Development of Fluorescent Ligands for CB$_2$ Receptor

A thesis submitted for the degree of Doctor of Philosophy at the University of Otago, New Zealand

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November 2017
CB₂ receptor plays an important role in inflammation and has been implicated in the pathologies of several diseases, including neurodegenerative disorders, cancer, and atherosclerosis. However, there is a lack of understanding of CB₂ receptor expression and signalling, which significantly hinders the development of CB₂ receptor-targeted therapies. The availability of a diverse range of advanced pharmacological tools for studying CB₂ receptor would lead to improved understanding of CB₂ receptor and thus aid drug development. Fluorescent ligands have been developed for other GPCRs and used to elucidate receptor expression and signalling roles. The aim of this PhD research was to develop a fluorescent ligand with high affinity and selectivity for CB₂ receptor which would be suitable for use as a pharmacological tool. The alkylindole class of cannabinoids are well characterised regarding CB₂ receptor affinity, selectivity and function, which was used to guide design of a series of fluorescent ligands. Compounds with linker and fluorophore substitution at the C5-, C6- or C7 indole positions were designed, synthesised and pharmacologically evaluated for CB₂ receptor affinity and function (Chapters Two and Three). No high affinity fluorescent ligands resulted from these alkylindole series, but a very high affinity, selective CB₂ receptor inverse agonist 3.7b was identified amongst the non-fluorescent ligands. Alkylindole 3.7b showed very promising properties in relation to other small ligand inverse agonists for CB₂ receptor. In addition, a lead pharmacophore-linker conjugate 3.13 was identified, which has potential for development into a fluorescent ligand. Fluorescent ligands were also developed from the 1,8-naphthyridin-2(1H)-one-3-carboxamide scaffold (Chapters Four and Five). A fluorescent ligand 5.9b with high affinity and selectivity for CB₂ receptor ($K_i = 467 \pm 20.0 \text{ nM}$ at hCB₂ receptor, $K_i > 10 \mu \text{M}$ at hCB₁ receptor) was identified amongst these compounds. This fluorescent ligand (5.9b) has potential as a pharmacological imaging tool with which to study CB₂ receptor and could also be further derivatised and optimised in the development of other fluorescent ligands for CB₂ receptor with a range of properties. CB₂ receptor homology models were generated and used in ligand docking studies to aid ligand design and to rationalise biological results.
Acknowledgements

Firstly, I would like to thank my primary supervisor, Andrea Vernall for all of the support she has provided during my PhD study. Andrea has always been very generous with her time and energy, which has been invaluable to my progress and is truly appreciated. I would also like to thank Sarah Hook for her encouragement and support and Joel Tyndall for his advice and efforts in teaching me modelling. The feedback my supervisors have provided on this thesis has been extremely helpful.

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### Abbreviations

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<th>Full Form</th>
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<tbody>
<tr>
<td>AAI</td>
<td>aminoalkylindole</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>Ac$_2$O</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>AEA</td>
<td>anandamide</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-arachidonylglycerol</td>
</tr>
<tr>
<td>AI</td>
<td>alkylnolide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>aq.</td>
<td>aqueous</td>
</tr>
<tr>
<td>Ar</td>
<td>aromatic</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>B$_{\text{max}}$</td>
<td>maximum specific binding</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>Boc$_2$O</td>
<td>di-tert-butyl dicarbonate</td>
</tr>
<tr>
<td>BODIPY</td>
<td>boron-dipyrromethene</td>
</tr>
<tr>
<td>BODIPY 630/650-X-OSu</td>
<td>6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl) aminohexanoic acid, succinimidy1 ester</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Bz</td>
<td>benzoyl</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>cannabinoid</td>
</tr>
<tr>
<td>CB$_1$</td>
<td>cannabinoid type 1</td>
</tr>
<tr>
<td>CB$_2$</td>
<td>cannabinoid type 2</td>
</tr>
<tr>
<td>Cbz</td>
<td>carboxylbenzyl</td>
</tr>
<tr>
<td>ccpm</td>
<td>corrected counts per minute</td>
</tr>
<tr>
<td>cLogP</td>
<td>calculated LogP</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COSY</td>
<td>homonuclear correlation spectroscopy</td>
</tr>
</tbody>
</table>
Cy7  cyanine 7
2D  two dimensional
DCM  dichloromethane
DBAD  di-tert-butyl azodicarboxylate
DIPEA  $N,N$-diisopropylethylamine
DMAP  4-dimethylaminopyridine
DMF  dimethylformamide
DMSO  dimethyl sulfoxide
DOPE  discrete optimised protein energy
EC$_{50}$  half maximal response concentration
ECL  extracellular loop
$E_{\text{max}}$  efficacy, maximum response
eq  equivalents
EA  ethyl acetate
EPAC  exchange protein directly activated by cAMP
FAAH  fatty acid amide hydrolase
FAF  fatty acid free
FCS  fluorescence correlation spectroscopy
FLIPR  fluorescence imaging plate reader
Fmoc  9-fluorenylmethoxycarbonyl
FRET  fluorescence resonance energy transfer
Fsk  forskolin
GDP  guanosine diphosphate
GFP  green fluorescent protein
GPCR  G protein-coupled receptor
GTP  guanosine triphosphate
h  hour(s)
HATU  1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxid hexafluorophosphate
HBTU  $N,N,N',N'$-Tetramethyl-$O$-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
hCB  human cannabinoid
HEK293  human embryonic kidney cells 293
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>HRMS-ESI</td>
<td>high resolution mass spectrometry electrospray ionisation</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence spectroscopy</td>
</tr>
<tr>
<td>HTS</td>
<td>high throughput screening</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICL</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inhibitory constant</td>
</tr>
<tr>
<td>MAGL</td>
<td>monoacylglycerol lipase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mCB</td>
<td>mouse cannabinoid</td>
</tr>
<tr>
<td>MHz</td>
<td>megahertz</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MVD</td>
<td>mouse vas deferens</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NBD</td>
<td>nitrobenzoxadiazole</td>
</tr>
<tr>
<td>NCC</td>
<td>non-classical cannabinoid</td>
</tr>
<tr>
<td>NIR</td>
<td>near-infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>nuclear Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>PDB</td>
<td>protein database</td>
</tr>
<tr>
<td>PE</td>
<td>petroleum ether</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission topography</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>ppm</td>
<td>part(s) per million</td>
</tr>
<tr>
<td>rCB</td>
<td>rat cannabinoid</td>
</tr>
<tr>
<td>RET</td>
<td>resonance energy transfer</td>
</tr>
<tr>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>retention factor</td>
</tr>
<tr>
<td>Rluc</td>
<td>&lt;i&gt;Renilla&lt;/i&gt; luciferase</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reverse phase-high performance liquid chromatography</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAR</td>
<td>structure activity relationships</td>
</tr>
<tr>
<td>sat.</td>
<td>saturated</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SCM</td>
<td>scanning confocal microscopy</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SI</td>
<td>selectivity index</td>
</tr>
<tr>
<td>SPPS</td>
<td>solid-phase peptide synthesis</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THC</td>
<td>tetrahydrocannabinol</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>THP</td>
<td>tetrahydropyran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMH</td>
<td>transmembrane helix</td>
</tr>
<tr>
<td>TFFH</td>
<td>tetramethylfluoroformamidinium hexafluorophosphate</td>
</tr>
<tr>
<td>UniProt</td>
<td>Universal Protein Resource</td>
</tr>
<tr>
<td>V</td>
<td>vehicle</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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### Amino acid codes

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<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>E</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
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<td>H</td>
<td>Histidine</td>
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<td>Isoleucine</td>
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<td>K</td>
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<td>L</td>
<td>Leucine</td>
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<td>P</td>
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<td>Serine</td>
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<tr>
<td>T</td>
<td>Threonine</td>
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<tr>
<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
</tbody>
</table>
Published works from this thesis

A review article:


A research article (of work discussed in Chapters Two and Three):


A paper discussing the results of Chapters Four and Five is in preparation.
Chapter One: Introduction

1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) comprise the largest family of membrane receptors in the human genome.¹ GPCRs consist of an integral membrane protein composed of seven transmembrane helices (TMH), three extracellular loops (ECLs), three intracellular loops (ICLs) and an N- and a C-terminus (Figure 1).² There are five classes of GPCR, of which Class A (the Rhodopsin family) is the largest and within Class A there are four groups (α, β, δ and λ) which are further classified into subfamilies (e.g. cannabinoid (CB) receptors) and then individual subtypes (e.g. cannabinoid type 1 (CB₁) and type 2 (CB₂) receptors).³

![Figure 1: Basic structure of GPCRs](image)

GPCRs are associated with a broad range of physiological processes, can bind a diverse variety of ligands at both the orthosteric (endogenous ligand binding site) and allosteric sites (other binding sites distinct from the orthosteric site),⁴ and exhibit aberrant expression and signalling in a multitude of pathologies.⁵⁻⁷ For these reasons, GPCRs are highly druggable and are the target of around 33% of current drugs in use.⁸ However, a dearth of detailed knowledge surrounding the complexity of GPCR structure and function, as well as challenges in identifying new lead ligands, has thus far restricted realisation of the true potential for targeting GPCRs.⁹
1.1.1 GPCR signalling

GPCRs mediate signals from both extracellular and intracellular stimuli to produce a broad range of cellular responses.¹⁰ The study of GPCR signalling pathways represents a large field of research and was the subject of the 2012 Nobel Prize in Chemistry.¹¹ The highly complex nature of GPCR signalling is reduced here to just a brief and simple overview.

GPCRs couple with intracellular heterotrimeric G proteins, which are composed of three subunits (α, β and λ), of which the Ga subunit acts to catalyse hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP).¹² Ligand binding induces conformational changes in GPCRs leading to exchange of GDP for GTP and disassociation of the GTP-bound Ga from Gβλ. GTP-bound Ga can then interact with effector molecules (e.g. adenylate cyclase or phospholipase C), initiating second-messenger signalling. There are multiple subforms of Ga proteins, which can be divided into four families: Gaₐ, Gaₐ/Ga₁₁, Ga₉/Ga₁₁ and Ga₁₂/Ga₁₃.¹³ A single GPCR subtype is often able to activate multiple different G proteins and thus elicit diverse biological responses. GPCR kinases phosphorylate GPCRs, causing binding of β-arrestin and subsequently receptor internalisation and recycling of receptor back to the cell surface.¹⁴ Some GPCRs are able to continue signalling whilst internalised and this mechanism enables ligand specific signalling by allowing cells to discriminate ligands which promote receptor endocytosis.¹⁵ The divergent nature of GPCR signalling means that on-target side effects can be a problem when developing therapies (i.e. undesirable effects mediated by the target receptor, as opposed to off-target side effects mediated by other targets). Functionally selective ligands which exhibit biased agonism and are able to elicit a specific set of signalling cascades are a potential means for targeting specific pathways without producing on-target adverse effects.¹⁶

Endogenously expressed GPCRs in native cells have typically low levels of expression and preferentially localise to lipid raft microdomains in the cell membrane.¹⁷ This leads to highly concentrated receptor regions, promoting oligomerisation through formation of GPCR homodimers and heterodimers. GPCR oligomerisation allows for diversification of receptor signalling and function and is important in the modulation of
various physiological processes.\textsuperscript{18} GPCR oligomerisation is also an avenue for selective drug design by developing bivalent ligands which are able to target heterodimers.\textsuperscript{19}

1.1.2 GPCR crystal structures

Crystallisation of GPCRs has furthered our understanding of GPCR structural biology, specifically ligand-receptor interactions and ligand-induced GPCR activation and has provided increased opportunity for rational, structure-based drug design.\textsuperscript{20} However, GPCRs are inherently difficult to crystallise due to poor solubility of lipophilic membrane-bound proteins, low native expressions levels, conformational flexibility and lack of stability.\textsuperscript{21} In 2000, the first GPCR crystal structure was solved for bovine rhodopsin\textsuperscript{22} and it was not until seven years later that the first crystal structure of a non-rhodopsin GPCR was reported for β\textsubscript{2} adrenoceptor.\textsuperscript{23} Methodological innovations in protein expression, protein engineering, GPCR isolation and crystallography techniques has enabled improved opportunities for solving GPCR crystal structures over the last decade.\textsuperscript{24} There are now crystal structures available for 44 unique GPCRs (as of November 2017), 37 of which belong to the Class A family.\textsuperscript{25}

1.1.3 GPCR conformational states

Protein crystallography and other biophysical techniques have enabled characterisation of conformationally distinct GPCR functional states and partial insight into mechanisms of receptor activation.\textsuperscript{26, 27} Traditional receptor theory states that GPCRs exist in an equilibrium between the inactive (R) state or active (R\textsuperscript{*}) state, however GPCRs are in reality highly flexible and dynamic and exhibit multistate conformations. Crystallography has captured the conformation of GPCRs during active states (R\textsuperscript{*}) and inactive states (R) as well as intermediate states between inactive and active, though GPCRs undoubtedly exhibit more conformational states beyond what have been characterised.\textsuperscript{28} When free from bound ligand, GPCRs can exist in varying equilibria of the different states, the distribution of which determines basal signalling levels (Figure 2).\textsuperscript{2} Agonist binding pushes the equilibria towards the R\textsuperscript{*} state, whilst inverse agonist-receptor interactions direct it towards the R state, reducing basal activity levels. In
contrast, neutral antagonists do not affect the equilibrium of basal receptor. Ligand-specific receptor conformations enable distinct and differentiated signalling pathways.\textsuperscript{29}

![Image of GPCR activation and multiple equilibria](image)

Figure 2: Illustration of the shift in helices associated with GPCR activation and the multiple equilibria in place between inactive (R), intermediate (R' and R") active (R*) and active signalling (R*G) conformational states. Figure republished from Katritch et al, 2013.\textsuperscript{29}

Ligands can behave as inverse agonists, reducing basal signalling levels, but also as antagonists by blocking receptor access to agonists. Protean ligands can act as agonists, inverse agonists and antagonists at the same receptor according to the constitutive activity of the receptor population.\textsuperscript{30} For example, in a receptor population with high levels of constitutively active receptors, a low efficacy agonist may lower the activity of the basal population, therefore behaving as an inverse agonist.\textsuperscript{31} Whereas, in a receptor population with low levels of constitutively active receptor, the same ligand could raise the activity of the basal population, acting as an agonist.

The diverse signalling pathways modulated by GPCRs provides a wealth of opportunity for drug development, yet produces distinct challenges in targeting specific downstream effects. A potential means for overcoming these challenges is by allosteric modulation, i.e. ligands that bind to allosteric sites and modulate the cellular responses produced by

\textsuperscript{*} The journal was contacted for permission to republish this figure in a thesis and responded to state that no permission was required as long as the source was fully acknowledged. This also applies to Figure 12 and Figure 16.
endogenous ligands,\textsuperscript{32, 33} or by developing ligands that exhibit signalling bias.\textsuperscript{34} Allosteric ligands can also be a means of achieving greater receptor subtype selectivity, since allosteric sites are not as highly conserved across GPCRs compared to orthosteric sites.\textsuperscript{35} Allosterism and biased signalling are vast and complex topics within GPCR research and are not discussed in depth here, as it is beyond the scope of this thesis.
1.2 The endocannabinoid system

The endocannabinoid system consists of the endocannabinoids, associated regulatory enzymes and the class A GPCR CB receptors. There are two known subtypes of CB receptors, CB₁ and CB₂ receptor. Two orphan receptors, GPR18 and GPR55, have been shown to be responsive to endocannabinoids and are suspected of being either further subtypes of CB receptor, or on the periphery of the endocannabinoid system.³⁶-³⁸ The major components of the endocannabinoid system were only elucidated relatively recently (within the last 30 years) and there are still considerable gaps in understanding of the complete system. The endocannabinoid system influences development and behaviour, reward processing, neural plasticity, lipid homeostasis and immune system processes.³⁹ Consequently, dysfunction of the endocannabinoid system can contribute to neurodegenerative disorders,⁴⁰ immune system disorders⁴¹ and cancer progression.⁴² A major role of the endocannabinoid system is regulation of various neurotransmitter systems.⁴³

The endogenous ligands of CB receptors are the endocannabinoids of which the most well characterised are anandamide ¹.¹ (AEA; identified in 1992)⁴⁴ and ²-arachidonylglycerol ¹.² (2-AG; identified in 1995; Figure 3).⁴⁵,⁴⁶ Endocannabinoids are lipids and can readily diffuse through cell membranes. They are synthesised on demand in response to elevations of intracellular calcium,⁴⁷ in contrast to many other neurotransmitters which are stored in vesicles. A selective membrane transport process is thought to rapidly remove endocannabinoids from the synaptic space.⁴⁸

![Figure 3: The endocannabinoids AEA ¹.¹ and 2-AG ¹.².](image-url)
A complex assortment of regulatory enzymes are responsible for the synthesis and metabolism of AEA 1.1 and 2-AG 1.2. Monoacylglycerol lipase (MAGL) is considered to be the most important enzyme for 2-AG 1.2 metabolism, whereas fatty acid amide hydrolase (FAAH) is primarily responsible for AEA 1.1 breakdown.

1.2.1 CB₁ receptor

Evidence of a specific binding site for the cannabinoids in the brain was reported in 1988 and CB₁ receptor was cloned in 1990. CB₁ receptor is one of the most abundant GPCRs in the brain, and is present in particularly high levels in the sensory and motor regions, playing a significant role in motivation and cognition. Cannabinoids can exert an affect through CB₁ receptor activation on synaptic plasticity, cell migration and perhaps neuronal growth. CB₁ receptor expression is predominantly in the central nervous system (CNS), primarily on the central and peripheral neurons in the presynapase, facilitating the endocannabinoid system’s role in inhibition of neurotransmitter release, but is also present in postsynaptic cells and astrocytes. CB₁ receptor has also been found in some peripheral organs, though at low levels. Like most GPCRs, CB₁ receptor exhibits multiplicity of G protein coupling by activating G_{i/o}, G_{s} and/or G_{q} proteins thereby initiating diverse downstream signaling events, of which the most commonly studied are inhibition of adenylyl cyclase or reductions in cyclic adenosine monophosphate (cAMP). G protein coupling of CB₁ receptor can be influenced by both receptor and G protein expression levels. CB₁ receptor can also activate mitogen-activated protein kinases (MAPKs) and recruit β arrestin 1 and 2. CB₁ receptor is a potential therapeutic target for neuropathic pain, neuroinflammation, cancer, gastrointestinal, cardiovascular, metabolic and psychiatric diseases. However, a key challenge in developing CB₁ receptor targeted therapies is avoiding unwanted psychotropic side effects resulting from the high CB₁ receptor expression levels in the CNS. For instance, the CB₁ receptor inverse agonist rimonabant 1.12 was licensed in the European Union as an obesity treatment, but was later withdrawn due to side effects of suicidal ideation and depression. Exploitation of biased signalling, whereby different ligands can differentially activate pathways, is a potential strategy for avoiding side effects in CB₁ receptor targeted drugs.
The crystal structure of CB1 receptor was recently determined by two research groups in 2016 and there are four high resolution crystal structures available, two in an active state\textsuperscript{65} and two in an inactive state.\textsuperscript{66, 67} This breakthrough represents a leap forward in the understanding of CB1 receptor structural biology as well as increased opportunities for structure based drug design.

### 1.2.2 CB\textsubscript{2} receptor

CB\textsubscript{2} receptor was cloned from spleen in 1993\textsuperscript{68} and in contrast to CB\textsubscript{1} receptor, is expressed mostly in peripheral tissue, particularly in immune cells and lymphoid tissues, such as spleen and tonsils.\textsuperscript{69} CB\textsubscript{2} receptor was originally assumed to be present only in cells of the immune system, but it has now been identified throughout the CNS, particularly in microglial, but overall in much lower levels than CB\textsubscript{1} receptor.\textsuperscript{70, 71} Functions of CB\textsubscript{2} receptor in the immune system include regulation of immune responses and inflammatory pathways via modulation of cytokine release and immune cell migration.\textsuperscript{72, 73} Evidence shows that endocannabinoid signalling through CB\textsubscript{2} receptor may be part of immune protection.\textsuperscript{74} Altered CB\textsubscript{2} receptor expression levels have been observed with many pathologies.\textsuperscript{75-78} Overall though, there is a lack of detailed knowledge of the role of peripheral CB\textsubscript{2} receptor. The function of neuronal CB\textsubscript{2} receptor has long been unknown and it is only recently that initial reports have suggested roles in basic neuronal transmission\textsuperscript{79} and the regulation of memory and anxiety.\textsuperscript{80} CB\textsubscript{2} receptor appears to be less promiscuous than CB\textsubscript{1} receptor, coupling with fewer G protein subforms, although this could be due to a lack of knowledge as CB\textsubscript{2} receptor signalling is not as well understood. CB\textsubscript{2} receptor has been shown to couple with G\textsubscript{i/o} proteins through which it inhibits adenylyl cyclase and reduces cAMP levels, as well as activate MAPKs and engage in \(\beta\)-arrestin 2 recruitment.\textsuperscript{64} Little is understood about CB\textsubscript{2} receptor trafficking, but receptor recycling has been shown to occur through alternating stimulation by agonists and inverse agonists.\textsuperscript{81} Differing chemical classes of CB\textsubscript{2} receptor ligands have demonstrated functional selectivity over CB\textsubscript{2} receptor internalisation, inhibition of adenylyl cyclase and arrestin recruitment, indicating potential for development of CB\textsubscript{2} receptor ligands with on-target signalling selectivity and therefore less adverse effects.\textsuperscript{82, 83}
1.2.2.1 CB2 receptor homology modelling and ligand binding

A number of homology models for CB2 receptor have been reported in the literature, most of which were generated using the crystal structures of human β2-adrenoceptor, bovine rhodopsin or sphingosine-1-phosphate receptor 1 (S1P1) as templates. Homology modelling has been used in combination with mutational and ligand binding studies to elucidate structural details of CB2 receptor and assist rational design of CB2 receptor selective ligands. There is currently no X-ray crystal structure reported for CB2 receptor, however the recently reported CB1 receptor crystal structures are helpful in understanding the structure of CB2 receptor as there is a 44% shared sequence identity between CB1 and CB2 receptors.

