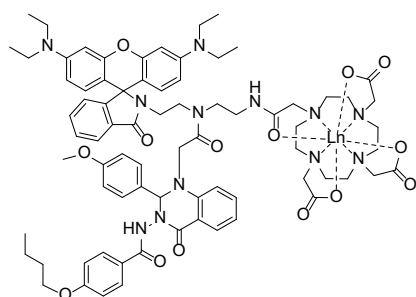
ES⁺: m/z 1173.6 {M+10}⁺ (~30 % relative intensity)

Compounds 61 and 62

61 (0.007 g, 39 %). HRMS: calculated: 1634.4798, found: 1634.4871 for $\{M+Na\}^+$ (~5 % relative intensity)

62 (0.011 g, 79 %). ^1H -NMR (400 MHz, D_2O) δ_H (ppm): broad, structureless resonances -18.93 to 15.28 and 28.88 to 37.80.

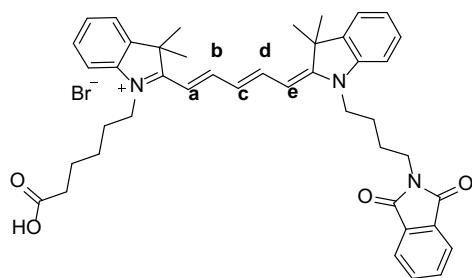
HRMS: calculated: 1598.4883, found: 1598.5522 for $\{M+H\}^+$ (~5 % relative intensity)

Compound Ln.67

Tb.67 (0.003 g, 51 %). MALDI-ToF m/z : 1588.9 $\{M+MeOH+H\}^+$ (~85 % relative intensity)

Eu.67 (0.004 g, 65 %). HRMS: calculated: 1567.6174, found: 1567.6248 for $\{M+H_2O\}^+$ (~10 % relative intensity)

Gd.67 (0.008 g, 40 %). HRMS: calculated: 1571.6124, found: 1571.6178 for $\{M+H_2O\}^+$ (~5 % relative intensity)

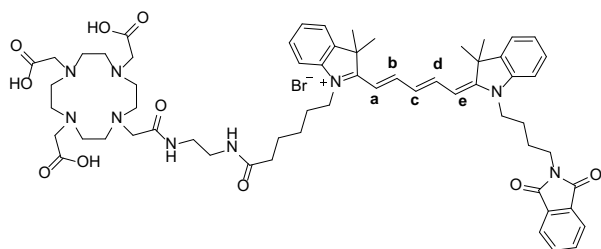
8.8.6 Synthesis of compounds in Chapter 7**Cy5, 1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(4-(1,3-dioxoisindolin-2-yl)butyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium (68)**

2,3,3-Trimethyl-1-(5-carboxypentyl)-3H-indolium bromide (1.2 g, 3.4 mmol) and malonaldehyde bis(phenylimine) monohydrochloride (1 g, 3.8 mmol) were heated at 120°C and under a nitrogen atmosphere in acetic anhydride (10 ml) for 30 minutes and then cooled to ambient temperature, before 2,3,3-trimethyl-1-[N-(phthalimidobutyl)]-3H-indolium bromide (2 g, 4.5 mmol) in pyridine (10 ml) was added. The

reaction was stirred overnight. Removal of the solvents *in vacuo* and repeated purification by silica column chromatography, first eluting with a dichloromethane/methanol mixture (9:1), then eluting with a dichloromethane/acetone (1:1) mix and a methanol gradient (up to 50 %) resulted in a metallic dark blue oil. (0.20 g, 9 %)

^1H -NMR (400 MHz, MeOD) δ_H (ppm): 1.52 (2H, m, CH_2), 1.71-1.73 (14H, m, CH_2 and CH_3), 1.85 (6H, m, CH_2), 2.32 (2H, t, $^3J_{HH} = 7.2$ Hz, NCH_2), 3.76 (2H, m, $COOH-CH_2$), 4.14 (4H, m, NCH_2), 6.29 (2H, d, $^3J_{HH} = 13.7$ Hz, $H_{a,e}$), 6.61 (1H, t, $^3J_{HH} = 12.3$ Hz, H_c), 7.21-7.51 (8H, m, Ph), 7.82 (4H, m, Phth-Ph), 8.20 (2H, m, $H_{b,d}$).