It is thought that ligand entry into CB receptors can be lipid-mediated. The endocannabinoids spend much of their life cycle at or in the lipid membrane where they are synthesised and metabolised and therefore it is not surprising that they would enter the receptor via the lipid bilayer. Molecular dynamics and nuclear magnetic resonance (NMR) studies on entry into CB receptors via lipid membrane have found this to be a plausible entry route for several cannabinoids, such as CP55,940 which was able to rapidly laterally diffuse through lipid membrane. Molecular dynamics with 2-AG suggested that 2-AG approaches CB2 receptor at the interface between TMH6 and 7, stimulating a movement in helices, allowing entrance of 2-AG into the orthosteric binding pocket between TMH6 and TMH7. Molecular dynamics simulations with (-)-Δ9-tetrahydrocannabinol (Δ9-THC) and AEA found a probable CB1 receptor entry route between TMH7 and TMH1 from the lipid membrane. It is important to consider receptor entry route when designing ligands and it is possible that there are multiple entry routes into the orthosteric binding site of CB2 receptor, as well as the allosteric sites.

The endocannabinoids bind to the orthosteric site, which is a binding pocket located within the receptor as demonstrated experimentally by Janero et al with a covalent endocannabinoid based probe binding at CB1 receptor. Cannabidiol has been demonstrated to bind to an allosteric binding site of CB2 receptor and act as a negative allosteric modulator of Δ9-THC and 2-AG at CB1 receptor.
1.2.3 CB₂ receptor as a therapeutic target

The endocannabinoid system is associated with numerous physiological processes and disorders.⁹⁵ However, the therapeutic potential of targeting CB₁ and CB₂ receptors has been largely unrealised due to the complex signalling pathways, poor physicochemical properties of typically lipophilic cannabinoids and psychotropic side effects. Due to their respective physiology, it is thought that the psychotropic side effects of cannabinoids are primarily mediated by CB₁ receptor in the CNS,⁹⁶ and that by targeting CB₂ receptor these can be minimised.⁹⁷ Although it is worth noting that there are current efforts to develop peripherally selective CB₁ receptor ligands (i.e. with low blood-brain barrier penetration) in an attempt to circumvent psychotropic effects.⁹⁸ CB₂ receptor is very important in mediating inflammatory processes and has been specifically shown to play a role in brain injury⁹⁹ and neurodegenerative disorders such as Alzheimer’s disease,¹⁰⁰ Parkinson’s disease,¹⁰¹ and Huntington’s disease,¹⁰² as well as atherosclerosis,¹⁰³ myocardial infarction and restenosis,¹⁰⁴ inflammatory bowel disease,¹⁰⁵ osteoporosis,¹⁰⁶ cancer,⁴²,¹⁰⁷ addiction,¹⁰⁸ and inflammatory and chronic pain.¹⁰⁹ In preclinical studies, both CB₂ receptor agonists and inverse agonists have been shown to have analgesic and anti-inflammatory effects,¹⁰⁹-¹¹² which could be reflective of the complex role of CB₂ receptor in these physiological processes. As yet, there are no CB₂ receptor selective drugs available on the market, but a number of candidates have undergone clinical trials.¹¹³,¹¹⁴ For example, the pyrimidine-based GW842166X 1.3 (Figure 4) completed phase II clinical trials for treatment of postoperative pain, but was found to show low efficacy.¹¹⁵ Pyridine-based S-777469 1.4 (Figure 4) completed phase II clinical trials evaluating safety and efficacy in treatment of atopic dermatitis.¹¹⁶ Some researchers have tried to synthesise cannabinoids with reduced lipophilicity in the pursuit of candidates with more favourable physicochemical properties.¹¹⁷,¹¹⁸ In addition, the divergent nature of GPCR signalling creates challenges in achieving signalling selectivity. Teasing out the intricacies of CB₂ receptor signalling pathways and ligand binding interactions could enable development of ligands with functional selectivity.
Allosteric modulation is a potential strategy for developing CB$_2$ receptor targeted drugs that produce a more nuanced fine-tuning of signalling pathways, reducing both off-target and on-target effects. In addition, a recent study found that allosteric modulation of muscarinic receptors produced inhibition of dopamine release and antipsychotic effects which were mediated through CB$_2$ receptor signalling. This is perhaps not surprising considering the role of the endocannabinoid system in neurotransmitter inhibition, but does point to the subtle mechanisms by which CB$_2$ receptor can be exploited to treat disease.

CB$_2$ receptor remains a promising pharmacological target for a multitude of disorders, but a more thorough understanding of the role CB$_2$ receptor plays in disease is required before effective CB$_2$ receptor targeted therapeutics can be developed. Greater appreciation of CB$_2$ receptor function as well as the subtleties of regulation and variation in receptor expression levels, including variation between cell types and healthy and diseased tissue would improve opportunities for drug development.

† Where error values are not included for literature binding affinities, it is because they were not given in the published report. This applies to the whole thesis.
1.3 Cannabinoids

Cannabinoids are defined in this thesis as any ligands that bind to CB receptors and can include endocannabinoids (Figure 3), synthetic ligands and phytocannabinoids derived from plants. Cannabinoids can be agonists, inverse agonists or antagonists.

1.3.1 Phytocannabinoids

Discovery of the endocannabinoid system was as a result of research into the plant *Cannabis sativa* L., from which the endocannabinoid system and its constitutive receptors derive their names. *Cannabis sativa* L. has been used as a medicinal therapy since at least the second millennium BC. However, elucidation of the chemical components of cannabis extracts was not achieved until the second half of the twentieth century, largely due to the complex mixture of over 60 closely related, lipophilic compounds, resulting in challenging isolation and characterisation of key components. The structure of the major mood-altering constituent, (-)-Δ⁹-THC 1.5 was elucidated in 1964 and has since been found to act as a partial agonist with high affinity ($K_i < 100$ nM) at both CB receptors, mediating antinociceptive effects through CB₁ receptor activation (Figure 5). (-)-Δ⁹-THC 1.5 (dronabinol, Marinol®) is licensed for the treatment of nausea in chemotherapy patients. Cannabidiol 1.6 is a major non-psychoactive constituent, significant for its anti-inflammatory properties and acts as a non-competitive antagonist at CB₁ receptor and an inverse agonist at CB₂ receptor, with micromolar affinity at both receptors (Figure 5). A 1:1 mixture of (-)-Δ⁹-THC 1.5 and cannabidiol 1.6 (nabiximols, Sativex®) is licensed for the treatment of neuropathic pain and spasticity in multiple sclerosis (MS) patients. Despite over sixty years of pharmacological research into phytocannabinoids, most of the other phytocannabinoids present in *Cannabis sativa* L. have not been thoroughly evaluated, yet show promising pharmacological properties. Phytocannabinoids have also been detected in plants other than *Cannabis sativa* L., for instance *Echinacea* spp.
The purported therapeutic effects of Cannabis sativa L. and subsequent appreciation for the physiological importance of CB receptors sparked intense interest in developing novel and diverse classes of synthetic cannabinoids. Numerous synthetic cannabinoids have been reported, but only a few of the most notable examples from each of the major chemical classes are described here, with particular focus on CB₂ receptor selective cannabinoids. Unfortunately, several synthetic cannabinoids that were developed with the aim of understanding more about CB receptors have now been appropriated as drugs of abuse, leading to increased legislation in many countries regarding research into novel CB₁ receptor ligands.

### 1.3.2 Synthetic cannabinoids

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#### 1.3.2.1 Endocannabinoid derivatives

CB₁ receptor selective agonists have been developed by introducing chlorine, fluorine, isopropyl or methyl substituents to the hydroxyl tail of AEA 1.1 as well as lengthening the alkyl chain and some of these ligands are stable to FAAH hydrolysis.

#### 1.3.2.2 Classical cannabinoids

Classical cannabinoids contain the same tricyclic dibenzopyran scaffold as the phytocannabinoid (-)-Δ⁹-THC 1.5. Stereoselectivity is a common feature amongst these
cannabinoids, for instance HU-210 1.7 (Figure 6) is a high affinity CB agonist, whereas the enantiomer HU-211 exhibits no significant affinity for CB receptors.\textsuperscript{133} Nabilone 1.8 is a CB receptor agonist marketed as Cesamet® for treatment of chemotherapy induced nausea and for appetite stimulation in acquired immunodeficiency syndrome (AIDS)-related anorexia.\textsuperscript{134} Methylation of the phenolic hydroxyl in classical cannabinoids generally favours CB\textsubscript{2} receptor selectivity, such as in the potent CB\textsubscript{2} receptor selective agonist L-759633 1.9.\textsuperscript{135}

![Figure 6: Classical synthetic cannabinoids HU-210 1.7, Nabilone 1.8 and L-759633 1.9.129, 135, 136](image-url)

1.3.2.3 Non-classical cannabinoids

Initial efforts at diversifying synthetic cannabinoid structures focused on derivatising and breaking the dibenzopyran ring of THC 1.5, giving rise to the non-classical cannabinoids (NCCs). Perhaps the most notable NCC is CP55,940 1.10, which is a high affinity agonist at both CB receptor subtypes (Figure 7). The tritiated analogue of CP55,940 1.10 is commonly used in radioligand binding assays at CB receptors (see 1.4.2 and 1.6.1) and is used in this thesis (Chapters Two - Five). HU-308 1.11 is a CB\textsubscript{2} receptor selective agonist with demonstrated anti-inflammatory and analgesic properties.\textsuperscript{137}
1.3.2.3.1 Diarylpyrazole cannabinoids

Most reported diarylpyrazole-based cannabinoids are selective for CB$_1$ receptor, e.g. Rimonabant 1.12 (SR141716A; see section 1.2.1; Figure 8). A notable exception is SR144528 1.13, which was the first highly potent, CB$_2$ receptor selective antagonist reported (Figure 8). SR144528 1.13 also exhibits inverse agonism at CB$_2$ receptor and has found widespread use as a pharmacological tool for characterising CB$_2$ receptor function.

1.3.2.3.2 Aminoalkylindole cannabinoids

The aminoalkylindoles (AAIs) represent a large chemical class of cannabinoids with extensive structure activity relationships (SAR) reported which will be discussed in more detail in Chapter Two. AM630 1.14 (Figure 8) is a CB$_2$ receptor selective AAI that has been characterised as an antagonist and inverse agonist at CB$_2$ receptor, and it has also been proposed that 1.14 behaves as a protean ligand, behaving as an agonist in certain receptor populations.

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‡ Where species of receptor is not listed for literature binding affinities, it is because it was not specified in the original report. This applies to the whole thesis. $K_i$ value for HU-308 1.11 at CB$_1$ receptor is at rat (r)CB$_1$ receptor.
Figure 8: Examples of diarylpyrazole, AAI, quinoline and 1,8-naphthyridin based cannabinoids.\textsuperscript{111, 135, 138, 140, 145}

1.3.2.3 Quinoline and 1,8-naphthyridin cannabinoids

Quinoline-based JTE-907 \textsuperscript{1.15} is a CB\textsubscript{2} receptor selective inverse agonist with demonstrated anti-inflammatory and anti-pruritic effects \textit{in vivo} (Figure 8).\textsuperscript{111, 146} 1,8-naphthyridin based cannabinoids (e.g. the antagonist \textsuperscript{1.16}; Figure 8) have emerged in recent years as providing a high degree of CB\textsubscript{2} receptor selectivity and reduced lipophilicity in comparison to other scaffolds.\textsuperscript{145} 1,8-naphthyridin-based cannabinoids will be reviewed in more detail in Chapter Four.
There are several other distinct cannabinoid scaffolds, such as imidazoles, benzimidazoles, pyridines and carbolines. Due to their therapeutic potential, there are many patent reports of novel CB₂ receptor ligands, which were recently reviewed by Morales et al. However these are not discussed further here because this is outside of the scope of this thesis.
1.4 Tools for studying CB$_2$ receptor

There are a variety of pharmacological tools which have been invaluable in the discovery and characterisation of CB$_2$ receptor thus far. However, each of these tools have their own distinct utilities and drawbacks. The further elucidation of complex CB$_2$ receptor processes requires a diverse set of tools with improved selectivity, pharmacokinetic profiles and utility in studying dynamic live cell receptor processes. A detailed review of radioligands, covalent ligands, antibodies and fluorescent ligands as tools for studying CB receptors and other lipid-binding Class A GPCRs can be found in the review article published during this PhD research (Anna Cooper, Sameek Singh, Sarah Hook, Joel D. A. Tyndall and Andrea J. Vernall, (2017) Chemical Tools for Studying Lipid-Binding Class A G Protein-Coupled Receptors, *Pharmacol Rev* 69:316-353). Therefore, the use of radioligands, covalent ligands and antibodies are summarised very briefly in this thesis. However, fluorescent ligands are the main focus of this thesis and so will be covered in more depth in the subsequent section (1.5).

1.4.1 Ligands

High affinity CB$_2$ receptor ligands are inherently useful for studying receptor function and have found substantial use in probing receptor signalling pathways and physiology. For instance, CP55,940 1.10 has been widely exploited in the study of CB receptor activation and physiology and is used in this PhD research in cAMP assays to test for antagonists (see 1.6.2). The CB$_2$ receptor selective inverse agonist SR144528 1.13 has been used to study CB$_2$ receptor ligand binding, phosphorylation and physiological roles, and in this PhD research was used as a reference ligand in both the radioligand binding assays and cAMP assays. CB$_2$ receptor selective agonist HU-308 1.11 has been used to elucidate CB$_2$ receptor trafficking mechanisms. In addition, high affinity ligands have been exploited as stabilising ligands in the crystallisation of CB$_1$ receptor.66, 67
1.4.2 Radioligands

Radioligands can be used to investigate phenomena such as receptor distribution,\textsuperscript{154} oligomerisation,\textsuperscript{155} signalling and allosteric modulation.\textsuperscript{156} Radioligands possessing radioisotopes such as $^3$H, $^{14}$C and $^{35}$S are frequently used in binding assays to identify or characterise CB$_2$ receptor ligands\textsuperscript{157} and determine ligand-receptor binding parameters, such as dissociation constant ($K_d$), inhibition constant ($K_i$; see section 1.6.1) or maximum specific binding ($B_{\text{max}}$). The CB receptor radioligand $[^3]$HCP55,940 1.17 (Figure 9) has proven a valuable tool in cannabinoid pharmacology, facilitating the discovery of CB$_1$ receptor\textsuperscript{51} and study of CB receptor expression in disease.\textsuperscript{158} $[^3]$HCP55,940 1.17 is used in this PhD research in competition radioligand binding assays (section 1.6.1). However, radioligands have significant drawbacks with their inherent safety hazards and disposal issues, expense and limited shelf life. Radioligands have been used to study the distribution of CB$_2$ receptor with in vivo positron emission topography (PET) imaging, by incorporating radioisotopes such as $^{11}$C and $^{18}$F into CB$_2$ receptor ligands.\textsuperscript{148} PET imaging offers deep tissue penetration and high sensitivity, but poor spatial resolution.\textsuperscript{159,160}

![Image of CP55,940 1.17](image)

Figure 9: The CB receptor radioligand $[^3]$HCP55,940 1.17

1.4.3 Covalent ligands

Covalent ligands possess electrophilic or photoactivatable functionality capable of forming a covalent bond with amino acid side chains.\textsuperscript{161,162} The primary utility of covalent ligands is in investigating receptor structure by identifying amino acid residues
key to ligand binding and receptor activation. Covalent ligands are often used in conjunction with site-directed mutagenesis studies and in silico modelling to characterise the topography of the ligand binding site(s). The electrophilic AM841 (Figure 10) has been used in multiple structure studies to elucidate CB receptor structure, function, physiological roles and ligand entry pathways, as well as a stabilising ligand in the crystallisation of CB1 receptor. Many covalent ligands developed for CB2 receptor lack the required functionality for use as imaging tools, however covalent fluorescent probes can be developed, enabling utility as imaging tools to study dynamic receptor processes.

![AM841 1.18](image)

Figure 10: CB receptor electrophilic ligand AM841 1.18

1.4.4 Antibodies

Antibodies are versatile and have been commonly used to study GPCRs in conjunction with several techniques, such as western blotting, immunohistochemistry, immunocytochemistry, immunofluorescence and flow cytometry. Indeed, much of our current understanding of CB1 and CB2 receptor expression has been gleaned through antibodies. However, there have been notable reports of lack of reproducibility and poor specificity of CB receptor antibodies, resulting in false positives and false negatives. Nanobodies are fragments of antibodies, derived from the variable domain and have been developed as diagnostics, therapeutics and as tools for studying GPCR structure and function. Allosteric nanobodies have been used to study
mechanisms of β2-adrenoceptor activation. Nanobodies have also been used to stabilise GPCRs for obtaining X-ray crystal structures.
1.5 Fluorescent ligands

1.5.1 Principles of fluorescence

Fluorescence occurs during vibrational relaxation of an excited state fluorophore. The first stage is excitation, in which the fluorophore absorbs a photon, raising the energy level from a singlet ground state ($S_0$) to an excited electronic singlet state ($S_1'$; Figure 11). During the excited-state lifetime (typically $10^{-8}$ seconds) partial dissipation of energy occurs from the excited singlet state ($S_1'$) to yield a relaxed excited singlet state ($S_1$). Subsequent emission of a photon produces a fluorescent signal and the fluorophore relaxes back to ground state ($S_0$).

![Jablonski diagram](image)

Figure 11: Jablonski diagram representing the shift in energies between ground state ($S_0$), excited electronic singlet state ($S_0'$) and relaxed excited singlet state ($S_1$).

A fluorophore emits light at a longer wavelength than it was excited at. This is due to the loss of energy between excitation and emission, during the excited-state lifetime, resulting in the emitted photon having lower energy than the absorbed photon. The Stokes shift describes the shift from high energy to low energy, i.e. the shift from the shorter absorption wavelength to the longer emission wavelength. The Stokes shift is important as it allows detection of emitted fluorophores to be distinguishable from absorbed photons. A fluorophore can repeatedly cycle through excitation (absorbance)
and relaxation (fluorescence) unless it is photobleached, whereby an over-excited fluorophore can react with oxygen to produce a nonfluorescent molecule.\textsuperscript{177}

Processes other than fluorescence can occur from the excited state, such as quenching leading to loss of photons. Quantum yield describes the efficiency of the fluorescence process and is equal to the number of emitted photons divided by the number of absorbed photons.\textsuperscript{178} Therefore, the maximum fluorescence quantum yield of a fluorophore is 1.0 and it can be as low as 0.05. The absorption efficiency of a fluorophore is described by the molar extinction coefficient, with typical values between 5000 – 200,000 cm\textsuperscript{-1}M\textsuperscript{-1}.\textsuperscript{174} The overall fluorescence output of a fluorophore is proportional to the product of the quantum yield and the molar extinction coefficient. Quenching can occur due to short-range interactions between the fluorophore and the local molecular environment, leading to loss of fluorescence signal.\textsuperscript{173, 179} For instance, when fluorophores are used in biological experiments, some fluorophores may be quenched in an aqueous environment, whereas others may be quenched in the membrane.

1.5.2 Applications of fluorescence for studying GPCRs

Fluorescence techniques, including fluorescent antibodies, autofluorescent proteins, tagged proteins and fluorescent ligands have found widespread use as non-invasive methods for visualising GPCR processes in real-time.\textsuperscript{180} This is a result of technological advances in fluorescence instrumentation as well as increased availability of diverse fluorophores. Fluorescent antibodies have been used to visualise membrane bound GPCRs in both living and fixed cells, or internalised GPCRs in fixed cells. Autofluorescent proteins, such as green fluorescent protein (GFP) and yellow fluorescent protein (YFP) are extensively used as reporter molecules for localising GPCRs and are utilised in fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) techniques to uncover molecular mechanisms of dimerisation, signalling and receptor recycling.\textsuperscript{181}

Fluorescent ligands are powerful tools for studying receptor structure and function in whole live cells.\textsuperscript{182} The versatility of fluorescent ligands means that they can be used to
study receptor localisation, oligomerisation, allostery and dynamic receptor processes such as activation and trafficking.\textsuperscript{183}

1.5.3 Fluorescent ligands for GPCRs

Fluorescent ligands are adaptable to many different fluorescence-based assay and imaging systems, including high throughput screening (HTS),\textsuperscript{184,185} FRET,\textsuperscript{186} confocal microscopy, scanning confocal microscopy (SCM), flow cytometry\textsuperscript{187} and fluorescence correlation spectroscopy (FCS).\textsuperscript{188} Fluorescent ligands can enable the analysis of single cells and single receptor-ligand interactions when utilised with SCM or FCS. Fluorescence is a highly sensitive spectroscopic method and is also a non-destructive process, so signal changes can be reliably measured as a function of time to determine receptor-ligand kinetics.\textsuperscript{180}

Near-infrared (NIR) fluorescent ligands have found \textit{in vivo} applications, due to relatively deep tissue penetration and low autofluorescence in the near infrared region (650-900 nm).\textsuperscript{189} NIR fluorescent ligands offer high sensitivity and resolution and have found applications as tumour-specific probes for guided surgery,\textsuperscript{190} as well as in optical biopsies.\textsuperscript{191} Overexpression of GPCRs on the surface of tumour cells renders them a potential target for receptor specific \textit{in vivo} tools.

High affinity, specific fluorescent ligands have been successfully designed for other class A GPCRs and utilised \textit{in vitro} in pharmacological studies.\textsuperscript{192} For instance, an $\alpha_{1L}$-adrenoceptor fluorescent ligand was able to localise receptor in the muscle layer of human prostate.\textsuperscript{193} Fluorescent ligands for A\textsubscript{3} adenosine receptor have been used to visualise receptor internalisation and arrestin recruitment,\textsuperscript{194} study single-cell ligand binding kinetics,\textsuperscript{195} as well as the effect of allosteric modulators in perfusion studies.\textsuperscript{196} Quantification of ligand-receptor complexes was enabled using a fluorescent ligand for histamine-H\textsubscript{1} receptor in conjugation with FCS.\textsuperscript{197}

Fluorescent ligands require high affinity and specificity to be put to use as biological tools in native cells with mixed receptor populations. Non-specific membrane binding of fluorescent ligands is usually determined by confocal imaging studies of cells
overexpressing the target receptor, incubated with the fluorescent ligand and in the presence or absence of an excess of a non-fluorescent high affinity receptor selective ligand (‘blocking experiments’, e.g. Figure 12).  

Figure 12: Example of a blocking experiment for determining non-specific binding of a GPCR fluorescent ligand. Confocal imaging of an A₃ receptor fluorescent ligand (‘28’, A₃ receptor selective pharmacophore-linker-BODIPY FL-X) in Chinese hamster ovary cells expressing human A₃ receptor. The fluorescent labelling of A₃ receptor by ‘28’ (top left panel) is blocked by a non-fluorescent high affinity, selective ligand for A₃ receptor (MRS1220, top right panel), therefore demonstrating that ‘28’ has minimal non-specific membrane binding.  

1.5.3.1 Fluorescent ligands for CB₂ receptor

There have been several reported fluorescent ligands for CB₂ receptor. However, many of these ligands have shown either poor affinity or selectivity for CB₂ receptor, or high non-specific membrane binding, limiting their utility as imaging tools. The challenge of developing a CB₂ receptor fluorescent ligand with little non-specific membrane binding is exacerbated by the lipophilic nature of many cannabinoids and fluorescent dyes.

---

The fluorescent ligand NMP6 1.19 was developed by replacing a portion of the pharmacophore with a nitrobenzoxadiazole (NBD) fluorophore (Figure 13). This approach is in contrast to most fluorescent ligand designs which append the fluorophore to the pharmacophore. NMP6 1.19 showed reasonable affinity and selectivity for CB\textsubscript{2} receptor (Table 1), although blocking experiments using NMP6 1.19 and a high concentration of non-fluorescent CB\textsubscript{2} receptor agonist still showed a degree of non-specific binding.

![NMP6 1.19 and Biotin-HU210-1 1.20](image)

Figure 13: Fluorescent CB\textsubscript{2} receptor ligands NMP6 1.19 and Biotin-HU-210-1 1.20.

The biotin-tagged HU210-1 1.20 showed high affinity for CB\textsubscript{2} receptor but no subtype selectivity over CB\textsubscript{1} receptor, limiting its utility in native cells (Table 1; Figure 13). In\textit{ situ} conjugation of biotin-HU210-1 1.20 with a fluorophore streptavidin-Alexa488 was demonstrated during\textit{ in vitro} experiments with neurons in primary culture and rat microglia.
Table 1: Literature affinity data for CB receptor fluorescent ligands.

<table>
<thead>
<tr>
<th>Fluorescent ligand</th>
<th>CB$_2$ receptor affinity $^a$</th>
<th>CB$_1$ receptor affinity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMP6 1.19 $^{201}$</td>
<td>$K_i = 387$ nM (h)</td>
<td>$&lt; 40%$ at 10 µM (h)</td>
</tr>
<tr>
<td>Biotin-HU210-1 1.20 $^{97}$</td>
<td>$K_i = 1.6 \pm 0.4$ nM (h)</td>
<td>$K_i = 2.4 \pm 0.4$ nM (h)</td>
</tr>
<tr>
<td>NIR-mbc94 1.22 $^{184}$</td>
<td>$K_i = 260$ nM (m)</td>
<td>-</td>
</tr>
<tr>
<td>NIR760-mbc94 1.23 $^{200}$</td>
<td>$K_d = 26.9 \pm 3.7$ nM (m)</td>
<td>-</td>
</tr>
<tr>
<td>ZW760-mbc94 1.24 $^{199}$</td>
<td>$K_d = 53.9 \pm 13.0$ nM (m)</td>
<td>-</td>
</tr>
<tr>
<td>IR700DX-mbc94 1.25 $^{202}$</td>
<td>$K_d = 42.0 \pm 19.6$ nM (m)</td>
<td>-</td>
</tr>
<tr>
<td>NIR760-Q 1.26 $^{203}$</td>
<td>$K_d = 75.5 \pm 28.0$ nM (h)</td>
<td>-</td>
</tr>
<tr>
<td>NIR760-XLP6 1.27 $^{204}$</td>
<td>$K_d = 169.1 \pm 66.1$ nM (m)</td>
<td>$K_d = &gt;10000$ nM (m)</td>
</tr>
</tbody>
</table>

$^a$ Affinity reported as either $K_i$ or $K_d$ value and for mouse (m)CB or human (h)CB receptor.

Most CB$_2$ receptor fluorescent ligands for in vivo applications have utilised SR144528 1.13 as the core pharmacophore. These studies illustrate the importance of selecting the appropriate position for linker conjugation, as linker attachment at the 4-chlorophenyl or pyrazole abolished CB$_2$ receptor affinity, whereas attachment at the benzylic position was tolerated in the linker conjugate mbc94 1.21. $^{184, 205}$ Conjugation of a NIR fluorophore, IRDye 800CWt (cyanine 7 (Cy7)) to mbc94 1.21, gave the fluorescent ligand NIR-mbc94 1.22, which demonstrated good affinity for mouse (m) CB$_2$ receptor (Table 1; Figure 14), but high non-specific membrane binding. $^{184}$ Changing the fluorophore to NIR760 (Cy7) resulted in a fluorescent ligand NIR760-mbc94 1.23 with high affinity for mCB$_2$ receptor, but still showed significant non-specific binding. $^{200}$ Conjugation of a zwitterionic NIR760 (Cy7) fluorophore to the same pharmacophore-linker to give ZW760-mbc94 1.24 resulted in some improvement in non-specific binding compared to NIR760-mbc94 1.23, but there was still enough non-specific binding to limit utility. $^{199}$ Fluorescent ligands have potential utility as photosensitiser therapies. For example, attaching the NIR fluorophore IR700DX to the pharmacophore-linker mbc94 1.21 yielded the photosensitiser IR700DX-mbc94 1.25, $^{202}$ which successfully inhibited CB$_2$-positive tumours in mice following irradiation. $^{206}$ It is important to note that none of the mbc94 1.21 conjugated fluorescent dyes have reported affinities for CB$_1$ receptor, so it is not yet demonstrated how selective they would be in mixed CB receptor populations.
Figure 14: Fluorescent CB2 receptor ligands NIR-mbc94 1.22, NIR760-mbc94 1.23, ZW760-mbc94 1.24 and IRD700DX-mbc94 1.25.
A quinolone-based ligand was used in the development of the fluorescent ligand NIR760-Q 1.26 (Table 1; Figure 15) possessing a Cy7 fluorophore, but it showed high non-specific binding in Jurkat cells. Conjugation of NIR760 to a pyrazolopyrimidine based pharmacophore yielded a fluorescent ligand NIR760-XLP6 1.27 with selectivity for CB₂ over CB₁ receptor, however non-specific binding was again observed.

Figure 15: Fluorescent CB₂ receptor ligands NIR760-Q 1.26 and NIR760-XLP 1.27.

The availability of a high affinity, selective fluorescent ligand for CB₂ receptor exhibiting little non-specific binding (meaning little non-specific membrane binding and little binding to other receptors) would enable a huge step forward in the in vitro study of CB₂ receptor expression and function, thus enabling exploitation of CB₂ receptor as a therapeutic target.

It is worth noting that there is also a lack of high affinity, selective fluorescent tools with favourable physicochemical properties for CB₁ receptor.
1.5.4 Fluorescent ligand design

Fluorescent ligands are usually designed by first selecting an appropriate pharmacophore, such as a well characterised ligand with high affinity and selectivity for the target receptor. It is worth considering the desired functionality of the fluorescent ligand when selecting a pharmacophore. There is a need for fluorescent ligands for CB2 receptor with a range of functionality to expand the pharmacological toolbox and enable study of diverse receptor processes. Fluorescent antagonists for other GPCRs have proven utility in binding assays and studies of receptor expression and localisation.\textsuperscript{207} Whereas, fluorescent agonists have been used to probe receptor kinetics,\textsuperscript{195} internalisation,\textsuperscript{187} and allosteric modulation.\textsuperscript{196} It is therefore of interest to develop both fluorescent agonists and antagonists, as well as inverse agonists for CB2 receptor.

A linker is often employed to create physical distance between the pharmacophore and the fluorophore, with the intention of reducing any detrimental effect of the fluorophore on pharmacophore-receptor binding.\textsuperscript{208} Conjugation of a fluorescent dye and linker to a pharmacophore introduces a large amount of steric bulk to the ligand, which can significantly alter the pharmacology and physicochemical properties compared to the unconjugated pharmacophore. An appropriate region of the pharmacophore for linker conjugation needs to be identified that is amenable to change and the introduction of steric bulk/length. The linker and fluorophore are likely to engage in binding (either specific and/or non-specific) interactions with receptor and/or lipid membrane. Therefore varying the site of linker attachment, linker length and composition and fluorophore can have a significant effect on linker affinity and efficacy.\textsuperscript{209} In addition, linker and fluorophore choice can greatly affect the overall physicochemical properties of the ligand, impacting non-specific membrane binding and non-specific cell accumulation.

There is a diverse selection of fluorophores commercially available that already contain a reactive group (e.g. sulfonyl chloride and activated esters), suitable for conjugation to amines. Important considerations when selecting a fluorescent dye include the excitation and emission wavelength, Stokes shift, molar extinction coefficient, quantum
yield, quenching environments, and susceptibility to photobleaching.\textsuperscript{210} The most commonly utilised fluorophores in GPCR fluorescent ligand design are borondipyrrromethene (BODIPY\textsuperscript{®}),\textsuperscript{211} Alexa Fluor\textsuperscript{®},\textsuperscript{212} Rhodamines\textsuperscript{213} and Cyanines.\textsuperscript{214}

BODIPY\textsuperscript{®} dyes are available across a range of wavelengths, which is determined by the substitutions on the core chromophore, with substitutions that extend the conjugated system producing longer wavelengths.\textsuperscript{215} As well as commercial sources, there are many published reports on the synthesis of novel BODIPY dyes.\textsuperscript{216-218}

Isothiocyanate containing fluorophores are not commonly commercially available due to long term instability. Sulfonyl chloride containing fluorophores are often unstable in water and therefore care has to be taken during conjugation reactions.\textsuperscript{219} Activated esters such as succinimidyl esters, tetrafluorophenyl esters and sulfodichlorophenol esters are generally stable and exhibit slow rates of hydrolysis\textsuperscript{220} and can react with primary amines to form highly stable amide bonds. Succinimidyl ester derivatives are the most widely available commercially.

Identifying a high affinity, selective pharmacophore-linker conjugate opens up opportunities for the development of tools other than fluorescent linkers. As well as a range of type and wavelength of fluorophore, other moieties can be attached to the linker, such as biotin,\textsuperscript{\textsuperscript{87}} covalent warheads, or a clickable handle for \textit{in vivo} labelling,\textsuperscript{221} or the pharmacophore-linker could be further developed into bitopic ligands for dual targeting of orthosteric and allosteric sites\textsuperscript{222} or bivalent ligands for targeting receptor oligomers.\textsuperscript{223}
1.6 Pharmacological characterisation

An array of assays have been reported for characterising ligand binding and function at CB2 receptor. The basic principles behind the assays utilised in this PhD research project will be briefly summarised here.

1.6.1 Radioligand binding assays

Competition radioligand binding assays measure the ability of a ligand to directly displace a known high affinity radioligand, such as [³H]CP55,940 1.17 from receptor. These assays are convenient for a 96-well plate format, meaning that multiple ligands can be tested in one plate. Each well usually contains three components - a fixed concentration of radioligand and receptor and either test ligand, vehicle control, or a hetero- or homologous competitor (e.g. CP55,940 1.10). The vehicle control determines the maximum radioligand binding level, whilst the hetero- or homologous competitor determines maximum specific displacement and together these values determine the specific binding window. Determining the specific binding window is important as cannabinoids often exhibit high levels of non-specific membrane binding. When a concentration response assay is carried out, a $K_i$ value of the test ligand can be determined using nonlinear regression to fit curves and then using the resultant half maximal inhibitory concentration (IC₅₀) in the Cheng-Prusoff equation:

$$K_i = IC_{50}/(1 + \frac{[\text{radioligand}]}{K_d}).$$

This equation requires the concentration and the $K_d$ of the radioligand, the latter of which is predetermined in a saturation binding assay. A $K_d$ value of a radioligand at a given receptor should theoretically be constant across cell lines and expression levels. However, there are sometimes minor differences in the reported values, possibly due to post-translational receptor modifications (e.g. phosphorylation or palmitoylation), assay conditions or the presence of native endocannabinoids in the serum used in binding buffers. Therefore it is important for a research group carrying out radioligand binding assays to determine the $K_d$ value of the radioligand themselves, rather than use literature reported $K_d$ values in $K_i$ calculations. As the $K_d$ value used effects the calculated $K_i$, it is also important to consider the $K_d$ value when comparing $K_i$ values across studies reported in the
literature. All pharmacological evaluation in this project was carried out in the laboratory of Professor Michelle Glass at the University of Auckland and therefore the $K_d$ values determined by her laboratory group for the radioligand ([$^3$H]CP55,940) 1.17 at human embryonic kidney (HEK)293 cell membranes overexpressing hCB$_1$ and hCB$_2$ receptor were utilised. The $K_i$ value determined in a radioligand binding assay is specific to the radioligand utilised and is therefore only a measure of the test ligand’s ability to displace that particular radioligand and assumes that the radioligand and the test ligand bind to the same or similar receptor site.

1.6.2 cAMP bioluminescence resonance energy transfer assays

BRET assays depend upon resonance energy transfer (RET) between a bioluminescent donor (Renilla luciferase (Rluc)) and a proximal fluorescent acceptor (e.g. YFP). Rluc catalysed oxidation of coelenterazine causes Rluc to emit light which can lead to excitation of proximal YFP by RET. The inverse rate of RET is determined by dividing the Rluc signal by the YFP signal and essentially measures the proximity of YFP to Rluc. An EPAC (exchange protein directly activated by cAMP) sensor contains the donor and acceptor in the same protein and therefore the RET is intramolecular. When EPAC comes into contact with cAMP, it changes conformation, forcing the donor and acceptor further apart, reducing BRET signal (Figure 16). This assay is useful for measuring the changing levels of cellular cAMP and therefore can determine whether a ligand is an agonist, antagonist or inverse agonist in the $G_i$ coupled CB receptor signalling pathway.
BRET assays are a popular technique for studying GPCRs and have found application in ligand binding assays, studying receptor-protein interactions (e.g. oligomerisation, G protein and β arrestin interactions) and receptor activation and signalling. BRET assays were carried out in this PhD research to characterise ligand function in cells overexpressing CB$_2$ or CB$_1$ receptor, monitoring cAMP signalling via the G$_i$/o coupled pathway (see Experimental 7.2.2).
1.7 Aims of the Thesis

The overall goal of this PhD research was to develop a high affinity, selective CB$_2$ receptor fluorescent ligand suitable for use as a pharmacological tool for studying CB$_2$ receptor *in vitro* or *ex vivo*. There were several objectives towards achieving this overall aim:

- Identify positions of a suitable pharmacophore that are amenable for conjugation of a linker and fluorophore.
- Synthesise a series of fluorescent ligands with varying linker positions, types and lengths.
- Pharmacologically evaluate the fluorescent ligands, linker-pharmacophore conjugates and pharmacophores to determine affinity and selectivity for CB$_2$ receptor and function in a cAMP signalling assay.
- Build a CB$_2$ receptor homology model for use in ligand docking studies to rationalise pharmacological results and aid in refinement of ligand design.
- Design a second generation of fluorescent ligands based on the first round results, to improve CB$_2$ receptor affinity and selectivity as necessary.
Chapter Two: Fluorescent ligands for CB$_2$ receptor based on 5 and 6 substituted alkyl-indoles

The first step in designing a fluorescent ligand is the selection of a suitable scaffold or ligand for derivatisation and conjugation with a fluorescent dye. Firstly, it is helpful for the scaffold to have well defined SAR patterns regarding high affinity and selectivity for CB$_2$ receptor. It is also advantageous that the scaffold has well characterised SAR indicating where the chemical change and steric bulk of a fluorescent dye could be tolerated. To this end, high affinity, selective CB$_2$ receptor ligand classes were examined and of these, AM630 1.14 of the AAI class of cannabinoids was selected as a promising candidate for development into a fluorescent ligand. The following section (2.1) outlines the literature SAR of the alkylindole (AI)** scaffold regarding affinity and selectivity for CB$_2$ receptor, which informed the fluorescent ligand design.

2.1 Indole-based cannabinoids

The AAI WIN 48,098 (Pravadoline) 2.1 (Figure 17) was developed by Sterling Winthrop as a new nonsteroidal anti-inflammatory drug (NSAID), and shortly afterwards was discovered to have cannabimimetic activity. Further work led to the conformationally constrained, high affinity, CB receptor agonist WIN 55,212-2 2.2, in which the morpholino group is restrained by tethering to C7 of the indole core (Figure 17). WIN 55,212 produces stereoselective cannabimimetic activity \textit{in vivo}, with the $R$ enantiomer (WIN 55,212-2 2.2) conferring higher potency than the $S$ enantiomer (WIN 55,212-3). The binding distribution of radiolabelled WIN 55,212-2 2.2 to rat brain was shown to be very similar to the binding patterns of $[^3]$H]CP55,940 1.17, helping to validate cannabinoid receptors as the target of AAIs. A study exploring the effects of over 60 compounds from multiple neurotransmitter systems on AAI

** AAIs are classified as indoles which possess an amino alkyl group, e.g. the morpholino group in AM630 1.14, whereas AIs possess alkyl groups, e.g. the pentyl group of JWH-007 2.3.
binding showed that only cannabinoids (e.g. Δ¹-THC, Δ⁶-THC, CP55,940) were able to inhibit binding of AAIs, demonstrating that AAIs bind to cannabinoid receptors.²³⁷

![Figure 17: Early AAI cannabinoids](image)

2.1.1 SAR of the N1, C2 and C3 positions of indole

A large number of AAIs and AIs have subsequently been synthesised, generating thorough SAR of the whole indole scaffold. In one of the earliest SAR studies, Huffman et al demonstrated that absence of an aromatic group at the C3 acyl resulted in loss of affinity at CB₁ receptor.²³⁸ The Winthrop group synthesised and studied over 100 related compounds and showed that a bicyclic substituent at C3, ideally a 1-naphthoyl or a substituted 1-naphthoyl, was preferred for CB₁ receptor affinity.²³⁹ The same study demonstrated that methyl was the largest group tolerated at C2 but that a C2-hydrogen atom was often preferable to a methyl. However, it is interesting to note that Shi et al recently reported that moving a C3-amidoadamantyl group to C2 resulted in high affinity and selective CB₂ receptor ligands, so it appears that bulky groups can be tolerated at C2, but only in the absence of C3 substituents.²⁴⁰ The Winthrop group also found that for the N1 amino alkyl group, an amino ethyl was optimal and should be connected to a cyclic amino such as morpholino, piperidine or thiomorpholine,²³⁹ later showing that the ethyl can also be connected to the α carbon of the cyclic amino group.
instead of the nitrogen.\textsuperscript{241} Exploration of N1 alkyl chain substituents have shown that the amino alkyl moiety is not necessary for CB\textsubscript{1} or CB\textsubscript{2} receptor affinity and in its place, an alkyl chain of three to six carbons is tolerated, generating AI compounds such as JWH-007 \textbf{2.3} and JWH-015 \textbf{2.4} (Figure 18).\textsuperscript{238, 242} Chains longer than hexyl were not tolerated, indicating limited opportunity for longer extension from this position. A propyl group at N1 appears to confer CB\textsubscript{2} receptor selectivity, as demonstrated by the CB\textsubscript{2} receptor selective agonists JWH-015 \textbf{2.4} and JWH-046 \textbf{2.5} (Figure 18),\textsuperscript{243} whilst N1-pentyl generally produces unselective or moderately selective CB\textsubscript{1} receptor ligands.\textsuperscript{136, 244} Many CB\textsubscript{1} receptor agonists belonging to the AI class have been identified as constituents in synthetic cannabis drugs of abuse, such as ‘spice’.\textsuperscript{157}

\begin{center}
\begin{tabular}{c c c}
\textbf{2.3} & \textbf{2.4} \\
\text{JWH-007} & \text{JWH-015} \\
\text{R\textsubscript{1} = C\textsubscript{6}H\textsubscript{11}, R\textsubscript{2} = CH\textsubscript{3}, R\textsubscript{3} = H} & \text{R\textsubscript{1} = C\textsubscript{6}H\textsubscript{7}, R\textsubscript{2} = CH\textsubscript{3}, R\textsubscript{3} = H} \\
\text{K\textsubscript{i} = 2.9 \pm 2.6 \text{nM} hCB\textsubscript{2}} & \text{K\textsubscript{i} = 14 \pm 4.6 \text{nM} hCB\textsubscript{2}} \\
\text{K\textsubscript{i} = 9.5 \pm 4.5 \text{nM} rCB\textsubscript{1}} & \text{K\textsubscript{i} = 336 \pm 36 \text{nM} rCB\textsubscript{1}} \\
\textbf{2.5} & \textbf{2.6} \\
\text{JWH-046} & \text{JWH-120} \\
\text{R\textsubscript{1} = C\textsubscript{3}H\textsubscript{7}, R\textsubscript{2} = CH\textsubscript{3}, R\textsubscript{3} = 7-CH\textsubscript{3}} & \text{R\textsubscript{1} = C\textsubscript{3}H\textsubscript{7}, R\textsubscript{2} = H, R\textsubscript{3} = 4-CH\textsubscript{3}} \\
\text{K\textsubscript{i} = 16 \pm 4.9 \text{nM} hCB\textsubscript{2}} & \text{K\textsubscript{i} = 6.1 \pm 0.7 \text{nM} hCB\textsubscript{2}} \\
\text{K\textsubscript{i} = 343 \pm 38 \text{nM} rCB\textsubscript{1}} & \text{K\textsubscript{i} = 1054 \pm 31 \text{nM} rCB\textsubscript{1}} \\
\end{tabular}
\end{center}

\textbf{2.7} & \\
\text{JWH-151} & \\
\text{R\textsubscript{1} = C\textsubscript{3}H\textsubscript{7}, R\textsubscript{2} = CH\textsubscript{3}, R\textsubscript{3} = 6-OME} & \\
\text{K\textsubscript{i} = 30 \pm 1.1 \text{nM} hCB\textsubscript{2}} & \\
\text{K\textsubscript{i} = \textgreater 10 \text{$\mu$M} rCB\textsubscript{1}} & \\

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{alkylindole_compounds.png}
\caption{Alkylindole compounds containing either an N1-pentyl or -propyl group and a substituted or unsubstituted C3-naphthoyl group.\textsuperscript{243, 245}}
\end{figure}

Two additional CB\textsubscript{2} receptor selective N1-propyl containing agonists were identified by incorporating substitutions onto the C3-naphthoyl moiety, either 4-methyl-1-naphthoyl (JWH-120 \textbf{2.6}) or 6-methoxy-1-naphthoyl (JWH-151 \textbf{2.7}; Figure 18).\textsuperscript{245} In addition to the aforementioned weak to moderate electron releasing groups, incorporation of electron withdrawing halogen groups on the C3-naphthoyl moiety has also been utilised as a method to modulate selectivity and affinity for CB\textsubscript{2} receptor.\textsuperscript{246} It is important to note that many of the aforementioned compounds have been pharmacologically
characterised using rat (r)CB₁ and human (h)CB₂ receptor. McPartland et al have demonstrated that interspecies variations can lead to significant differences in ligand binding affinity at cannabinoid receptors. Therefore, the selectivity of these compounds between hCB₁ and hCB₂ may differ to that reported for rCB₁ and hCB₂ receptor.