HRMS m/z : calculated: 670.3645, found: 670.3651 for $\{M\}^+$ (100 % relative intensity)

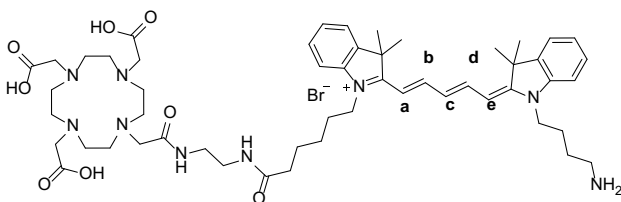
Cy5 phthalimide DO3A (69)

To a stirring solution of Cy5 dye (0.050 g, 75 μ mol) in acetonitrile (2 mL) at room temperature, *N,N,N',N'*-Tetramethyl-O-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate, O-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (0.038 g, 100 μ mol) and

N,N diisopropylethylamine (0.019 g, 100 μ mol) were added. The reaction mixture was stirred for 10 minutes before addition of a solution of XX (0.022 g, 50 μ mol) in acetonitrile (2 mL). After 4 hours, borate buffer (pH 8.5, 4 mL) was added and the reaction was stirred for another 18 hours. Residual solvents were evaporated under reduced pressure and the crude product was purified by dialysis. The compound was carried forward in the presence of impurities.

$^1\text{H-NMR}$ (400 MHz, MeOD), in presence of residual reagents δ_{H} (ppm): 1.25- 4.26 (br, m, aliphatic protons), 6.28 (2H, m, $\text{H}_{\text{a,e}}$), 6.61 (1H, m, H_{c}), 7.16- 7.54 (8H, m, Ph dye), 7.66- 7.91 (6H, m, phth Ph and residual HBTU), 8.02 (2H, m, residual HBTU), 8.10-8.27 (3H, m, $\text{H}_{\text{b,d}}$ and HBTU).

MALDI ToF m/z : 1099.3 $\{\text{M}\}^+$ (~30 % relative intensity)

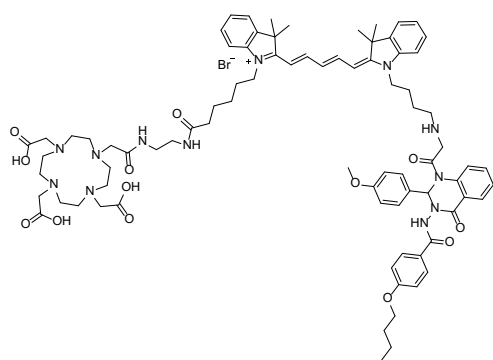
Cy5 DO3A (70)

Methyl amine (2 M in methanol, 0.5 mL) was added to a solution of **69** (approx. 0.100 g, 0.09 mmol) in methanol (5 mL) and the mixture was heated for 4 hours at 65 $^{\circ}\text{C}$. Subsequently, solvents were removed under reduced pressure

and the resulting solid was purified by silica column chromatography eluting with dichloromethane and a methanol gradient of 5 % to 50 %. The final fraction was eluted in methanol with 0.1 % trifluoroacetic acid to yield a dark blue hygroscopic solid (0.011 g, 13 %).

$^1\text{H-NMR}$ (400 MHz, MeOD), δ_{H} (ppm): 1.23- 3.79 (br, m, aliphatic protons), 2.54 (s, residual methylamine), 4.14 (4H, m, NCH_2), 6.30 (2H, m, $\text{H}_{\text{a,e}}$), 6.63 (1H, t, $^3J_{\text{HH}} = 12.5$ Hz, H_{c}), 7.21- 7.54 (8H, m, Ph), 8.27 (2H, m, $\text{H}_{\text{b,d}}$). IR (solid) in cm^{-1} : 3356, 1675, 1453, 1195, 1130.

LCMS (ES) peak found m/z : 969 $\{\text{M}\}^+$.

Cy5 DO3A Quin C1 (71)

Compound **70** (0.011 g, 11 μmol), potassium carbonate (0.075 g, 54 μmol) and **7** (0.005 g, 9 μmol) were suspended in acetonitrile (2 mL) and heated at 60 $^{\circ}\text{C}$ for 18 hours. Solvents were removed *in vacuo*, the crude was resuspended in ethanol and inorganic solids were removed by filtration to yield a pale green solid (0.010 g, 69 %).