A study exploring substitution of a variety of non-aromatic and aromatic heterocycles at N1 showed a degree of tolerance for varying chemical groups, but again with strict size limitations. For instance, propylmorpholino substitution led to a greater than 200 fold loss in affinity at CB₂ receptor when compared to the ethylmorpholino analogue. N1-methyl-tetrahydropyran (THP) and methyl-tetrahydrofuran (THF) were identified as privileged groups, conferring high CB₂ receptor affinity (and extremely high selectivity in the case of methyl-THF; e.g. 2.8 versus 2.9, Figure 19). The same study also demonstrated that non-aromatic groups can be utilised at the C3-acyl position, with retained CB₂ receptor affinity, with particular preference given to tetramethylcyclopropyl (e.g. 2.8, 2.9, Figure 19 and 2.13, Figure 21).

![Figure 19: High affinity, selective CB₂ receptor ligands containing methyl-THP or methyl-THF at N1.]

Two CB₂ receptor selective ligands (L-768,242 2.10 and L-759,787 2.11; Figure 20) were identified by swapping the N1 cyclic amino groups with the C3 acyl groups, further showing that non-aromatic groups are tolerated at C3. Huffman et al found that substitution of the C3 carbonyl with a CH₂ resulted in compounds with only slightly diminished CB₁ receptor affinity, implying that AAIs may interact with CB₁ receptor primarily by aromatic stacking, whilst the presence of a C3 carbonyl slightly enhances binding through hydrogen bonding. Variation of the C3 acyl to an amido group
demonstrated some sensitivity in this region to chemical change and led to identification of a high affinity, selective CB$_2$ receptor agonist 2.12 (Figure 20).\textsuperscript{251} Modifying the C3 acyl to an acetyl, by synthesis of a series of 3-phenylacetylindoles, led to unselective, moderate CB$_2$ receptor affinity compounds.\textsuperscript{252} However, the identification of high affinity CB$_2$ receptor selective ligands substituted at C3 with either an adamantylcarboxamide or adamantyloxalamide (e.g. 2.14; Figure 21), demonstrated that chemical change at the C3 position is somewhat tolerated.\textsuperscript{253}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig20.png}
\caption{CB$_2$ receptor selective ligands with varying N1 and C3 groups.\textsuperscript{249, 251}}
\end{figure}

Yates \textit{et al} have already explored extension and fluorescent dye conjugation at the C3 acyl position of the AI scaffold.\textsuperscript{254} Starting from the agonist JWH-015 2.4 (Figure 18), an amino group was substituted at the 3-position of the naphthoyl, to which glycine and then the fluorophore 7-NBD were conjugated. This approach was unsuccessful and resulted in a \textgreater 250 fold loss in affinity for CB$_2$ receptor compared to the parent pharmacophore and implied that there is a strict size limitation on the C3 acyl substituent critical for retaining affinity.
2.1.2 SAR of the C4-7 positions of indole

Iodopravadoline (AM630) \textbf{1.14} (Figure 21) was initially investigated for its ability to antagonise CB receptor agonist induced twitch response in the mouse vas deferens (MVD).\textsuperscript{255} Significant differences in the ability of AM630 \textbf{1.14} to antagonise different cannabinoids indicated the presence of multiple CB receptors in the MVD and that the primary receptor target of AM630 \textbf{1.14} may not be CB\textsubscript{1} receptor. AM630 \textbf{1.14} was subsequently confirmed as being CB\textsubscript{2} receptor selective and was found to act as an inverse agonist and antagonist at CB\textsubscript{2} receptor and a weak partial agonist at CB\textsubscript{1} receptor.\textsuperscript{135}

![Figure 21: CB\textsubscript{2} receptor selective ligands with bulky substitutions at the C5-7 positions of indole.\textsuperscript{135, 253, 256}](image)

The presence of an iodine atom at C6 in AM630 \textbf{1.14} is well tolerated with regards to CB\textsubscript{2} receptor affinity when compared with the uniodinated analogue pravadoline \textbf{2.1}. Substitution of bulky groups on the indole ring has been explored by Frost \textit{et al.}\textsuperscript{256} Indoles with tetramethylcyclopropyl at the C3 acyl and either ethylmorpholino or methyl-THP at N1, and with varying substitutions at C4-7 with either halogen atoms, CF\textsubscript{3}, SO\textsubscript{2}CH\textsubscript{3}, hydroxyl, methoxy, methanol, $O$-benzyl, NH\textsubscript{2}, alkylamines, $O$-butanol or $O$-butylbromine, nitrile, methoxymethane or phenyl groups were synthesised. In general, these substitutions were well tolerated, producing high CB\textsubscript{2} receptor affinity,
especially in the N1-methylTHP derivatives, e.g. 2.13 (Figure 21). In the hydroxyl substituted derivatives, C5-hydroxyl substitution provided the highest CB$_2$ receptor affinity (Table 2; entry 2), whilst C6 substitution gave the best selectivity (entry 3), whereas in the methoxy derivatives, 7 substitution provided the best CB$_2$ receptor affinity and selectivity (entry 8). In the O-benzyl derivatives, C6 substitution provided the highest CB$_2$ receptor affinity (entry 11), whereas C4 substitution was the most selective (entry 9). C5 substitutions appeared to impair CB$_1$ receptor affinity, in every instance but one, resulting in a decrease in CB$_1$ affinity when compared with the C6-substituted analogues (entries 6, 10, 13, 15, 17 and 19 cf. to 7, 11, 14, 16, 18 and 20).

Pasquini et al have also demonstrated that indole ring substitutions, such as furan in the high affinity CB$_2$ receptor selective inverse agonist 2.14 (Figure 21), can be tolerated. Small substitutions of hydroxyl, methoxy or methyl ester groups at C4-7 of the indole ring have also been demonstrated in a number of other CB$_2$ receptor ligands as well as in compounds 2.10 and 2.12 (Figure 20).
Table 2: Literature reported radioligand binding data for C4-7 substituted indoles from Frost et al.256

![Chemical Structure](image)

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a Data represent mean values from at least three experiments performed in duplicate. Kᵢ values determined using HEK293-hCB₂ or CHO-hCB₁ membranes and [³H]CP55,940 (Kᵢ values unreported).

b SI = selectivity index for CB₂ receptor, calculated by Kᵢ(CB₁)/Kᵢ(CB₂).

2.1.3 *In vitro* and *in vivo* effects of indole-based cannabinoids

Indole-based cannabinoids with a range of functions have been developed, including agonists, inverse agonists, antagonists and neutral antagonists. Of these, the most
commonly identified have been agonists, which could be attributed to the increased interest in cannabinoid receptor agonists driven by drug discovery efforts.\textsuperscript{113} CB receptor agonist WIN 55,212-2 \textsuperscript{2.2} has been shown to increase intracellular calcium, through CB\textsubscript{1} receptor coupling to G\textsubscript{q}, independent of other G proteins.\textsuperscript{259} The less potent \textit{S}-enantiomer, WIN 55,212-3 has been shown to act as a competitive neutral antagonist at CB\textsubscript{2} receptor and an inverse agonist at CB\textsubscript{1} receptor.\textsuperscript{260} WIN 55,212-2 \textsuperscript{2.2}, as well as some other AI cannabinoid agonists can elicit off-target effects through interaction with AI-sensitive GPCRs and microtubules, binding to which has been shown to enhance the anti-proliferative effects of AI cannabinoids.\textsuperscript{261} It is necessary to put any \textit{in vivo} effects into context by examining the possibility of off-target effects. The most promising \textit{in vivo} effect observed with AI-based CB\textsubscript{2} receptor ligands is analgesia, for example AI CB\textsubscript{2} receptor selective agonist AM1241 (\textit{K}\textsubscript{i} = 7.1 nM at mCB\textsubscript{2} receptor, 580 nM at rCB\textsubscript{1} receptor) inhibits pain through CB\textsubscript{2} receptor binding, independent of CB\textsubscript{1} receptors in the CNS.\textsuperscript{262} The high affinity CB\textsubscript{2} receptor selective agonist A-796260 has demonstrated efficacy in inflammatory, post-operative, neuropathic and osteoarthritic pain models.\textsuperscript{112}

\subsection*{2.1.4 Conclusions on literature SAR of the indole scaffold}

A fluorescent dye is very bulky and so it is important that the position of linker and dye attachment on the core ligand or pharmacophore is tolerant to chemical change. Existing SAR around N1 substituents of the indole scaffold shows quite clearly that there is a strict size and length limitation in this region (Figure 22).\textsuperscript{238, 248} In comparison, the chemistry and size of C3 groups may be slightly more amenable to change.\textsuperscript{248, 253} However, Yates \textit{et al} have already explored extension and fluorescent dye conjugation at the C3 acyl position of the AI scaffold, which was unsuccessful.\textsuperscript{254} In addition, tolerability of substitution at the C2 position is limited to methyl groups.\textsuperscript{239}
Therefore, the only region of the indole scaffold left for possible extension and development into a fluorescent dye is C4-7. This portion of the indole ring is amenable to the substitution of some bulky groups\textsuperscript{253, 256} and this region is as yet unexplored with regards to development of longer linkers and fluorescent ligands. It is also worth noting that there are also numerous high affinity CB\textsubscript{2} receptor ligands without substituents at C4-7,\textsuperscript{248, 263} demonstrating that C4-7 substituents are not necessary for binding and therefore extension at this region may avoid disturbing key binding regions.
2.2 Fluorescent ligand design

2.2.1 Core ligand

Various N1-alkyl or -aminoalkyl R¹ and C3-acyl R² groups were selected (Figure 23) based on previously reported optimal CB₂ receptor affinity and selectivity as discussed in section 2.1.1. At the N1 (R¹) position, the ethylmorpholino group was selected as a substituent due to its presence in multiple CB₂ receptor ligands, such as AM630 1.14. In addition, methyl-THP and methyl-THF were selected due to the high CB₂ receptor affinity and selectivity conferred by these groups (e.g. 2.8 and 2.9, Figure 19).  

![Figure 23: A library of fluorescent compounds were designed based on various combinations of R and X groups.](image)

For ease of synthesis methyl substitution was not used at C2, as the SAR shows this methyl to be non-essential for CB₂ receptor affinity and selectivity with in some cases C2-H being preferred. A range of functional groups are tolerated at C3, including, acyl, aminoalkyl, amido, carboxamide and oxalamide. In this series an acyl group was utilised as it is present in AM630 1.14 and has well characterised SAR being the most pervasive C3 functionality amongst CB₂ receptor ligands.
At the C3 acyl position (R²; Figure 23), the 4-methoxyphenyl moiety of AM630 1.14 was selected as an R² substituent, as well as the 1-naphthoyl utilised in ligands such as WIN 55,212-2 2.2 and JWH-015 2.4. In an attempt to sample non-aromatic groups at the C3 acyl, a cyclohexane was also selected as an R² substituent, as it has been shown to offer reasonable CB₂ receptor affinity²⁴⁸ and the requisite starting materials are easily available. As tetramethylcyclopropyl substituents at the C3 acyl position (e.g. 2.8, 2.9, Figure 19 and 2.13, Figure 21) have shown high CB₂ receptor affinity in combination with numerous different N1 groups,²⁴⁸,²⁵⁶ utilising tetramethylcyclopropyl in this series was initially of interest. However, the synthetic route determined (discussed below in 2.3.1) required the nitriles of substituents for C3 acylation. As the tetramethylcyclopropyl nitrile was not commercially available and synthesis of it would be time consuming, expensive and require unstable reagents,²⁶⁴ it was not considered as a substituent in this series.

### 2.2.2 Linker

In this series, substitution of linkers was explored at the C5 position, as well as an analogue at the C6 position. Existing SAR shows that substitution at these positions generally provides high CB₂ receptor affinity, while substitution at C5 impairs CB₁ receptor affinity ²⁵⁶ and could therefore be anticipated to result in CB₂ receptor selective ligands. A linker is usually employed in the design of a fluorescent ligand to create separation between the fluorescent dye and the core ligand, to help reduce fluorophore impact on ligand efficacy and affinity. In this series, a ‘mini-linker’ was first installed on the pharmacophore as a means to introduce a carboxylic acid handle for coupling with amine linkers (Figure 23).

Three different linkers were included in the compound series: an alkyl chain (1,8-diaminooctane), a polyethylene glycol (PEG) chain (1,8-diamino-3,6-dioxaoctane) and a short peptide (Ala-Ala-ethylamine; Figure 23). These linkers were chosen with the aim of generating tools with varied polarity for studying CB₂ receptor with the diacetylated (i.e. AcNHXNHAc) alkyl chain, PEG chain and short peptide linkers
having calculated (c)LogP values of 0.54, -2 and -3, respectively.†† These cLogP values do not represent the linker-indole conjugates as a whole, but provide an indication of the rank lipophilicity of the linker components in isolation. Chain length was preserved at 10 atoms in each linker in order to allow evaluation of the effect of linker chemistry on CB₂ receptor affinity and activity. It was expected that the alkyl chain would deliver ligands with higher affinity for CB₂ receptor due to greater lipophilicity, as favoured in cannabinoids. It is possible that the lipophilicity of the linker could affect ligand entry into CB₂ receptor as it is postulated that cannabinoids gain entry via the lipid membrane.⁹⁰ This might potentially be more likely for the lipophilic alkyl linked conjugates than the PEG or peptide linked derivatives. In contrast, the hydrophilic short peptide was anticipated to produce a probe with improved physicochemical properties via decreased overall compound hydrophobicity, therefore reducing non-specific membrane binding which is typically problematic in GPCR fluorescent ligands.¹⁹⁹ This approach of using peptide linkers has been successfully demonstrated with A₃ adenosine receptor fluorescent ligands.¹⁹⁸ Di-alanine was chosen as the peptide linker, to be synthesised from the naturally occurring L-alanine, which has more affordable commercial availability than the D-isomer. The fluorescent ligands were designed as tools for in vitro use. However, in the event of identifying a good fluorescent ligand amongst the peptide-linked conjugates, it could potentially be resynthesised with the more metabolically stable D-alanine to facilitate in vivo use. The PEG linker was expected to provide a balance between the alkyl and peptide linkers, having moderate lipophilicity and therefore retaining affinity, but with diminished non-specific binding.

2.2.3 Fluorescent dye

The fluorophore selected can have a huge impact on the overall ligand properties and its use as an imaging tool. The fluorescent ligand design utilised here, where the dye is attached to a linker moiety, is highly versatile, as a promising linker-indole conjugate could be derivatised by attachment of different dyes, therefore varying physicochemical properties and excitation and emission wavelengths. This is in contrast to some published CB₂ receptor fluorescent ligand designs,²⁰¹ where the fluorophore is

†† cLogP values calculated using Crippen's fragmentation,²⁶⁵ in ChemDraw Prime 15.1, PerkinElmer Informatics, Inc
incorporated into the pharmacophore and so cannot be altered without a complete redesign of the compound. Fluorophore lipophilicity is an important consideration as this can affect non-specific membrane binding and cell accumulation. It is also advantageous in a GPCR probe that the fluorophore is quenched when the ligand is in an aqueous environment, but not when bound to the receptor or membrane.\textsuperscript{192}

\[ \text{Figure 24: Structure of BODIPY 630/650-X-OSu 2.15} \]

BODIPY 630/650-X-OSu 2.15 was chosen as the fluorescent dye (Figure 24). The commercially available succinimidyl ester can easily be reacted with a primary terminal amine of a linker to form a chemically stable peptide bond. This reaction is easier to control in comparison with reactions at some of the other dye groups such as isothiocyanates and sulfonyl chlorides.\textsuperscript{266} BODIPY 630/650-X-OSu 2.15 is a popular choice in fluorescent ligand design and has been successfully utilised in the design of other Class A GPCR fluorescent probes.\textsuperscript{194, 198, 207} BODIPY 630/650-X-OSu 2.15 also benefits from high photo and chemical stability, intense absorption and good fluorescence quantum yield while the red-emitting wavelength means minimal detection interference from cellular autofluorescence.\textsuperscript{215}
2.3 Synthesis

2.3.1 Synthesis of core ligand

2.3.1.1 N1-alkylation with R¹ groups

All compounds were synthesised from the commercially available 5- or 6-benzyloxyindole (2.18a-b; Scheme 1). N-alkylation of 2.18a-b required the mesylates (2.17a-c) of the requisite R¹ groups, which were prepared from the commercially available alcohols (2.16a-c, Scheme 1, step i) and used without further purification. Initially, mesylation of 4-(2-hydroxyethyl)-morpholine 2.16a with methanesulfonyl chloride and triethylamine was attempted in dry THF. However, the reaction was not successful and ¹H NMR confirmed that the starting material 4-(2-hydroxyethyl)-morpholine 2.16a was still present and that the expected singlet peak of the methyl in product 2.17a was missing. It was thought that perhaps the THF had not been completely dry and that presence of water had either resulted in no reaction occurring or led to formation of mesic acid instead of the R¹-mesylate 2.17a. Therefore, the mesylation reaction to form 2.17a was repeated using a slightly different procedure, which utilised dry dichloromethane (DCM), a much less hygroscopic solvent than THF. Under these conditions, formation of 2.17a was successful and the same reaction conditions were repeated to form 2.17b and 2.17c (Scheme 1, step i). Triethylamine hydrochloride salt formed as a by-product of the mesylation reaction and was sparingly soluble in DCM (but not THF) so it was not completely filtered out before reaction of 2.17a-c with the 5- or 6-benzyloxyindoles 2.18a-b. Therefore, a quantitative yield was assumed for R¹-mesylates 2.17a-c and they were used the following day in the next step due to poor long term stability.

Indoles 2.18a-b were N-alkylated with R¹-mesylates 2.17a-c using sodium hydride in dimethylformamide (DMF) to give 2.19a-d (Scheme 1, step ii). As the triethylamine hydrochloride salts were not soluble in DMF, a wide gauge needle (to prevent needle blockage) was used when adding the mesylates 2.17a-c to the benzyloxyindoles 2.18a-b (whilst taking care to avoid excessive addition of salt). Alkyl-indoles 2.19a-d were
purified by flash silica chromatography and purification yields were correlated to the polarity of the product. The most non-polar 2.19c (retention factor (Rf) 0.71, 1:2 petroleum ether (PE)/EA; 66%) eluted close to the non-polar fraction of unreacted starting material 5-benzyloxyindole 2.18a (Rf 0.81, 1:2 PE/EA), making separation more difficult, whereas 2.19d (Rf 0.63, 1:2 PE/EA; 70%) and the most polar 2.19a (Rf 0.17, 1:2 PE/EA 76%), were more easily isolated in pure form. However, the 6-benzyloxy analogue, 2.19b had the lowest purified yield (54%), possibly due to the large scale synthesis, making work up of the reaction more arduous.

Scheme 1: Synthesis of core ligand. Reagents and conditions: (i) MsCl, Et$_3$N, DCM, room temperature (rt), 2 h, quantitative; (ii) NaH, DMF, 45°C, 2 h, 54-76%; (iii) 1,10-phenanthroline, acetone, rt, 1 h, 57%; (iv) R$_2$-nitrile, H$_2$O, CH$_3$COOH, 1,4-dioxane, 140°C, 42 h, 18-83%; (v) Pd/C, H$_2$, EtOH, EA, rt, 20 h, 12-67%.
2.3.1.2 C3-acylation with R² groups

Friedel-Crafts acylation has commonly been used for C3 acylation of the indole class\textsuperscript{257} however, this reaction typically uses hazardous reagents and generates large quantities of acidic and aluminium containing waste, as well as being prone to side reactions.\textsuperscript{269} There have been several recently reported attempts at developing cleaner, safer methods for acylation of indoles, for example a solvent-free acid catalysed acylation method utilising 1,3-diones.\textsuperscript{270} Alternatively, another recently reported procedure\textsuperscript{271,272} utilises [(Phen)Pd(OAc)\textsubscript{2}] (2.21) to catalyse the addition of nitriles to indoles, providing 3-acylindoles. This latter method was chosen to acylate N-alkylindoles \textbf{2.19a-d}, due to the thorough validation of the reaction, which was reported with a range of nitrile substituents and varyingly substituted indoles.\textsuperscript{272} The palladium-ligand catalytic complex \textbf{2.21} was pre-formed\textsuperscript{273} (57\% yield) from Pd(OAc)\textsubscript{2} \textbf{2.20} and 1,10-phenanthroline (Scheme 1, step iii) rather than generated \textit{in situ} as this was reported to give superior acylation yields.\textsuperscript{272}

In the mechanism put forward by Jiang and Wang (for indoles without N-substituents),\textsuperscript{271} acetic acid protonates the carbon-carbon double bond at C3 of the starting indole, which then forms the iminium salt, before palladation by the catalyst (Scheme 2, steps i and ii). The nitrile coordinates to the cationic complex (step iii) and then forms a ketimine complex via carbopalladation of the nitrile (step iv). Protonation releases the free ketimine, regenerating [(Phen)Pd(OAc)\textsubscript{2}] \textbf{2.21} (step v). Finally, hydrolysis of the ketimine yields the 3-acylindole (step vi).
The reaction mechanism suggests acylation occurs at C3 as this is the favoured position for protonation of indoles. Proof that acylation occurred at C3 of 2.19a-d was determined using two dimensional (2D)-NMR experiments that were carried out on several of the series and is discussed here using the example of 5-(benzyloxy)-3-(4-methoxybenzoyl)-1-[(oxolan-3-yl)methyl]-1H-indole 2.22h (Figure 25), which was formed by acylation of 2.19d.
Figure 25: 5-(Benzyloxy)-3-(4-methoxybenzoyl)-1-[(oxolan-3-yl) methyl]-1H-indole 2.22h, with HMBC or NOESY spectra correlations (red arrows).

Homonuclear correlation spectroscopy (COSY) and heteronuclear single quantum coherence spectroscopy (HSQC) NMR experiments assisted in the partial assignment of the $^1$H NMR and $^{13}$C NMR spectra of 2.22h. The heteronuclear multiple bond correlation (HMBC) NMR spectrum corroborated these assignments and was used to identify the remaining unassigned peaks and along with the nuclear Overhauser effect spectroscopy (NOESY) spectrum was used to confirm acylation at indole-C3 (see appendix for spectra, section 8).

The HMBC spectrum of 2.22h showed a strong cross peak across three bonds between the N-CH$_2$ protons and C2 carbon and as it is the only protonated indole carbon coupled to the N-CH$_2$ protons, it must indeed be C2 (Figure 25). A cross peak was also present between C2-H and the three closest quaternary carbons of the indole (C3, C3a and C7a) as well as long range cross peaks across four bonds to C4 and C7. Crucially, the C2 proton is the only indole proton to correlate with the downfield carbonyl carbon at 189.9 ppm. The carbonyl carbon also shows a cross peak with the proximal benzoyl aromatic protons (C2"-H and C6"-H). Therefore, the HMBC correlations for 2.22h, cement C2 as being located directly adjacent to the acyl group, confirming that C3 must have been acylated.
The NOESY spectrum of 2.22h showed that the C2-H and C4-H of the indole are in close proximity to benzoyl aromatic protons (C2''-H and C6''-H; Figure 25). There are also NOE correlations between C2-H and the N-CH2 protons as well as with C2'-H and C3'-H of the oxolane. This confirms that C2-H of indole is located between the N-alkyl and the acyl group and that acylation has occurred at C3.

Indoles 2.19a-d were reacted in a pressure tube with the requisite R2-nitrile and catalytic [(Phen)Pd(OAc)2] 2.21 in glacial acetic acid, H2O and 1,4-dioxane to give 2.22a-i (Scheme 1, step iv). Products were isolated with a generally robust yield (18-83%). The reaction conditions used successfully converted the majority of starting material to product, for example, the crude 1H NMR spectra of 2.22a, showed a 5:1 mixture of 2.22a and 2.19a. However, separating 2.22a, 2.22c and 2.22d from the small amount of unreacted 2.19a proved difficult due to similar Rf values and often required either multiple column purifications, or recrystallisation after column purification, leading to reduced isolated yield following purification of derivatives 2.22a (51%) and 2.22c (18%). Compound 2.22d showed a high reaction yield in the crude 1H NMR spectra, but could not be isolated from the starting material and was taken forward as a 4:1 mixture of desired product 2.22d and starting material 2.19a.

### 2.3.1.3 Hydrogenolysis of O-benzyl

The benzyl ether of 2.22a-f and 2.22h was cleaved using palladium on carbon and hydrogen (balloon) to reveal hydroxyindoles 2.23a-g (Scheme 1, step v). Hydrogenolysis of the benzyl ethers of 2.22g and 2.22i was not attempted and these two compounds were not progressed to pharmacological evaluation. The option of resuming development was kept open if SAR results indicated that the combination of R groups in 2.22g and 2.22i might be favourable. Poor conversion rates of starting material to product led to generally disappointing isolated yields (2.23a-d, 2.23f and 2.23g 12-39%). Strategies recognised to improve hydrogenolysis, such as a small amount of glacial acetic acid and fast stirring to improve mixing of phases, were trialled but had minimal effect. However, successful reaction optimisation was later achieved when larger quantities of 2.23e were required (for synthesis of compounds discussed in Chapter 3) and made multiple repeated syntheses necessary. Hydrogenation of 2.22e
using a 5:2 solvent mixture of CHCl$_3$ and EtOH, instead of 2:1 EtOH and ethyl acetate (EA), as well as utilising a three-way tap for vacuum and hydrogen balloon connections, rather than a rubber bung and needle system, led to an increased yield of 2.23e from 17 to 67%. It has been previously shown that the velocity of hydrogenation reactions is effected by solvent choice with the reaction rate being faster in chloroform than in ethanol, possibly due to the decreased viscosity of chloroform improving transport of dissolved hydrogen to catalyst.\textsuperscript{274} The three-way tap may have improved conversion of starting material to product by helping to maintain the hydrogen atmosphere better than that facilitated by a needle connection. Another option to increase reaction yields could be use of a Parr apparatus with higher hydrogen gas pressure or utilising a different catalyst, such as Pearlman’s catalyst (Pd(OH)$_2$/C), rhodium or Raney nickel catalysts.\textsuperscript{275} Several of the hydroxyindoles were purified by precipitation, which also reduced the isolated yield obtained, but gave high purity and enough material for progression to the next step.

Hydroxyindoles 2.23a-g were all evaluated for CB$_2$ receptor binding (see section 2.4.1), however, not all of them were progressed further through the synthetic route. Three compounds (2.23a, 2.23c, 2.23e) were selected for progression, all of which possessed both well characterised N1 ($R^1$) groups (ethylmorpholino and methyl-THP) and C3-acyl ($R^2$) groups ($p$-methoxyphenyl and 1-naphthalene).

### 2.3.2 Linker and fluorophore conjugation

Hydroxyindoles 2.23a, 2.23c and 2.23e were alkylated using \textit{tert}-butylbromoacetate via a Williamson ether reaction, thereby installing a short spacer moiety (2.24a-c; 45-76%; Scheme 3, step i). Subsequent cleavage of the \textit{tert}-butyl using trifluoroacetic acid (TFA) gave the 5-\textit{O}-ethanoic acids 2.25a-c (Scheme 3, step ii), which all underwent pharmacological evaluation (see section 2.4.1).
Scheme 3: Attachment of linker and fluorophore to the core ligand. Reagents and Conditions: (i) tert-butylbromoacetate, NaH, DMF, 60°C, 3 h, 45-76%; (ii) TFA, DCM, rt, 1 h, quantitative; (iii) 1. Fmoc-Ala-Ala-trityl resin (2.26, see Scheme 4), piperidine, DMF, 2. HATU, DIPEA, DMF, 3. TFA, DCM, rt, 1 h, 27-30%; (iv) Boc₂O, dioxane or DCM, 0°C, 5–15 h, 69%; (v) HATU, DIPEA, DMF, rt, 12 h, 50-94%; (vi) TFA, DCM, rt, 1 h, 27-77%; (vii) Ac₂O, DCM, rt 1 h, 97%. (viii) BODIPY 630/650-X-OSu 2.15, DIPEA, DMF, rt 12 h, 36-88%.
The 5-O-ethanoic acids 2.25a-c were coupled to three different linkers, either N-Boc-1,8-diaminooctane (2.28a), N-Boc-1,8-diamino-3,6-dioxaoctane (PEG linker; 2.28b) or Ala-Ala-1,2-diaminoethane-resin-bound (9-fluorenylmethoxycarbonyl (Fmoc) deprotected 2.26). tert-Butoxycarbonyl (Boc)-protected linkers 2.28a and 2.28b were prepared at a 69% yield from either mono-Boc protection of 1,8-octanediamine 2.27a or 1,8-diamino-3,6-dioxaoctane 2.27b respectively (Scheme 3, step iv). Dilute addition of 0.2 equivalents (eq.) of di-tert-butyl dicarbonate (Boc2O) via a pressure equalised dropping funnel over a prolonged period with vigorous stirring minimised di-Boc protection.

Solid-phase peptide synthesis (SPPS) was used to prepare the resin-bound dipeptide linker 2.26. Fmoc-Ala-OH was pre-activated with HBTU and N,N-diisopropylethylamine (DIPEA), thereby preventing guanidine side products forming between HBTU and the resin-bound amine (Scheme 4; step 1). HBTU is a commonly used activating reagent in SPPS, providing reduced racemisation and good peptide coupling efficiency. Coupling of the activated ester of Fmoc-Ala with 1,2-diaminoethane trityl resin was performed twice to maximise yield and was followed by capping of unreacted resin amines with acetic anhydride (Scheme 4; steps 2 & 3). An Fmoc loading test was performed to calculate the degree of resin substitution. Fmoc cleavage to reveal the amine was followed by a second coupling of activated Fmoc-Ala to give the Fmoc protected, resin-bound dipeptide 2.26 (Scheme 4; steps 4 & 5), after which another Fmoc loading test was performed.
Scheme 4: SPPS of dipeptide linker 2.26. Reagents and conditions: (1) HBTU, DIPEA, DMF, added as a solution to (2); (3) Ac₂O, DIPEA, DMF; (4) piperidine, DMF; (5) DIPEA, DMF.

Fmoc cleavage of 2.26 and on-resin coupling to 2.25a-c using HATU and DIPEA was followed by resin cleavage with TFA to give primary amines 2.30e-g (Scheme 3, step iii). Time limitations resulted in only 2.25a and 2.25b being derivatised with linkers 2.28a and 2.28b, which was achieved using HATU and DIPEA in DMF to give 2.29a-d, which were purified by column chromatography to give typically good yields (50-94%; Scheme 3; step v). Boc-deprotection of 2.29a-d using TFA yielded primary amines 2.30a-d (Scheme 3, step vi) Small quantities of the crude amines 2.30a-g were purified by semi-preparative RP-HPLC (reverse phase-high performance liquid chromatography) in order to ensure maximum purity before dye conjugation. Therefore, the percentages reported for the Boc-deprotection (2.30a-d: 27-77%) and synthesis of 2.30e-g (27-30%) are reflective of the small amount of product purified using semi-preparative RP-HPLC and not the overall reaction conversion.
Coupling with BODIPY 630/650-X-OSu 2.15 gave 2.32a-g which were also purified by semi-preparative RP-HPLC (36-88%; Scheme 3; step viii). The small quantities of 2.30a-g purified meant that only small excesses of 2.30a-g (1.4 – 2.1 eq., av. 1.7 eq.) relative to mmol of dye could be used, which may have reduced the reaction yields. In addition, purification of 2.32g was very challenging due to the close elution of 2.32g to an unidentified peak (possibly hydrolysed BODIPY 630/650-X or an impurity present in the commercial dye) by semi-preparative RP-HPLC, resulting in a significantly reduced isolated yield (36%).

The final fluorescent ligands 2.32a-g, as well as the pharmacophore-linker conjugates 2.30a-g were pharmacologically evaluated (see section 2.4). Additionally, the amine 2.30b was acetylated to give 2.31 (Scheme 3; step vii), which also underwent pharmacological testing to allow for evaluation of the effect of the primary amine on binding. All compounds pharmacologically tested had >95% purity as determined by analytical RP-HPLC.

### 2.3.3 Synthetic summary

The aim of this synthesis was to generate a small library of compounds exploring varying groups at N1 (R1) and C3 (R2), of which selected derivatives would have a linker and fluorophore appended. The starting indoles 2.18a-b possessed an O-benzyl group at C5 or C6 to allow for linker substitution at these positions. The synthetic route started with N-alkylation of the indole, followed by acylation at C3. Cleavage of the O-benzyl group revealed an alcohol which was reacted with a brominated mini-linker. The tert-butyl group of the mini-linker was cleaved to reveal a carboxylic acid for coupling with a mono-Boc protected diamine linker or a peptide linker. Finally, BODIPY 630/650-X-OSu 2.15 was coupled to the deprotected amine of the linker to give the final fluorescent ligand.
2.4 Pharmacological evaluation

Seven derivatives of 5- or 6-hydroxyindoles (2.23a-g) representing the core pharmacophore, three 5-O-ethanoic acid substituted derivatives (2.25a-c), seven pharmacophore-linker conjugates (2.30a-g), one acetylated pharmacophore-linker derivative (2.31) and seven final fluorescent ligands (2.32a-g) were subjected to pharmacological evaluation. Radioligand binding assays were carried out to determine affinity at hCB₂ and hCB₁ receptor. Compounds with high affinity at CB₂ receptor were then tested in cAMP assays to evaluate function (refer to section 1.6). All pharmacological assays were carried out by Anna Cooper, except where noted specifically. Refer to section 7.2 for a detailed list of experimental contributions by others, including membrane preparations.

2.4.1 Radioligand binding assays

2.4.1.1 Competition binding screens at 10 µM

A competition binding screen was used to identify which compounds could displace more than 50% of bound [³H]CP55,940 1.17 at hCB₂ and hCB₁ receptors at a fixed concentration of 10 µM. In all binding assays (including concentration response assays in 2.4.1.2), there were fixed concentration controls of unlabelled CP55,940 1.10 (10 µM) as a homologous competitor to determine maximum specific binding which, together with vehicle control, defined the specific binding window.

Six of the seven hydroxyindole compounds (2.23a, 2.23c-g) showed close to or greater than 50% displacement of [³H]CP55,940 1.17 at CB₂ receptor (Figure 26). The O-ethanoic acid derivative 2.25c showed high displacement (80%) at CB₂ receptor and two fluorescent ligands (2.32b and 2.32g) reached close to 50% displacement of radioligand at CB₂ receptor.
Figure 26: Competition binding screen at CB₂ receptor showing displacement of [³H]CP55,940 (2.5 nM) at HEK293 hCB₂ (10 µg/point) membranes by a fixed concentration (10 µM) of test compound. Raw data was expressed in corrected counts per minute (cpm) and normalised to unlabelled CP55,940 (100%) and vehicle control (0%). Data shown is the mean value ± standard error of the mean (SEM) of three independent experiments conducted in triplicate. The dashed line indicates the 50% displacement criteria for progression to concentration response assay.

6-Hydroxyindole 2.23b, 5-O-ethanoic acids 2.25a-b, pharmacophore-linker conjugates 2.30a-g and 2.31 and fluorescent ligands 2.32a, 2.32c-f all had lower levels of displacement of [³H]CP55,940 1.17 at hCB₂ receptor (Figure 26). This means that these compounds are unlikely to have high enough affinity to be able to accurately determine a $K_i$ value. Therefore, statistical analysis was used to determine whether CB₂ receptor binding occurred. The percentage displacement values (n = 3, in triplicate) of 2.23b, 2.25a-b, 2.30a-d, 2.31, 2.32d-f at CB₂ receptor all passed the D’Agostino & Pearson normality test and showed significant difference from the vehicle control (0%) in a one sample $t$-test and were thus determined to have a $K_i$ value >10 µM at CB₂ receptor (Table 3, Table 4). The percentage displacement values (n = 3, in triplicate) of 2.30e-g, 2.32a and 2.32c all passed the D’Agostino & Pearson normality test, but did not show significant difference from the vehicle control (0%) in a one sample $t$-test and were therefore determined to not show significant binding to CB₂ receptor (Table 3, Table 4).
Three 5-hydroxyindoles (2.23c, 2.23e and 2.23f) showed greater than 50% displacement of [3H]CP55,940 1.17 at CB₁ receptor (61%, 74% and 78% respectively; Figure 26). The 5-hydroxyindoles 2.23a, 2.23d and 2.23g, 6-hydroxyindole 2.23b and 5-O-ethanoic acids 2.25a-c all showed less than 50% displacement of [3H]CP55,940 1.17 at CB₁ receptor, so the D’Agostino & Pearson normality test and a one sample t-test were used to determine whether significant CB₁ receptor binding had occurred (Table 3). CB₁ receptor binding of 2.30a-g, 2.31 and 2.32a-g could not be determined with statistical analysis, as n = 1 for these compounds. Repeat 10 µM binding screens were not carried out for 2.30a-g, 2.31 and 2.32a-g as they showed low displacement in the initial screen and were therefore a low priority for pharmacological testing.

Figure 27: Competition binding screen at CB₁ receptor, showing displacement of [3H]CP55,940 (2.5 nM) at HEK293 hCB₁ (7.5 µg/point) membranes by a fixed concentration (10 µM) of test compound. Raw data was expressed in ccppm and normalised to unlabelled CP55,940 (10 µM) (100%) and vehicle control (0%). Data shown is the mean value ± SEM of three independent experiments conducted in triplicate, except for 2.30a-g, 2.31 and 2.32a-g, where data is mean ± SEM of one experiment conducted in triplicate.
2.4.1.2 Concentration response assays

Compounds that displaced approximately 50% or more of bound $[^3]$HCP55,940 1.17 at hCB$_2$ (2.23a, 2.23c-g, 2.25c, 2.32b and 2.32g) or hCB$_1$ (2.23c, 2.23e-f) receptors were subjected to concentration response competition binding assays to determine $K_i$ values (Table 3).

![Figure 28: Competition binding curve for SR144528 1.13 at hCB$_2$ receptor, determined using $[^3]$HCP55,940 (2.5 nM, $K_d$ = 3 nM) and HEK293-hCB$_2$ membranes (10 µg/point). Data is representative of one independent experiment conducted in triplicate and data shown are the mean ± SEM.](image)

The binding affinities of CB receptor inverse agonist SR144528 1.13 at CB$_2$ and CB$_1$ receptors were also determined as a literature compound control. SR144528 1.13 displayed a $K_i = 51.0 \pm 3.0$ nM at CB$_2$ and $K_i = 5682 \pm 2890$ nM at CB$_1$ receptor with a selectivity of 110-fold for CB$_2$ receptor (Figure 28, Table 3). All the binding affinities reported here were calculated using the $[^3]$HCP55,940 1.17 $K_d$ values (3 nM at CB$_2$ and 2 nM at CB$_1$ receptor) previously determined by the Glass laboratory, where pharmacological evaluation was carried out (refer to section 1.6.1). Varying $K_d$ values for $[^3]$HCP55,940 1.17 at hCB$_2$ and hCB$_1$ receptors have been reported in the literature and so it is important to note what the $K_d$ value is when comparing results from different studies. The binding affinities determined in this study are comparable to the recently reported$^{277}$ SR144528 1.13 $pK_i = 7.88 \pm 0.06$ at hCB$_2$ ($K_i = 13.2$ nM) and $pK_i =$
5.77 ± 0.09 at hCB₁ receptor ($K_i = 1698.2$ nM), with a selectivity of 129-fold for CB₂ receptor. The literature study used $[^3]H$CP55,940 1.17 $K_d$ values of 0.33 nM at hCB₂ and 0.1 nM at hCB₁ receptor. If these $[^3]H$CP55,940 1.17 $K_d$ values are used with the raw data in this study, the recalculated CB₂ receptor $K_i$ value (10.9 nM) is very close to the literature reported value (13.2 nM). This validation of the binding assay gives confidence in the $K_i$ values reported for the compounds tested.

The 5-hydroxyindole 2.23g and the fluorescent ligands 2.32b and 2.32g showed low affinity at hCB₂ receptor ($K_i = 22.3$ μM, 1.9 mM and 48.7 μM, respectively; all $n = 1$) and did not approach a plateau for maximum radioligand displacement at the highest concentration (126.4 μM; Figure 29). Thus, an accurate $K_i$ value could not be calculated, so concentration response assays were not repeated for these compounds and 2.23g, 2.32b and 2.32g were all determined to have a $K_i > 10$ μM at hCB₂ receptor (Table 3, Table 4), using the D’Agostino & Pearson normality test and one sample $t$-test as described for other low affinity compounds.

Figure 29: Competition binding curves for 2.23g, 2.32b and 2.32g at hCB₂ receptor, determined using $[^3]H$CP55,940 (2.5 nM, $K_d = 3$ nM) and HEK293-hCB₂ membranes (10 μg/point). Data is representative of one independent experiment conducted in triplicate and data shown are the mean ± SEM. CP55,940 (10 μM) was a homologous competitor to determine specific binding.
Table 3: Affinity of 5- and 6-hydroxyindoles and 5-O-ethanoic acid derivatives for hCB\textsubscript{2} and hCB\textsubscript{1} receptors. \textsuperscript{a}

![Chemical structures](image.jpg)

<table>
<thead>
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<th>Compound</th>
<th>R\textsuperscript{1}</th>
<th>R\textsuperscript{2}</th>
<th>hCB\textsubscript{2} receptor</th>
<th>hCB\textsubscript{1} receptor</th>
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<td>(10 \mu M \text{ displ.}) [(^3\text{H})CP\textsubscript{1.17} \text{d}] (% ± SEM)</td>
<td>(K\text{\textsubscript{i}} \text{e} ) (nM ± SEM)</td>
<td>(10 \mu M \text{ displ.}) [(^3\text{H})CP\textsubscript{1.17} \text{d}] (% ± SEM)</td>
<td>(K\text{\textsubscript{i}} \text{e} ) (nM ± SEM)</td>
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<tr>
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<td>SR144528</td>
<td>Me-OPh</td>
<td>80.0 ± 5.6</td>
<td>1660 ± 114</td>
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</table>

\(\text{SI} = K\text{\textsubscript{i} (CB\textsubscript{1})}/K\text{\textsubscript{i} (CB\textsubscript{2})}\).
The 5-hydroxyindole 2.23e had the highest CB2 receptor affinity ($K_i = 120.1 \text{ nM} \pm 23.4$) of all the compounds and was followed by the 5-hydroxyindoles 2.23f, 2.23a and 2.23d (Figure 30; Table 3). 6-Hydroxyl substitution resulted in a $>13$ fold loss in CB2 receptor affinity (2.23b) when compared with the 5-hydroxyl analogue (2.23a) (Table 3). Only one C6 substituted derivative (2.23b) was synthesised (possessing ethylmorpholino at R1 and methoxyphenyl at R2), so a general trend cannot be extrapolated from these results. It is possible that the 5-hydroxyl derivative 2.23a was able to form more favourable interactions with CB2 receptor. Frost et al found 6-OMe substitution gave a $>6$ fold loss in CB2 receptor affinity in comparison to the 5-OMe analogue in a series also possessing CB2 receptor affinity in comparison to the 5-OMe analogue in a series also possessing ethylmorpholino at R1, but with tetramethylcyclopropyl at R2, though this trend was not observed with the 5/6-O-benzyl and 5/6-OH analogues. The low CB2 receptor affinity observed for 2.23b was particularly surprising given that it is an analogue of AM630 1.14, which has very high affinity for CB2 receptor. These two compounds only differ by the C6-substituents (hydroxyl in 2.23b, iodine in AM630 1.14) and the C2-Me present in AM630 1.14. A key difference between the binding of 2.23b and AM630 1.14 to CB2 receptor, could be the ability of AM630 1.14 to participate in intermolecular halogen bonding with amino acid residues. Halogen bonding is orthogonal to hydrogen bonding and thus can form without interfering with the intramolecular hydrogen bonding of CB2 receptor and local residue conformation. Whilst halogen bonds are weak, they are highly specific and iodine substitutions in particular have been shown to have a large effect on affinity across a broad spectrum of targets.