$^1\text{H-NMR}$ (400 MHz, MeOD), δ_{H} (ppm): 0.77-4.19 (br, m, aliphatic protons), 6.10 (1H, s, aminal proton), 6.60-8.13 (25H, aromatic protons).

IR (solid) in cm^{-1} : 3381, 2958, 1674, 1642, 1431, 1400, 1373, 1304, 1250, 1205, 1175, 1131, 1070.

ES $^{+}$: m/z 1454 $\{M\}^{+}$ (~30 % relative intensity)

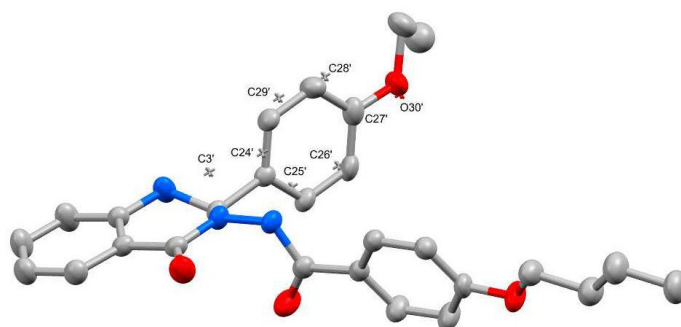
8.9 Crystallographic data

Figure 8.1: X-ray molecular structure of **6** obtained by X-ray crystallography with 50% thermal ellipsoids (hydrogen atoms omitted for clarity) showing the modelled disorder within the compound introduced by the second orientation.

Crystal data and structure refinement

Formula	C ₂₆ H ₂₇ N ₃ O ₄	
Formula weight	445.50	
Temperature	173(2) K	
Diffractometer, wavelength	Agilent Xcalibur 3 E, 0.71073 Å	
Crystal system, space group	Monoclinic, P2 ₁ /c	
Unit cell dimensions	a = 14.1692(8) Å	a = 90°

	b = 13.5603(6) Å	b = 110.407(7)°
	c = 12.7578(7) Å	g = 90°
Volume, Z	2297.4(2) Å ³ , 4	
Density (calculated)	1.288 Mg/m ³	
Absorption coefficient	0.088 mm ⁻¹	
F(000)	944	
Crystal colour / morphology	Colourless tabular needles	
Crystal size	0.642 x 0.258 x 0.069 mm ³	
q range for data collection	3.004 to 28.277°	
Index ranges	-17<=h<=15, -12<=k<=17, -11<=l<=16	
Reflns collected / unique	7767 / 4582 [R(int) = 0.0174]	
Reflns observed [F>4s(F)]	3195	
Absorption correction	Analytical	
Max. and min. transmission	0.994 and 0.960	
Refinement method	Full-matrix least-squares on F ²	
Data / restraints / parameters	4582 / 72 / 316	
Goodness-of-fit on F ²	1.033	
Final R indices [F>4s(F)]	R1 = 0.0527, wR2 = 0.1210	
R indices (all data)	R1 = 0.0850, wR2 = 0.1405	
Largest diff. peak, hole	0.419, -0.253 eÅ ⁻³	
Mean and maximum shift/error	0.000 and 0.001	

Bond lengths [Å] and angles [°] for NL1710

C(1)-O(1)	1.228(2)
C(1)-N(2)	1.377(2)
C(1)-C(10)	1.468(3)
N(2)-N(11)	1.390(2)
N(2)-C(3)	1.496(3)

N(2)-C(3')	1.502(7)
C(3)-N(4)	1.395(3)
C(3)-C(24)	1.509(3)
C(3')-N(4)	1.414(7)
C(3')-C(24')	1.506(8)
N(4)-C(5)	1.378(2)
C(5)-C(6)	1.394(3)
C(5)-C(10)	1.408(3)
C(6)-C(7)	1.375(3)
C(7)-C(8)	1.389(3)
C(8)-C(9)	1.374(3)
C(9)-C(10)	1.396(3)
N(11)-C(12)	1.366(2)
C(12)-O(12)	1.219(2)
C(12)-C(13)	1.486(3)
C(13)-C(18)	1.385(3)
C(13)-C(14)	1.393(3)
C(14)-C(15)	1.370(3)
C(15)-C(16)	1.391(3)
C(16)-O(19)	1.365(2)
C(16)-C(17)	1.382(3)
C(17)-C(18)	1.388(3)
O(19)-C(20)	1.423(3)
C(20)-C(21)	1.521(3)
C(21)-C(22)	1.499(3)
C(22)-C(23)	1.522(3)
C(24)-C(25)	1.3900
C(24)-C(29)	1.3900