The three derivatives with the 5-hydroxy and 4-methoxyphenyl R2 groups held constant, but with varying R1 groups, revealed that 4-methyl-THP (2.23e) had higher affinity for CB2 receptor compared to 4-ethylmorpholino (2.23a) and 3-methyl-THF (2.23g). Frost et al also found that for 5-hydroxyindole compounds 4-methyl-THP at R1 provides a higher $K_i$ at CB2 receptor than ethylmorpholino (when in combination with a tetramethylcyclopropyl group at R2). The 3-methyl-THF containing derivative 2.23g showed minimal binding to CB2 receptor and no significant binding to CB1 receptor. Whilst N1-methyl-THF is not a common substituent amongst AI CB2 receptor ligands, a previous report has resulted in very high CB2 receptor affinity and selectivity. It is possible that in combination with other R2 groups, methyl-THF may be more
favourable, for instance in the 5-O-benzyl derivative 2.22i (containing R\textsuperscript{2} naphthoyl), which did not undergo hydrogenolysis and was not evaluated.

By comparing 5-hydroxy derivatives possessing the same R\textsuperscript{1} group it appeared that the optimal R\textsuperscript{2} group for CB\textsubscript{2} receptor affinity was either 1-naphthoyl (compare 2.23c to 2.23a and 2.23d; Figure 30a) or 4-methoxyphenyl (compare 2.23e to 2.23f; Figure 30b), whilst cyclohexane (2.23d) showed the lowest affinity. It was perhaps to be expected that developing an AI-based CB\textsubscript{2} ligand with a non-aromatic R\textsuperscript{2} group, such as cyclohexane would be more challenging. Whilst there are some notable high affinity examples of CB\textsubscript{2} receptor ligands possessing non-aromatic R\textsuperscript{2} groups,\textsuperscript{248, 253} aromatic groups are much better characterised at this position and there is strong evidence that aromatic π-stacking is key to AI interactions with CB\textsubscript{2} receptor.\textsuperscript{250, 280, 281}

Extension of 5-hydroxyl 2.23e to the 5-O-ethanoic acid 2.25c led to a 14 fold loss in CB\textsubscript{2} receptor affinity (Figure 30b; Table 3). This loss in affinity may be due to the increased size of the ethanoic acid substituent or to the carboxylic acid functionality itself. Larger groups than an ethanoic acid, such as O-benzyl or furan have been tolerated at C5 in published AI derivatives,\textsuperscript{253, 256} albeit with differing R\textsuperscript{1} and R\textsuperscript{2} groups to those used here. The carboxylic acid would be ionised at physiological pH, which could be detrimental to lipid-mediated entry to CB\textsubscript{2} receptor (section 1.2.2.1) and may be the cause of the lower affinity observed. This could be investigated further by synthesising and testing the methylated ester analogue 5-O-CH\textsubscript{2}COOMe, however this was not carried out in this project. The weak binding of 5-O-ethanoic acid derivatives 2.25a and 2.25b compared to 2.25c further indicated a preference for R\textsuperscript{1} group 4-methyl-THP and R\textsuperscript{2} group 4-methoxyphenyl.
Figure 30: Competition binding curves at hCB₂ receptor for (A) hydroxyindoles 2.23a, 2.23c and 2.23d which all contain ethylmorpholino at R¹, but varying R² groups, and (B) hydroxyindoles 2.23e, 2.23f and 5-O-ethanoic acid 2.25c, which all contain methyl-THP at R¹. Assays were carried out with [³H]CP55,940 (2.5 nM, Kᵰ = 3 nM) and HEK293-hCB₂ membranes (10 µg/point). [³H]CP55,940 bound (cpm) raw values are normalised to the specific binding window (%). Data shown is representative of one independent experiment conducted in triplicate and data shown are the mean ± SEM. CP55,940 at 10 µM was used as a homologous competitor to determine specific binding.

The 5-hydroxyindoles 2.23c, 2.23e and 2.23f underwent concentration response assays at CB₁ receptor to evaluate receptor subtype selectivity (Figure 31). The most CB₂ receptor selective compound of the three was 2.23c (Kᵰ,CB₁/Kᵰ,CB₂ = 17), followed by 2.23e and 2.23f (Kᵰ,CB₁/Kᵰ,CB₂ = 12 and 3, respectively; Table 3), which suggests that ethylmorpholino at R¹ confers better CB₂ selectivity than methyl-THP. 5-
Hydroxyindole 2.23f showed the highest affinity for CB$_1$ receptor ($K_i = 823.8 \pm 215$ nM).

![Figure 31: Competition binding curves for 2.23c, 2.23e and 2.23f at hCB$_1$ receptor, determined using $[^3]$H]CP55,940 (2.5 nM, $K_d = 2$ nM) and HEK293-hCB$_1$ membranes (7.5 µg/point). Data is representative of one independent experiment conducted in triplicate and data shown are the mean ± SEM. CP55,940 (10 µM) was used as a homologous competitor to determine specific binding.](image)

Unfortunately, conjugation of a linker and fluorescent dye to the C5 position of the indole scaffold failed to produce a high affinity CB$_2$ receptor fluorescent ligand (Table 4). The tolerability of bulky 5-substituents such as furan and O-benzyl in published indole SAR studies suggests that extension from C5 indole beyond the atom length that was achieved here (4 atoms, 2.25c) should be possible. All of the fluorophore-linker conjugates evaluated extended from the indole scaffold with the same functional group, an O-ethanamide. This strategy enabled divergence from one common derivative to give three different linker conjugates (e.g. synthesis of 2.30a, 2.30b, and 2.30e from 2.25a). However, the ethanamide group is very polar and being located so close to the indole scaffold may well disrupt the binding of the core ligand to the lipid CB$_2$ receptor. In particular, the ethanamide could possibly impede potential lipid-mediated entry of the indole core into CB$_2$ receptor.
Table 4: Affinity of 5-substituted indole-based linker and fluorescent conjugates at hCB$_2$ receptor. 

![Diagram of indole-based linker](attachment:image.png)

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<tr>
<th>Compound</th>
<th>Linker</th>
<th>R$^3$</th>
<th>10 µM displ. [³H]CP 1.17 at hCB$_2$ (% ± SEM)$^b$</th>
<th>$K_i$ at hCB$_2$ (nM)$^c$</th>
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<td>Peptide</td>
<td>BODIPY 630/650-X</td>
<td>45.7 ± 5.5</td>
<td>&gt;10000</td>
</tr>
</tbody>
</table>

$^a$ Radioligand binding assays performed with [³H]CP55,940 (2.5 nM) and HEK293-hCB$_2$ membranes. Data is the mean ± SEM of at least three individual experiments performed in triplicate.

$^b$ Percentage displacement of [³H]CP55,940 by test compound (10 µM). Raw data was normalised to the specific binding window (unlabelled CP55,940 (10 µM), 100%; vehicle control, 0%).

$^c$ D’Agostino & Pearson normality test and a one sample t-test was used to determine if significant competition with [³H]CP55,940 occurred ($K_i >$10 µM) or not (no binding).

Linkers of identical atom length were chosen to allow direct comparison of the effect of linker chemistry on CB$_2$ receptor binding. However, the limitation of this design was that varying the atom length of linkers was not explored and could have a large impact on receptor binding. For example, a lengthier linker may allow greater flexibility, enabling favourable positioning of the fluorophore and the indole core. A linker that is too short may prevent the indole core from favourable positioning within the ligand.
binding pocket. Therefore, it would be interesting to explore variations in linker length as well as polarity at the 5 position of indole, in the pursuit of fluorescent ligands for CB$_2$ receptor (although this was outside of the scope of this thesis).

The primary amines ($pK_a = \sim 9-10$) of the linker conjugates (2.30a-g) would all be predominantly ionised at physiological pH and so may impede ligand entry via the lipid bilayer. However, the shared low CB$_2$ receptor affinity of acetylated analogue 2.31 and the corresponding primary amine 2.30b demonstrated that the primary amine is not solely responsible for the lack of affinity (Table 4). It would likely be more difficult for the linker conjugates and fluorescent ligands to gain receptor entry via the lipid membrane than for the smaller hydroxyindoles, due to the large size of the molecules. Therefore these larger compounds may possibly have to gain access to the receptor through the extracellular loops.

The lack of detectable CB$_2$ receptor binding observed for compounds containing a dipeptide linker (2.30e-g) may be due to a variety of reasons. As discussed in the linker design section (2.2.2), it was anticipated that the greater polarity of the peptide and PEG linkers (cLogP = -3 and -2, respectively)$\dagger\dagger$ may impede lipid membrane-mediated ligand entry when compared to the alkyl linker (cLogP = 0.54). It is also worth considering that perhaps the greater steric bulk of the alanine moieties (such as the methyl and amide) over the alkyl and PEG linkers may not be tolerated, especially if the linker resides in a narrow access point to the ligand binding pocket. It seems apparent that the ability of the di-alanine to engage in hydrogen bonding with receptor residues has not improved ligand binding in these derivatives. Conjugation of fluorophore to the peptide linked conjugates restored minimal CB$_2$ receptor binding ($K_i = >10 \mu$M, 2.32e-g; Table 4), which may be due to the fluorophore acting as a lipid anchor, thereby improving affinity at receptor. Vernall et al reported that fluorophore conjugation increased the affinity of peptide-ligand conjugates for adenosine A$_3$ receptor, and that varying the amino acids of the peptide linker had a significant effect on receptor affinity.$^{198}$ Altering the amino acid sequence and length of the peptide

$\dagger\dagger$ cLogP values calculated using Crippen's fragmentation,$^{265}$ in ChemDraw Prime 15.1, PerkinElmer Informatics, Inc. Values were calculated for the diacetylated (i.e.AcNHCHXNHAc) linkers in isolation and are not representative of the linker-indole conjugates as a whole.
linkers utilised in this study may be worth exploring for improved ligand affinity at CB
receptor.

The improved affinity of peptide linker containing fluorescent ligands was contrasted
by the loss of discernible binding for the alkyl linker containing fluorescent ligands
(2.32a, 2.32c; Table 4). This may be due to the very high lipophilicity of the overall
fluorescent-alkyl linker conjugates (2.32a, 2.32c) which might result in high
accumulation at the lipid membrane and only minimal ligand gaining entry to CB
receptor.

2.4.2 Forskolin-stimulated cAMP assays

Compounds that showed micromolar or nanomolar affinity for CB2 receptor were
analysed for functional activity using a BRET sensor to measure modulation of
forskolin-stimulated cAMP in whole HEK293 S4 low cells overexpressing hCB2 or
hCB1 receptors (section 1.6.2). Potency (EC50) is determined in concentration response
functional assays and is the concentration of compound required to produce a half
maximal response, thus a highly potent compound can produce a significant response at
a low concentration. Efficacy (maximum response, Emax) is a measure of the maximum
response evoked by the compound and is determined at the 10 µM concentration in this
study. Since both CB1 and CB2 receptor are G1 coupled, agonists cause a decrease in
cellular cAMP in this assay. Emax values for agonists will therefore be <100% and the
more efficacious an agonist, the closer Emax will be to 0%. In contrast, antagonists can
prevent the inhibition produced by an EC90 concentration of a full agonist (i.e.
CP55,940 1.10) and inverse agonists lead to an increase in cellular cAMP (Emax >
100%). It is important to report both potency and efficacy as without one, the other can
lack context. For instance whilst a compound may exhibit high potency, it could have
low efficacy meaning that it will not invoke a large effect.

The 5-hydroxyindoles 2.23a, 2.23c-f and 5-ethanoic acid 2.25c were initially tested in a
10 µM screen under three conditions: in the absence of forskolin (to evaluate the effect
on basal cAMP), in the presence of forskolin (to screen for agonism or inverse
agonism), and in the presence of both forskolin and CP55,940 1.10 (to test for their
ability to antagonise CP55,940 1.10). All compounds tested at 10 µM acted as agonists at both CB$_2$ and CB$_1$ receptors, producing a decrease in cAMP, whilst failing to antagonise an EC$_{90}$ concentration (20 nM at CB$_2$ and 4 nM at CB$_1$ receptor) of CP55,940 1.10 (Figure 32).

---

§§ 10 µM BRET screen at hCB$_2$ receptor in presence and absence of CP55,940 1.10 (n = 1) carried out by Christa Macdonald.
Figure 32: BRET assay screen measuring effect of compound (10 µM) on forskolin (Fsk)-stimulated (5 µM) cellular cAMP levels in HEK293 cells overexpressing (A) hCB₂ receptor (assay carried out by Christa Macdonald) or (B) hCB₁ receptor. Compounds were checked for effect on basal cAMP (compound/vehicle (V)/V; light grey) and in the presence (compound/Fsk/CP; dark grey) and absence of CP55,940 (compound/Fsk/V; mid grey). Data is the mean ± SEM of (A) one or (B) three independent experiments conducted in duplicate. Data has been normalised to a percentage of forskolin only response (V/Fsk/V; 100%) and basal response (V/V/V; 0%).
All of the identified agonists (2.23a, 2.23c-f and 2.25c) were then subjected to a concentration response assay at CB$_2$ receptor to determine potency (Figure 33; Table 5). The potency of CP55,940 1.10 at CB$_2$ receptor was also determined as a literature agonist for comparison. Only the lead compound (in terms of CB$_2$ receptor affinity), 2.23e was analysed for potency at CB$_1$ receptor (Table 5).

![Figure 33: Forskolin-stimulated cAMP concentration response curves for CP55,940 1.10, 2.23e and 2.25c at hCB$_2$ receptor. Area under the curve (AUC) values are normalised so that forskolin only response = 100% and basal response = 0%. Data shown is representative of a single experiment, conducted in duplicate and data shown are the mean ± SEM.](image)

In contrast to the results observed, it was anticipated that these compounds would behave as inverse agonists or antagonists because of the structural similarities to AM630 1.14. However, there are several reported C5-substituted AI-based CB$_2$ receptor ligands which behave as agonists. The highest affinity compound for CB$_2$ receptor (2.23e) also translated into the most potent agonist (CB$_2$ EC$_{50}$ = 4.4 ± 0.35 nM). There appeared to be a trend for methoxyphenyl at R$^2$ (2.23e, 2.23a) conferring higher potency than naphthoyl (2.23c, 2.23f), followed by cyclohexane. The aromatic R$^2$ groups might engage in aromatic π-stacking interactions with CB$_2$ receptor, resulting in a different degree of signalling to that caused by the non-aromatic cyclohexane group. It was curious that efficacy amongst the 5-hydroxyindoles seemed to be determined by the R$^1$ group, with methyl-THP containing derivatives (2.23f, 2.23e),
producing the greatest maximal response, followed by ethylmorpholino derivatives (2.23a, 2.23d, 2.23c). The small sample size means that these trends lack rigour, especially in the absence of much literature SAR describing indole substituent effect on functional potency and efficacy at CB2 receptor. 5-Hydroxyindole 2.23e showed similar potency to the agonist CP55,940 1.10 at CB2 receptor, but it had significantly lower efficacy (Table 5; Figure 33). Whilst all of the compounds evaluated exhibited reasonable potencies, none of them demonstrated especially high efficacy when compared to CP55,940 1.10.

Table 5: Potency (EC50) and efficacy (Emax) of 5-hydroxyindoles and a 5-O-ethanoic acid derivative at hCB2 and hCB1 receptors, determined using a forskolin-stimulated cAMP assay. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (nM ± SEM)</th>
<th>Emax ( % ± SEM)</th>
<th>EC50 (nM ± SEM)</th>
<th>Emax ( % ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.23a</td>
<td>5.4 ± 2.7</td>
<td>67.5 ± 2.9</td>
<td>n.d.</td>
<td>79.1 ± 6.3</td>
</tr>
<tr>
<td>2.23c</td>
<td>8.7 ± 1.6</td>
<td>77.4 ± 6.1</td>
<td>n.d.</td>
<td>55.6 ± 4.1</td>
</tr>
<tr>
<td>2.23d</td>
<td>18.6 ± 5.1</td>
<td>74.1 ± 1.2</td>
<td>n.d.</td>
<td>74.3 ± 4.2</td>
</tr>
<tr>
<td>2.23e</td>
<td>4.4 ± 0.35</td>
<td>65.7 ± 3.7</td>
<td>n.d.</td>
<td>62.9 ± 29.7</td>
</tr>
<tr>
<td>2.23f</td>
<td>14.7 ± 5.3</td>
<td>64.4 ± 1.5</td>
<td>n.d.</td>
<td>53.1 ± 1.3</td>
</tr>
<tr>
<td>2.25c</td>
<td>70.2 ± 6.2</td>
<td>57.4 ± 3.2</td>
<td>n.d.</td>
<td>66.7 ± 4.4</td>
</tr>
<tr>
<td>CP55,940 1.10</td>
<td>5.6 ± 2.3</td>
<td>41.0 ± 1.4</td>
<td>n.d.</td>
<td>56.5 ± 1.4</td>
</tr>
</tbody>
</table>

*a cAMP levels measured in a BRET assay using a CAMYEL sensor, performed in HEK293-hCB2 or hCB1 S4 low cells. Data is the mean ± SEM of three individual experiments performed in duplicate.

b Emax is the response at 10 µM, normalised to basal (0%) and forskolin only (100%) levels of cAMP and the lower the value, the greater the efficacy of the agonist.

Extension of the 5-hydroxyl position of 2.23e with an ethanoic acid moiety retained agonist activity (2.25c), but with reduced potency (Table 5; Figure 33). Despite the 14-fold loss in CB2 receptor affinity of 2.25c compared to 2.23e, 2.25c is still able to elicit a signalling response at CB2 receptor. This may be because 2.25c is still able to make the necessary interactions with CB2 receptor to induce/stabilise an active receptor conformation. This illustrates how different lead compounds may be selected from the competition binding assay versus a forskolin-stimulated cAMP assay. The radioligand
binding assay measures the ability of the test compound to compete with \([^3]H\)CP55,940 1.17, which is more complex than simply the affinity of the compound for receptor, as it is also affected by binding and competition kinetics. For instance, a larger compound may have a slower on rate, resulting in less competition with the radioligand. However, in the BRET cAMP assay, there is no competition and it is simply measuring the ability of the compound to produce a response, although the response would be effected by the on-rate of the compound.

The lead compound 2.23e also showed agonist activity at CB\(_1\) receptor, but at a lower potency (EC\(_{50}\) = 62.9 ± 29.7 nM) than at CB\(_2\) receptor, demonstrating CB\(_2\) receptor selectivity. Two compounds (2.23c and 2.23f) showed similar efficacy to CP55,940 1.10 at CB\(_1\) receptor and four out of the seven compounds evaluated (2.23c-f) showed similar or higher efficacy at CB\(_1\) receptor than at CB\(_2\) receptor, despite all showing low CB\(_1\) receptor affinity (Table 5). It is difficult to fully evaluate this trend without potency data, but it does further illustrate the very different parameters that are measured in a competition binding versus a signalling response assay.

All of the compounds (2.23a, 2.23c-f and 2.25c) analysed for function were also screened in a cAMP BRET assay in wild type (WT) HEK293 cells, to verify that the observed effects at CB\(_2\) and CB\(_1\) receptors were receptor mediated (Table 6). WT HEK293 cells express some Class A GPCRs, such as chemokine, lysophospholipid and prostanoid receptors, but do not typically express cannabinoid receptors. The compound cAMP responses were normalised to basal (0%) and forskolin only (100%) responses and were then analysed in a one sample \(t\)-test to determine if the compound responses were significantly different from the forskolin only response. Compounds 2.23a, 2.23d-f and 2.25c showed no significant response in the WT cells, verifying that the agonist activity observed for these compounds was indeed CB receptor mediated. Compound 2.23c invoked a small but significant response at 10 μM but not at 1 μM. As 2.23c demonstrated a response in HEK293-hCB\(_2\) cells at 1 μM, it can be concluded that the WT HEK cell response is not large enough to have significantly affected the calculated CB\(_2\) receptor potency. However, as 2.23c was not tested at 1 μM in HEK293 cells overexpressing hCB\(_1\) receptor, it cannot be definitively concluded that the agonist activity observed at CB\(_1\) receptor is receptor mediated.
Table 6: Forskolin stimulated cAMP response in WT HEK293 cells. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>10 µM (% ± SEM)</th>
<th>1 µM (% ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.23a</td>
<td>105.2 ± 5.9</td>
<td>101.9 ± 1.8</td>
</tr>
<tr>
<td>2.23c</td>
<td>118.7 ± 3.8 *</td>
<td>101.9 ± 3.2</td>
</tr>
<tr>
<td>2.23d</td>
<td>105.1 ± 4.0</td>
<td>105.9 ± 2.6</td>
</tr>
<tr>
<td>2.23e</td>
<td>109.6 ± 7.7</td>
<td>105.5 ± 3.5</td>
</tr>
<tr>
<td>2.23f</td>
<td>108.1 ± 3.2</td>
<td>95.0 ± 5.0</td>
</tr>
<tr>
<td>2.25c</td>
<td>100.1 ± 6.2</td>
<td>98.8 ± 3.7</td>
</tr>
<tr>
<td>WIN 55,212-2 2.2</td>
<td>102.6 ± 2.1</td>
<td>96.1 ± 1.0</td>
</tr>
<tr>
<td>CP55,940 1.10</td>
<td>98.0 ± 4.0</td>
<td>99.2 ± 2.7</td>
</tr>
</tbody>
</table>

* Assay carried out in WT HEK293 cells measuring forskolin-stimulated (5 µM) cAMP. Data is normalised, so that forskolin only response is 100% and vehicle only response is 0%. Data is the mean ± SEM of three individual experiments performed in duplicate. A one sample t-test was used to determine if values were significantly different from forskolin only response (100%), and where values are significantly different, they are marked with *.

### 2.4.3 Off target activity

Compounds 2.22e, 2.22f, 2.22g, 2.22h, 2.22i, 2.23e and 2.23f were accepted into the Eli Lilly and Company Open Innovation Drug Discovery Program and the Tres Cantos Open Lab Foundation and did not show significant proprotein convertase subtilisin kexin type 9 (PCSK9) inhibition, GPR120 agonism, disruption to IL-17 protein-protein interaction, *Leishmania donovani* growth inhibition, voltage-gated potassium channel KCNQ2/3 agonism or nicotinamide N-methyltransferase (NNMT) inhibition.
2.5 Conclusions and future directions

A review of the published SAR informed the decision to explore the C5 and C6 positions of the indole scaffold for tolerability of linker attachment, with the goal of developing a high affinity, selective CB$_2$ receptor fluorescent ligand. A library of seven hydroxyindoles, three O-ethanoic acid derivatives, eight linker conjugates and seven linker-fluorophore conjugates were synthesised and underwent pharmacological evaluation. Varying R$^1$ and R$^2$ groups were trialled at the N1 and C3 acyl position of indole, with methyl-THP and p-methoxyphenyl (R$^1$) or 1-naphthoyl (R$^2$) identified as the preferred groups at N1 and C3 respectively (Table 3). Notably, 5-hydroxyl substitution was favoured over 6-hydroxyl. A high affinity CB$_2$ receptor agonist 2.23e ($K_i = 120.1 \pm 23.4$ nM at hCB$_2$, $K_{iCB1}/K_{iCB2} = 12$) was identified amongst the 5-hydroxyl derivatives. However, extension of 2.23e to include a 5-O-ethanoic acid moiety (2.25e) led to a 14 fold loss in CB$_2$ receptor affinity. It is not clear from these results whether this loss in affinity was due to intolerance to substituent size or carboxylic acid functionality, or both. All of the high affinity CB$_2$ receptor compounds identified behaved as agonists at CB$_2$ receptor. It remains to be seen if the low CB$_2$ receptor affinity of linker fluorophore conjugates was due to poor choice of linker length and functionality, or if the C5 position of indole is unsuitable for extension. In addition, the polarity of the O-ethanamide group so close to the core indole ligand may be unfavourable for entry and binding to the lipid CB$_2$ receptor. Therefore, it would be interesting to explore varying the polarity of moieties adjacent to the indole scaffold, as well as linker length and position of linker appendage (as discussed in Chapter Three), in the pursuit of fluorescent ligands for CB$_2$ receptor.
Chapter Three: Fluorescent ligands for CB₂ receptor based on 5 and 7 substituted alkyl-indoles

Chapter Two explored 5- and 6-substituted AIs, in the pursuit of CB₂ receptor fluorescent ligands. A lead compound 2.23e was identified amongst the 5-substituted AIs, whereas, the 6-substituted AIs were not progressed further because of the poor CB₂ receptor affinity attained. The findings of Chapter Two raised two key questions regarding the development of higher affinity 5-substituted AI fluorescent ligands. Firstly, was the polar O-ethanamide group adjacent to the indole ring responsible for the low CB₂ receptor affinity of the linker-AI conjugates 2.30a-g and 2.31 or was the C5 position of the indole ring simply unsuitable for further extension? Secondly, would varying the linker length at the C5 position result in fluorescent ligands with improved affinity for CB₂ receptor? The work described in sections 3.1, 3.2 and 3.3 of this chapter aimed to answer the first of these questions through synthesis and evaluation of linker-AI conjugates and subsequently opened up new avenues for development of AI-based fluorescent ligands, which were explored further in the work described in sections 3.4, 3.5 and 3.6.

3.1 AI and linker-AI conjugate design exploring varying polarity and substitution position of the indole ring

The lead compound identified in Chapter Two was the 5-hydroxyindole agonist 2.23e, which showed the highest affinity for CB₂ receptor out of the novel compounds evaluated \( (K_i = 120.1 \pm 23.4 \text{ nM}; \text{Table 3}) \). However, extension of the hydroxyl group of 2.23e to the more polar ethanoic acid (2.25c) considerably reduced CB₂ receptor affinity, which was then reduced even further upon linker and dye conjugation (2.32g; Figure 34). It was not clear whether this loss of affinity from 2.23e to 2.25c was due to intolerance to extension at the C5 position, or whether the presence of the increasingly polar, ionised at physiological pH carboxylic acid of 2.25c and then amide of 2.32g in
such close proximity to the indole scaffold disrupted ligand binding (or whether both of these factors were detrimental). Substitution of large, bulky groups at the C5 position of indole has led to several high affinity CB2 receptor ligands reported in the literature,\textsuperscript{253, 256} therefore it would not be expected that the size of the O-ethanoic acid in \textbf{2.25c} would be a hindrance to CB2 receptor binding. Therefore, a small library of compounds was designed, exploring reduction of the polarity of the linker moiety immediately adjacent to the indole ring.

![Diagram](image)

**Figure 34**: Extension of \textbf{2.23e} to \textbf{2.25c} and \textbf{2.32g} led to a considerable reduction in hCB2 receptor affinity, which led to the suspicion that presence of the polar carboxylic acid and amide groups (circled) adjacent to the core indole scaffold could be detrimental to CB2 receptor binding.

All derivatives of this small library were designed as analogues of the lead compound \textbf{2.23e}, possessing both the N1-methyl-THP and C3 acyl methoxyphenyl groups. An O-propyl group with increased lipophilicity and no charge at physiological pH (\textbf{3.7a}) was designed as an equal atom-length replacement of the O-ethanoic acid of \textbf{2.25c}.

In addition, extension of \textbf{2.23e} with a long PEG linker attached directly to the 5-hydroxyl (\textbf{3.12}) was designed in order to explore replacement of the polar amide
immediately adjacent to the pharmacophore. The PEG linker was designed to possess the same length as the linker-AI conjugates (2.30a-g, 2.31) synthesised in Chapter Two, to allow meaningful comparison. It was decided that the amine terminus of 3.12 (and all subsequent amino linker-pharmacophore conjugates designed) should be acylated to produce ligands with no charge at physiological pH. Acylation was chosen over Boc-protection due to lesser steric bulk and closer structural similarity to the amide bond that would be produced by conjugation with the fluorophore.

There are several literature reports of 7-substituted indole derivatives with high affinity and selectivity for CB2 receptor.\textsuperscript{251, 256, 257} Substitution at the C7 position of indole had not yet been explored in this study, so three 7-substituted analogues were designed to investigate whether linker extension from this position would be favoured over the C5 position. The derivatives designed included a 7-hydroxyl analogue of 2.23e (3.4), a 7-O-ethanoic acid analogue of 2.25e (3.6) and a 7-O-propyl analogue of 3.7a (3.7b). Ligand designs in this small series did not incorporate a fluorophore, as the primary aim was to investigate linker polarity and substitution position before further development of fluorescent ligands.
3.2 Synthesis

Synthesis of the 7-hydroxyindole (3.4) and 7-O-ethanoic acid (3.6) derivatives followed the same pathway as that for the 5 substituted analogues (2.23e and 2.25c; Scheme 1 and Scheme 3). Briefly, 7-benzyloxyindole 3.1 was N-alkylated using the mesylate of methyl-THP (2.17b; Scheme 1), to give 3.2 in high yield (87%; Scheme 5, step i), which was then acylated at C3 with methoxybenzonitrile via palladium catalysis with 2.21, to give 3.3 (58%; step ii). The 7-O-benzyl 3.3 was benzyl deprotected by palladium catalysed hydrogenolysis to reveal 7-hydroxyindole 3.4 (55%; step iii). Reaction with tert-butylbromoacetate gave 3.5 in low yield (33%; step iv), probably due to the very small scale of the reaction (11 mg of 3.4), which meant any small loss of product during work-up and silica column purification had a significant impact on yield. Cleavage of the tert-butyl of 3.5 with TFA revealed 7-O-ethanoic acid 3.6 (53%; step v).

Scheme 5: Synthesis of 7-hydroxyindole (3.4) and 7-O-ethanoic acid (3.6). Reagents and conditions: (i) NaH, DMF, 45°C, 2 h, 87%; (ii) methoxybenzonitrile, H2O, CH3CO2H, 1,4-dioxane, 140°C, 42 h, 58%; (iii) Pd/C, H2, EtOH, CHCl3, rt, 20 h, 55%; (iv) tert-butylbromoacetate, NaH, DMF, 60°C, 3 h, 33%; (v) TFA, DCM, rt, 1 h, 53%.
Hydroxyindoles 2.23e and 3.4 were reacted by Williamson ether procedure with 1-bromopropane and NaH to give the O-propyl derivatives 3.7a and 3.7b respectively in moderate yield (57-83%; Scheme 6).

Scheme 6: Synthesis of 5- and 7-O-propyl derivatives 3.7a and 3.7b. Reagents and conditions: (i) 1-bromopropane, NaH, DMF, rt, 20 h, 57-83%.

The first step in the synthesis of long PEG linker-AI conjugate 3.12 was Boc protection of the PEG-amine alcohol linker, 2-(2-(2-aminoethoxy)ethoxy)ethanol 3.8 to give 3.9 (Scheme 7, step i). Reaction of the two alcohols 3.9 and 2.23e was initially attempted by Mitsunobu coupling, using triphenylphosphine and di-tert-butyl azodicarboxylate (DBAD) in THF, following a literature procedure for preparation of sulfamoylphenyl aminoalkyl ethers (Scheme 7, step ii). However, 2.23e was not soluble in THF, so a small amount of DMF (0.5 mL) was added to dissolve 2.23e. There is an inverse relationship between the polarity of the solvent used and the rate of a Mitsunobu reaction. Therefore it was expected that addition of polar DMF would result in a slower reaction than in just THF alone. However, after four days (and addition of extra triphenylphosphine), no product formation could be detected by thin layer chromatography (TLC), mass spectrometry or ¹H NMR spectra. Mitsunobu reactions require the nucleophile to have a \( pK_a < 15 \), therefore the 5-hydroxy of 2.23e (\( pK_a = 9.11^{***} \)) should be suitable for coupling under these conditions. It was reasoned that the reagents were too dilute, as Mitsunobu reactions generally require a concentration of at least 0.1 M, so the reaction was repeated with all reagents at a concentration of >0.1 M in anhydrous DMF. Also, the order of addition of reagents can greatly effect Mitsunobu reactions. In the original attempt, triphenylphosphine was

*** \( pK_a \) value calculated with MarvinSketch 17.2.27.0, ChemAxon Ltd.
added to the linker 3.9, followed by addition of the hydroxyindole 2.23e and the reaction mixture was then put on ice for dropwise addition of DBAD. This order of addition was used as it is reported to reduce by-product formation, but if it is unsuccessful, literature reports recommend altering the order of addition to pre-form the betaine between the azodicarboxylate and triphenylphoshine. Therefore, in the repeated reactions, DBAD was added to triphenylphosphine at 0°C and stirred for 15 min, after which the linker (3.9) was added and after another 15 min stirring, the hydroxyindole 2.23e was added. The reaction mixture was sonicated for 30 min after addition of all reagents, as sonication has also been shown to improve Mitsunobu reaction yields. After three days an unsatisfactory amount of product (3.11) had formed, insufficient for progression to the next synthetic step. As the synthesis schedule was under strict time restrictions, it was decided that Mitsunobu coupling would not be an efficient method for coupling these two alcohols (2.23e and 3.9) with a satisfactory yield. If the schedule had not been so restrictive, it would have been interesting to pursue further variations to the order of addition, or increasing reagent concentration and sonication time or possibly changing the azodicarboxylate used e.g. to diethyl azodicarboxylate (DEAD) or diisopropyl azodicarboxylate (DIAD).
Subsequently, synthesis of \(3.11\) was attempted using the Williamson ether reaction. The alcohol of the Boc-protected linker \(3.9\) was brominated to give \(3.10\), which was not easily visible on TLC by UV or permanganate staining, making purification by silica chromatography challenging and leading to a reduced isolated yield (Scheme 7, step iii). Brominated \(3.10\) was then reacted with 5-hydroxyindole \(2.23e\) and NaH to give \(3.11\) (step iv). Linker-pharmacophore \(3.11\) was Boc-deprotected and then acetylated to give \(3.12\) (step v).
3.3 Radioligand binding assays

Concentration-response radioligand binding assays were carried out using HEK293-hCB₂ and hCB₁ membranes in order to obtain $K_i$ values for 3.4, 3.6, 3.7a, 3.7b and 3.12 (Table 7). The binding affinities of these five compounds were analysed and compared to compounds reported in Chapter Two (Table 3) in the following section.

The most striking pattern observed was the clear preference for substitution at the C7 position over the C5 position of indole. 7-Hydroxyindole 3.4 had the highest hCB₂ receptor affinity and selectivity ($K_i = 53.9 \pm 7.6$ nM, $K_{CB1}/K_{CB2} = 66$; Table 7) of all the hydroxyindole compounds synthesised thus far and showed a two-fold increase in CB₂ receptor affinity over the analogous 5-hydroxyl 2.23e and a five-fold increase in selectivity for CB₂ over CB₁ receptor. Similarly, 7-O-ethanoic acid 3.6 showed a three-fold increase in CB₂ receptor affinity over the analogous 5-O-ethanoic acid 2.25e. Most remarkably though, 7-O-propyl 3.7b showed around 230 fold higher affinity for CB₂ receptor than the analogous 5-O-propyl analogue 3.7a. This preference for 7-substitution appears to be specific to the R¹ and R² groups utilised (i.e. 4-methyl-THP and methoxyphenyl), as Frost et al found a 5-hydroxy derivative with 4-methyl-THP at R¹ and tetramethylcyclopropyl at R² to possess higher affinity than the analogous 7-hydroxyl. Notably, 7-O-propyl 3.7b showed both the highest affinity ($K_i = 5.7 \pm 1.4$ nM at CB₂ receptor) and most pronounced selectivity (89 fold CB₂ over CB₁ receptor) of all the indole-based compounds tested (including those discussed in Chapter Two). It was also exciting to observe that 3.7b showed a 9-fold higher affinity for CB₂ receptor than the inverse agonist SR144528 1.13.

It is possible that 7-substituted indoles had a higher affinity for CB₂ receptor because they form more favourable interactions with the ligand binding site than 5-substituents. Changing the position of substitution may also cause a subtle repositioning of the indole core and N1 and C3 groups of the molecule, altering the interactions of these moieties with the CB₂ receptor orthosteric binding site. This surprising favourability of

††† Radioligand binding assays at hCB₂ and hCB₁ receptors on four (3.4, 3.7a, 3.7b and 3.12) out of the five compounds were carried out by Christa Macdonald.
7-substituted derivatives was explored further using homology modelling and ligand docking studies and is discussed in section 3.7.3.

Table 7: Affinity of 5- and 7-substituted indole derivatives at hCB$_2$ and hCB$_1$ receptors. $^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>5 or 7 Substitution</th>
<th>$K_i$ at hCB$_2$ (nM ± SEM)</th>
<th>$K_i$ at hCB$_1$ (nM ± SEM)</th>
<th>SI $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.23e $^c$</td>
<td>H</td>
<td>5</td>
<td>120.1 ± 23.4</td>
<td>1406 ± 464</td>
<td>12</td>
</tr>
<tr>
<td>3.4</td>
<td>H</td>
<td>7</td>
<td>53.9 ± 7.6</td>
<td>3583 ± 427</td>
<td>66</td>
</tr>
<tr>
<td>2.25e $^c$</td>
<td>CH$_2$CO$_2$H</td>
<td>5</td>
<td>1660 ± 114</td>
<td>&gt;10000</td>
<td>&gt;6</td>
</tr>
<tr>
<td>3.6</td>
<td>CH$_2$CO$_2$H</td>
<td>7</td>
<td>581.8 ± 70.8</td>
<td>&gt;10000</td>
<td>&gt;17</td>
</tr>
<tr>
<td>3.7a  $^b$</td>
<td>n-propyl</td>
<td>5</td>
<td>1350 ± 399.3</td>
<td>4527 ± 676</td>
<td>3</td>
</tr>
<tr>
<td>3.7b  $^b$</td>
<td>n-propyl</td>
<td>5</td>
<td>1660 ± 114</td>
<td>1406 ± 464</td>
<td>66</td>
</tr>
<tr>
<td>3.12</td>
<td>(C$_2$H$_4$O)$_2$C$_2$H$_4$NHAc</td>
<td>5</td>
<td>1406 ± 464</td>
<td>&gt;10000</td>
<td>no binding</td>
</tr>
<tr>
<td>SR144528 1.13</td>
<td>-</td>
<td>-</td>
<td>51.0 ± 3.0</td>
<td>5628 ± 2890</td>
<td>110</td>
</tr>
</tbody>
</table>

$^a$ Radioligand binding assays performed with $[^3]$HCP55,940 (1 nM or 2.5 nM) and HEK293-hCB$_2$ or -hCB$_1$ membranes. Data are the mean ± SEM of at least three individual experiments performed in triplicate. $K_i$ values calculated with a $[^3]$HCP55,940 $K_a$ = 3 nM for hCB$_2$ receptor or $K_a$ = 2 nM for hCB$_1$ receptor. For compounds showing low displacement of $[^3]$HCP55,940 in the 10 µM screens, the D’Agostino & Pearson normality test and a one-sample t-test were used to determine if significant competition with $[^3]$HCP55,940 occurred ($K_i$ > 10 µM) or not (no binding). Binding assays (n = 4 or 5) of 3.4, 3.7a, 3.7b and 3.12 were performed by Christa Macdonald.

$^b$ SI = $K_i$CB$_1$/$K_i$CB$_2$.

$^c$ Binding affinities of 2.23e and 2.25e were reported in Chapter Two, but are repeated here to allow easy comparison of 5- and 7-substituted derivatives.

Extension of the 7-hydroxyl 3.4 to the 7-O-propyl 3.7b led to a nine-fold increase in affinity for CB$_2$ receptor, as well as a slight increase in selectivity (Table 7; Figure 35). Also, comparison of the 7-O-propyl 3.7b to the 7-O-ethanoic acid 3.6 showed that the non-polar alkyl was vastly preferred over the polar carboxylic acid at this position, with
over a hundred-fold difference in CB$_2$ receptor affinity. The presence of the lipophilic propyl group may allow for favourable hydrophobic interactions with the ligand binding pocket, leading to the observed increase in CB$_2$ receptor affinity. Increasing the overall lipophilicity of the molecule may also improve ligand entry into the receptor. The high affinity of 7-substituted derivatives 3.4 and 3.7b, as well as the apparent tolerance for extension at this position indicated that 7-substituted indoles are a promising lead for development of fluorescent ligands for CB$_2$ receptor (as discussed in sections 3.4, 3.5 and 3.6).

![Graph showing competition binding curves for compounds 3.4 and 3.7b at hCB$_2$ receptor.](image)

Figure 35: Competition binding curves for 3.4 and 3.7b at hCB$_2$ receptor, determined using [$^3$H]CP55,940 (1 nM, $K_d$ = 3 nM). Data are representative of one independent experiment conducted in triplicate and data points shown are the mean ± SEM. CP55,940 (10 µM) was used as a homologous competitor to determine specific binding. Assay carried out by Christa Macdonald.

The poor binding affinities observed for the 5-substituted compounds 3.7a and 3.12 did not support the hypothesis that the polar carboxylic acid and amide groups adjacent to the indole ring in the 5-substituted compounds from Chapter Two were detrimental to affinity for CB$_2$ receptor. It was anticipated that replacing the 5-O-ethanoic acid of 2.25c with a 5-O-propyl group would increase affinity for CB$_2$ receptor. However, the 5-O-propyl derivative 3.7a (Table 7) showed only a small improvement in CB$_2$ receptor affinity over the 5-O-ethanoic acid derivative 2.25c ($K_i$ = 1350 ± 399.3 nM versus 1660 ± 114.2 nM at CB$_2$ receptor, respectively), which was shown to be non-significant by an unpaired t-test with Welch’s correction (p = 0.4934). Extension of 5-hydroxyindole
2.23e to the 5-O-propyl 3.7a led to an 11 fold loss in affinity for CB$_2$ receptor. The intolerability of extension with a small, relatively benign group such as propyl, suggests that linker extension from the C5 position of 2.23e derivatives is likely not tolerated. It seems that the lack of success with the linker-AI conjugates in Chapter Two was not due to polarity alone, but intolerance to steric bulk, as demonstrated by the propyl group in 3.7a or ethanoic acid in 2.25c. However, the success of bulky group substitution at the C5 position of indoles in a number of publications$^{253,256}$ suggests that the findings observed here may be specific to the R groups utilised in the 2.23e derivatives (N1-methyl-THP and C3-acyl-methoxyphenyl). A key point of difference is that the bulky 5-substituted literature compounds possess non-aromatic groups at the C3 position, such as adamantyl and tetramethylcyclopropyl (Figure 21).$^{253,256}$ Indoles are thought to interact with CB$_2$ receptor through aromatic stacking$^{152}$ and so these literature compounds 2.13 and 2.14 may have a considerably altered orientation in the ligand binding pocket, especially in the derivatives where aromatic groups are included at the C5 position of indole. The aromatic methoxyphenyl group employed in the 2.23e derivatives may ‘lock’ the ligands into an orientation whereby the methoxyphenyl forms aromatic interactions with the aromatic rich regions of the CB$_2$ receptor between TMH3 and TMH6. Indeed, ligand docking studies with 2.23e, 2.25c and 3.7a (section 3.7.3.1) did result in the methoxyphenyl group positioned in the aromatic regions between TMH3 and TMH6. This orientation may not allow for steric bulk at the C5 position of the indole and might be key to the different findings of 5-extension tolerability observed for the 2.23e derivatives and literature compounds. In agreement with the results for 3.7a, conjugation of a long PEG linker to 2.23e was not well tolerated and resulted in a ligand (3.12) with only minimal affinity for CB$_2$ receptor, showing no improvement on the Chapter Two linker-AI conjugates.

At this stage 3.4, 3.6, 3.7a, 3.7b and 3.12 were evaluated only by radioligand binding assay and not analysed for function as the main criteria for a fluorescent ligand lead linker position was high CB$_2$ receptor affinity. The primary aim of this small compound set was to elucidate the influence of the linker polarity and the position of attachment, in order to inform synthesis of a refined series of fluorescent ligands. Functional evaluation of 3.4, 3.6, 3.7a and 3.7b was carried out in conjunction with the next series and is discussed in section 3.6.2.
3.4 Ligand design of 7-substituted indole-based fluorescent ligands

Extension from the C7 position of the indole scaffold with non-polar groups was identified as a promising lead for development of fluorescent ligands because of the high affinity of 7-O-propyl 3.7b. Thus, 3.7b, was used as the basis for the design of all subsequent indole-based fluorescent ligands. The intention was to maintain the lipophilic propyl moiety of 3.7b and keep any amide bond(s) or other polar groups of the linker at a distance from the indole scaffold. Varying the linker length was of interest, as it would determine where the fluorophore resides (at receptor, membrane or extracellular environment) and therefore has a large effect on CB₂ receptor affinity as well as the utility of the fluorescent ligand.