C(25)-C(26)	1.3900
C(26)-C(27)	1.3900
C(27)-O(30)	1.380(4)
C(27)-C(28)	1.3900
C(28)-C(29)	1.3900
O(30)-C(31)	1.432(5)
C(24')-C(25')	1.3900
C(24')-C(29')	1.3900
C(25')-C(26')	1.3900
C(26')-C(27')	1.3900
C(27')-O(30')	1.354(12)
C(27')-C(28')	1.3900
C(28')-C(29')	1.3900
O(30')-C(31')	1.442(13)
O(1)-C(1)-N(2)	121.91(17)
O(1)-C(1)-C(10)	123.46(17)
N(2)-C(1)-C(10)	114.59(16)
C(1)-N(2)-N(11)	117.04(15)
C(1)-N(2)-C(3)	117.80(16)
N(11)-N(2)-C(3)	116.51(15)
C(1)-N(2)-C(3')	123.3(3)
N(11)-N(2)-C(3')	117.9(3)
N(4)-C(3)-N(2)	108.41(19)
N(4)-C(3)-C(24)	113.13(19)
N(2)-C(3)-C(24)	110.69(19)
N(4)-C(3')-N(2)	107.0(4)
N(4)-C(3')-C(24')	117.7(5)

N(2)-C(3')-C(24')	112.9(5)
C(5)-N(4)-C(3)	116.80(17)
C(5)-N(4)-C(3')	122.8(3)
N(4)-C(5)-C(6)	122.42(17)
N(4)-C(5)-C(10)	118.59(17)
C(6)-C(5)-C(10)	118.98(18)
C(7)-C(6)-C(5)	120.35(19)
C(6)-C(7)-C(8)	121.0(2)
C(9)-C(8)-C(7)	119.2(2)
C(8)-C(9)-C(10)	121.04(19)
C(9)-C(10)-C(5)	119.37(17)
C(9)-C(10)-C(1)	120.12(17)
C(5)-C(10)-C(1)	120.50(17)
C(12)-N(11)-N(2)	118.71(15)
O(12)-C(12)-N(11)	120.80(17)
O(12)-C(12)-C(13)	122.53(18)
N(11)-C(12)-C(13)	116.67(17)
C(18)-C(13)-C(14)	118.46(18)
C(18)-C(13)-C(12)	124.26(18)
C(14)-C(13)-C(12)	117.22(18)
C(15)-C(14)-C(13)	121.1(2)
C(14)-C(15)-C(16)	119.8(2)
O(19)-C(16)-C(17)	124.90(19)
O(19)-C(16)-C(15)	114.96(18)
C(17)-C(16)-C(15)	120.13(18)
C(16)-C(17)-C(18)	119.41(19)
C(13)-C(18)-C(17)	121.07(19)
C(16)-O(19)-C(20)	119.11(17)

O(19)-C(20)-C(21)	106.12(19)
C(22)-C(21)-C(20)	113.9(2)
C(21)-C(22)-C(23)	111.4(2)
C(25)-C(24)-C(29)	120.0
C(25)-C(24)-C(3)	119.4(2)
C(29)-C(24)-C(3)	120.6(2)
C(26)-C(25)-C(24)	120.0
C(25)-C(26)-C(27)	120.0
O(30)-C(27)-C(28)	125.8(3)
O(30)-C(27)-C(26)	114.0(3)
C(28)-C(27)-C(26)	120.0
C(27)-C(28)-C(29)	120.0
C(28)-C(29)-C(24)	120.0
C(27)-O(30)-C(31)	117.8(4)
C(25')-C(24')-C(29')	120.0
C(25')-C(24')-C(3')	122.5(6)
C(29')-C(24')-C(3')	117.3(6)
C(24')-C(25')-C(26')	120.0
C(25')-C(26')-C(27')	120.0
O(30')-C(27')-C(28')	124.6(8)
O(30')-C(27')-C(26')	115.4(8)
C(28')-C(27')-C(26')	120.0
C(27')-C(28')-C(29')	120.0
C(28')-C(29')-C(24')	120.0
C(27')-O(30')-C(31')	117.2(11)

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