Two short linkers (methyl valerate and hexylamine) were selected to extend the indole scaffold 3.4. A diamine PEG linker (2.28b), rather than an alkyl linker, was chosen for further extension with the intention of reducing non-specific membrane binding. A dipeptide linker was not utilised in this series, as the polarity may inhibit ligand entry and binding to the lipid CB₂ receptor. In an attempt to sample dramatically varying linker length and polarity, a compound in which the fluorophore was directly attached to a shorter hexylamine linker was also designed.
3.5 Synthesis

Synthesis of fluorescent ligands 3.18 and 3.25 started from 7-hydroxyindole 3.4 (the synthesis of which is described in Scheme 5). Alkylation of 3.4 with methyl 5-bromovalerate via Williamson ether synthesis gave 3.13 (Scheme 8, step i). Methyl ester hydrolysis of 3.13 using lithium hydroxide gave carboxylic acid 3.14 (step ii), which underwent an amide coupling with N-Boc-1,8-diamino-3,6-dioxaoctane 2.28b (Scheme 3) to yield 3.15 (step iii). Boc-deprotection of 3.15 revealed the amine 3.16 (step iv), which was purified by semi-preparative RP-HPLC to ensure maximum purity. The purified amine 3.16 was then either acetylated to give 3.17 or used in four-fold excess for coupling to BODIPY 630/650-X-OSu 2.15, affording fluorescent ligand 3.18 (steps v-vi). All reactions were achieved in high yield, apart from the first step (3.13, 30%).

Scheme 8: Reagents and conditions: (i) methyl 5-bromovalerate NaH, DMF, rt, 20 h, 30%; (ii) 0.2 M aq. LiOH.H₂O, THF, 0°C, 12 h, 88%; (iii) N-Boc-1,8-diamino-3,6-dioxaoctane (2.28b), HATU, DIPEA, DMF, rt, 12 h, 88%; (iv) TFA, DCM, rt, 1 h, 95%; (v) Ac₂O, DIPEA, DCM, rt, 1 h, 96%; (vi) BODIPY 630/650-X-OSu 2.15, DIPEA, DMF, rt 12 h, 93%.
Synthesis of an alkyl chain-linked series began with Boc-protection of 6-amino-1-hexanol 3.19 to give 3.20 (Scheme 9, step i), which was then brominated to give 3.21 (step ii). Coupling of 7-hydroxyindole 3.4 with 3.21 via Williamson ether reaction afforded 3.22 (step iii). Subsequent Boc cleavage revealed the amine 3.23 (step iv), which was purified by semi-preparative RP-HPLC prior to conjugation as a five-fold excess with BODIPY 630/650-X-OSu 2.15 to give the fluorescent ligand 3.25 (step vi). The amine 3.23 was also acetylated to give 3.24 (step v).

Scheme 9: Reagents and conditions: (i) Boc₂O, dioxane, rt, 18 h, 79%; (ii) Br₂, Ph₃P, Et₃N, DCM, 0°C, 2 h, 57%; (iii) NaH, DMF, rt, 20 h, 79%; (iv) TFA, DCM, rt, 1 h, 92%; (v) Ac₂O, DIPEA, DCM, rt, 1 h, 98%; (vi) BODIPY 630/650-X-OSu 2.15, DIPEA, DMF, rt 12 h, 77%.
3.6 Pharmacological evaluation

3.6.1 Radioligand binding assays

Radioligand binding assays were carried out on 3.13, 3.14, 3.17, 3.18, 3.24 and 3.25 at a fixed concentration of 10 µM, to evaluate the ability of the compounds to displace \[^{3}\text{H}]\text{CP55,940} \text{1.17}\) at hCB\textsubscript{2} and hCB\textsubscript{1} receptor (Figure 36).

![Figure 36: Combined results of competition binding screens showing displacement of \[^{3}\text{H}]\text{CP55,940} (2.5 nM) at HEK293 hCB\textsubscript{2} (10 µg/point) (black) and HEK293 hCB\textsubscript{1} (7.5 µg/point) (grey) membranes by a fixed concentration (10 µM) of test compound. Raw data were expressed in ccpp and normalised to unlabelled CP55,940 (10 µM) (100%) and vehicle control (0%). Data shown are the mean value ± SEM of three independent experiments conducted in triplicate, except for 3.13, 3.14 and 3.24 at hCB\textsubscript{2} receptor where n = 2. The dashed line indicates the 50% displacement criteria for progression to concentration-response assay.](image)

The long linker-AI conjugate 3.17 and the two fluorescent ligands 3.25 and 3.18 showed less than 50% displacement of \[^{3}\text{H}]\text{CP55,940} \text{1.17}\) at both hCB\textsubscript{2} and hCB\textsubscript{1} receptor (Figure 36), therefore affinity would be too low to accurately determine \(K_i\) values by concentration response assays. The 7-\(O\)-pentanoic acid 3.14 also showed less than 50% displacement of \[^{3}\text{H}]\text{CP55,940} \text{1.17}\) at hCB\textsubscript{1} receptor. Subsequent statistical analysis with the D’Agostino and Pearson normality test and the one sample \(t\)-test was
carried out and showed that 3.17, 3.25 and 3.18 at hCB2 and hCB1 receptors and 3.14 at hCB1 receptor demonstrated statistically significant competition with [3H]CP55,940 1.17 (Table 8).

The 7-O-methyl valerate conjugate 3.13, the 7-O-pentanoic acid 3.14, and the acetylated 7-O-hexylamine 3.24 all showed greater than 50% displacement of [3H]CP55,940 1.17 at hCB2 receptor and 3.13 and 3.24 also showed greater than 50% displacement at hCB1 receptor (Figure 36; Table 8). Therefore, these compounds were subjected to concentration-response assays at the respective receptors to ascertain $K_i$ values.

The highest affinity compound was the 7-O-methyl valerate conjugate 3.13, which demonstrated a $K_i$ of 200.7 ± 4.9 nM at CB2 receptor (Table 8). Whilst this represents a 35-fold reduction in CB2 receptor affinity compared to 3.7b (Table 7), 3.13 still retained reasonable CB2 receptor affinity and showed that extension of the 7-O-propyl-chain of lead compound 3.7b was tolerated. The larger size of 3.13 perhaps reduced flexibility of the core ligand orientation, disrupting formation of favourable receptor interactions compared to the smaller 3.7b. The carboxylic acid 3.14 showed approximately a 6-fold loss in affinity for CB2 receptor compared to the methyl ester analogue 3.13. Carboxylic acid 3.14 and methyl ester 3.13 should be able to form very similar hydrogen bond interactions with CB2 receptor, therefore the loss of affinity demonstrated in 3.14 may be due to the ionised carboxylic acid which could impede lipid mediated ligand entry into the receptor. Prior to carrying out radioligand binding assays, it was considered a remote possibility that hydrolysis of the methyl ester might occur during the course of the assay. However, it is unlikely that this occurred as if it had, the binding affinities found for 3.14 and 3.13 would likely have been very similar.
Table 8: Affinity of 7-substituted indole linker-AI conjugates and fluorescent ligands at hCB$_2$ and hCB$_1$ receptors. $^a$

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3.13</td>
<td>COOme</td>
<td>97.2 ± 1.5</td>
<td>200.7 ± 4.9</td>
<td>77.1 ± 2.0</td>
<td>1583 ± 242.7</td>
<td>8</td>
</tr>
<tr>
<td>3.14</td>
<td>CO$_2$H</td>
<td>85.1 ± 1.6</td>
<td>1307 ± 302.5</td>
<td>29.8 ± 3.2</td>
<td>&gt;10000</td>
<td>&gt;7</td>
</tr>
<tr>
<td>3.24</td>
<td>C$_2$H$_4$NHAc</td>
<td>74.6 ± 1.8</td>
<td>1969 ± 351.9</td>
<td>65.0 ± 3.7</td>
<td>3918 ± 800.4</td>
<td>2</td>
</tr>
<tr>
<td>3.17</td>
<td>CONH(C$_2$H$_4$O)$_2$ C$_2$H$_4$NHAc</td>
<td>14.8 ± 1.5</td>
<td>&gt;10000</td>
<td>13.4 ± 5.4</td>
<td>&gt;10000</td>
<td>-</td>
</tr>
<tr>
<td>3.25</td>
<td>C$_2$H$_4$NHAc BODIPY630/650-X</td>
<td>13.2 ± 2.9</td>
<td>&gt;10000</td>
<td>13.4 ± 3.7</td>
<td>&gt;10000</td>
<td>-</td>
</tr>
<tr>
<td>3.18</td>
<td>CONH(C$_2$H$_4$O)$_2$ C$_2$H$_4$NH BODIPY630/650-X</td>
<td>27.3 ± 1.4</td>
<td>&gt;10000</td>
<td>11.1 ± 4.8</td>
<td>&gt;10000</td>
<td>-</td>
</tr>
<tr>
<td>SR144528 1.13</td>
<td>-</td>
<td>-</td>
<td>51.0 ± 3.0</td>
<td>-</td>
<td>5628 ± 2890</td>
<td>110</td>
</tr>
</tbody>
</table>

$^a$ Radioligand binding assays performed with [3$^3$H]CP55,940 (2.5 nM) and HEK293-hCB$_2$ or -hCB$_1$ membranes. Data are the mean ± SEM of at least three individual experiments performed in triplicate.

$^b$ Percentage displacement of [3$^3$H]CP55,940 by test compound (10 µM). Raw data were normalised to the specific binding window (unlabelled CP55,940 (10 µM), 100%; vehicle control, 0%).

$^c$ $K_i$ calculated using [3$^3$H]CP55,940 $K_d = 3$ nM at hCB$_2$ receptor or 2 nM at hCB$_1$ receptor. For compounds showing low displacement of [3$^3$H]CP55,940 in the 10 µM screens, the D’Agostino & Pearson normality test and a one sample $t$-test were used to determine if significant competition with [3$^3$H]CP55,940 occurred ($K_i > 10$ µM) or not (no binding).

$^d$ $SI = K_iCB_1/K_iCB_2$.

Extension of the 7-O-ethanoic acid 3.6 (Table 7) to the 7-O-pentanoic acid 3.14 led to a two-fold loss in affinity for CB$_2$ receptor (Table 8). It had already been demonstrated that a substituent length of eight atoms at the C7 position of indole is tolerated in 3.13,
therefore, the physical size of the pentanoic acid should not prohibit CB$_2$ receptor binding. It is therefore possible that unfavourable placement of the carboxylic acid moiety in 3.14 contributed to the loss in CB$_2$ receptor affinity. On the contrary, the carboxylic acid of 3.6 may form favourable hydrogen bonds with residues within the ligand binding site and therefore movement of the carboxylic acid moiety three atoms further along from the indole core (as in 3.14) may be disruptive. In addition, the larger size of 3.14 compared to 3.6 might reduce ligand entry via the lipid membrane.

The linker-indole conjugate 3.24 was the longest linker to show appreciable binding to CB$_2$ receptor ($K_i = 1969 \pm 351.9$ nM; Table 8) of all the indole compounds synthesised thus far (Chapters Two and Three). Whilst 3.13 and 3.24 had the highest affinity for CB$_2$ receptor of all the linker-indole conjugates, they both showed only minimal selectivity ($K_i$CB$_2$/$K_i$CB$_1 = 8$ and 2, respectively). This was disappointing as one of the key criteria for developing a successful fluorescent ligand was good subtype selectivity for CB$_2$ receptor. The considerable binding affinities of 3.13 and 3.24 for CB$_2$ receptor show that polar groups such as an ester and an amide are tolerated in the linker when at a distance of six to eight atoms away from the indole scaffold.

PEG-linked 3.17, which is an extension of 3.14, showed minimal affinity for CB$_2$ receptor ($K_i > 10$ µM). Similarly, fluorescent ligands 3.18 and 3.25, which are extended from 3.17 and 3.24, also showed low affinity for CB$_2$ receptor ($K_i > 10$ µM). This disappointing affinity may be due to unfavourable linker length or positioning of the amide bonds and fluorophore. In addition, the polarity of the PEG linker in 3.17 may disrupt binding. Whilst two considerably different linker lengths were trialled in 3.18 and 3.25, these are only two derivatives on a large spectrum of possible linker lengths. As previously discussed, linker length dictates where the fluorophore resides upon ligand binding and can have a significant impact on positioning of the core ligand. The considerable difference in affinity between 7-O-ethanoic acid 3.6 and 7-O-pentanoic acid 3.14 indicates that polar groups such as amide may be better tolerated closer to the indole core. However, in order to retain the high affinity of the lead compound 7-O-propyl 3.7b, it may be the case that an amide linker has to be located somewhere in between the two positions of the carboxylic acids in 3.6 and 3.14, say at four or five atoms away from the indole core. Using a different fluorophore other than
BODIPY 630/650-X is another potential strategy for improving CB$_2$ receptor affinity. It is also possible that extension from the C7 position of indole beyond the eight and ten atoms (3.13 and 3.24) achieved here is not tolerated. Indeed, a literature search of 7-substituted indole ligands for CB$_2$ receptor failed to find any compounds with groups as long as those described here. The closest found in length, though bulkier in size, was O-benzyl substitution.  

### 3.6.2 Forskolin-stimulated cAMP assays

Compounds 3.13, 3.14, and 3.24, as well as 3.4, 3.6, 3.7a and 3.7b from the series discussed in sections 3.1-3.3 were all able to displace CP55,940 1.10 at CB$_2$ receptor by more than 50% in the 10 µM binding screen and so were also analysed for functional activity in the cAMP BRET assay. Compounds were subjected to a concentration-response assay at CB$_2$ receptor to determine potency and efficacy and the two compounds, 3.4 and 3.7b, with the highest affinity for CB$_2$ receptor were also analysed for potency at CB$_1$ receptor (Table 9). In contrast to agonists, the E$_{\text{max}}$ of inverse agonists will be >100% and higher percentages represent greater efficacy for inverse agonists.

Previously, all of the compounds evaluated by cAMP assay that were 5-substituted (for example 2.23e) showed agonist activity (Table 5). However, 5-O-propyl 3.7a showed no functional response at CB$_2$ or CB$_1$ receptors under the assay conditions (Table 9), in contrast to the analogous 5-O-ethanoic acid 2.25c (Table 5). It is curious that substituting the ethanoic acid for a propyl group would result in such a loss of function as these substituents are the same length, and it is suggestive that hydrogen bonding (which could be formed by the carboxylic acid and hydroxyl groups, but not the alkyl chain), may be responsible for the agonist activity of 5-O-ethanoic acid 2.25c and 5-hydroxyls 2.23a and 2.23c-f.
Table 9: Potency (EC$_{50}$) and efficacy (E$_{\text{max}}$) of 5- and 7-substituted indoles at hCB$_2$ and hCB$_1$ receptors, determined using a forskolin-stimulated cAMP assay. $^a$

![Diagram of 3.7a and 3.4, 3.7b, 3.6, 3.13, 3.14, 3.24](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>EC$<em>{50}^b$ or IC$</em>{50}^c$ (nM ± SEM)</th>
<th>E$_{\text{max}}^f$ (% ± SEM)</th>
<th>Func. $^g$</th>
<th>EC$_{50}^b$ (nM ± SEM)</th>
<th>E$_{\text{max}}^f$ (% ± SEM)</th>
<th>Func. $^g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7a</td>
<td>-</td>
<td>-</td>
<td>NR</td>
<td>-</td>
<td>-</td>
<td>NR</td>
<td>-</td>
</tr>
<tr>
<td>3.4</td>
<td>H</td>
<td>271.7 ± 131 $^c$</td>
<td>189.8 ± 13.8</td>
<td>IA</td>
<td>632.5 ± 315</td>
<td>64.3 ± 8.1</td>
<td>A</td>
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<tr>
<td>3.7b</td>
<td>C$_2$H$_2$</td>
<td>30.6 ± 13.9 $^c$</td>
<td>263.3 ± 14.4</td>
<td>IA</td>
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<td>-</td>
<td>NR</td>
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<tr>
<td>3.6</td>
<td>CH$_2$CO$_2$H</td>
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<td>84.9 ± 3.3</td>
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<td>n.d.</td>
<td>80.5 ± 5.5</td>
<td>A</td>
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<tr>
<td>3.13</td>
<td>C$_6$H$_5$CO$_2$Me</td>
<td>&gt;10000 $^c,d$</td>
<td>165.8 ± 7.1</td>
<td>IA</td>
<td>n.d.</td>
<td>77.8 ± 3.6</td>
<td>A</td>
</tr>
<tr>
<td>3.14</td>
<td>C$_6$H$_5$CO$_2$H</td>
<td>&gt;10000 $^c,d$</td>
<td>123.6 ± 7.7</td>
<td>IA</td>
<td>n.d.</td>
<td>82.5 ± 1.6</td>
<td>A</td>
</tr>
<tr>
<td>3.24</td>
<td>C$_6$H$_5$NHAc</td>
<td>&gt;10000 $^c,d$</td>
<td>150.9 ± 10.7</td>
<td>IA</td>
<td>n.d.</td>
<td>74.3 ± 4.9</td>
<td>A</td>
</tr>
<tr>
<td>SR144528</td>
<td>-</td>
<td>760 ± 61.7 $^d,e$</td>
<td>212.2 ± 2.5</td>
<td>IA</td>
<td>-</td>
<td>-</td>
<td>NR</td>
</tr>
<tr>
<td>CP55,940</td>
<td>-</td>
<td>5.6 ± 2.3 $^b$</td>
<td>41.0 ± 1.4</td>
<td>A</td>
<td>n.d.</td>
<td>56.5 ± 1.4</td>
<td>A</td>
</tr>
</tbody>
</table>

$^a$ cAMP levels measured in a BRET assay using a CAMYEL sensor, performed in HEK293-hCB$_2$ or hCB$_1$ S4 low cells. Data are the mean ± SEM of three individual experiments performed in duplicate, except $^e$ which is two individual experiments performed in duplicate.

$^b$ EC$_{50}$ for compounds showing agonist activity. $^c$ IC$_{50}$ for compounds showing inverse agonist activity.

$^d$ compounds did not reach a plateau for maximum efficacy.

$^f$ E$_{\text{max}}$ is the response at 10 µM, normalised to basal (0%) and forskolin only (100%) levels of cAMP and or agonists, the lower the value, the greater the efficacy, whereas for inverse agonists, the higher the value, the greater the efficacy.

$^g$ Compounds were characterised as either agonists (A) or inverse agonists (IA). t-Tests were used to determine if the E$_{\text{max}}$ observed was significantly different from the forskolin only response and if no significant difference was found, the compounds were deemed to show no response (NR).

$^h$ n.d. = value was not determined.
In contrast to the 5-substituted agonists, derivatisation of the AI core in the C7 position led to inverse agonist activity at CB$_2$ receptor in five out of six compounds ($3.4$, $3.7b$, $3.13$, $3.14$, $3.24$) analysed (Table 9). Of note is the dramatic difference in function between the regioisomers 5-hydroxy-$2.23e$ (Table 5) and 7-hydroxy-$3.4$ (Figure 37; Table 9), which showed agonism or inverse agonism at CB$_2$ receptor respectively. However, bucking this trend, 7-$O$-ethanoic acid derivative, $3.6$, showed weak agonist activity at CB$_2$ receptor.

![Figure 37: Forskolin-stimulated cAMP concentration response curves for $2.23e$ and $3.4$ at hCB$_2$ receptor. Area under the curve values are normalised so that forskolin only response = 100% and basal response = 0%. Data shown is representative of a single experiment, conducted in duplicate and data points shown are the mean ± SEM.](image)

It remains to be determined if this regioisomer functional switch between 5- and 7-substituted indoles is a more general trend since there is no additional SAR available in the literature directly comparing the function of 5- and 7-substituted analogues with regards to cAMP signalling. Pasquini et al identified 5-aryl-substituted indoles that displayed inverse agonism with cAMP signalling,$^{253}$ however with very different N1 and C3 indole substituents than those utilised in this study. In an interesting study by Frost et al$^{256}$ using 4-, 5-, 6- and 7-hydroxyl, $O$-benzyl and methoxy substituted indoles, the 7-substituted analogues failed to show agonism in a fluorescence imaging plate reader (FLIPR) calcium flux assay (but were not tested for antagonism or inverse
agonism), whilst two out of three 5-substituted analogues did display agonism through $G_{\alpha q/o5}$ coupling. It is important to note that this study only analysed the $G_i$ pathway via cAMP production and not any other CB signalling pathways such as $\beta$-arrestin recruitment, $G_q$ coupling (intracellular calcium release), or $G_s$ coupling (adenyl cyclase stimulation). GPCR ligands are well known to exhibit signalling bias,\textsuperscript{64, 290} and therefore the new ligands reported here could possess varying functions via signalling pathways other than cAMP. If a more comprehensive trend for a functional switch between 5- and 7-substituted indoles does indeed exist, this might be due to the 5- or 7-substituents inducing different interactions with receptor residues which might cause a conformational switch in the receptor to either the inactive R or active $R^*$ state. Such a trend has been reported in the literature with another scaffold, the 1,8-naphthyridin class of cannabinoids (see Chapters Four and Five), in which a functional switch has been shown between analogues with or without C6 substituents.\textsuperscript{145}

The compound with the highest hCB$_2$ receptor potency of the inverse agonists, \textbf{3.7b} (IC$_{50} = 30.6 \pm 13.9$ nM), was also the compound with the highest affinity for CB$_2$ receptor. In fact, \textbf{3.7b} represents a uniquely high affinity CB$_2$ receptor inverse agonist amongst the AI scaffold. In comparison to previously reported AI inverse agonists, \textbf{3.7b} showed improved hCB$_2$ receptor affinity over AM630 \textbf{1.14} (hCB$_2$ $K_i = 32.1$ nM, $K_{ihCB_2}/K_{ihCB_1} = 165$)\textsuperscript{135} but with less subtype selectivity. There are only very few higher affinity AIs that have been reported as inverse agonists, one example being the inverse agonist \textbf{2.14} ($K_i = 0.37$ nM at hCB$_2$, $K_i = 344.9$ nM at hCB$_1$ receptor, calculated using $[^3H]CP55,940$ \textbf{1.17} hCB$_2$ $K_d = 0.31$ nM, hCB$_1$ $K_d = 0.18$ nM) reported by Pasquini \textit{et al.}\textsuperscript{253} However there are many higher affinity CB$_2$ receptor AI-based agonists reported, which could be attributed to the increased interest in CB receptor agonists driven by drug discovery efforts.\textsuperscript{113} It was very exciting to see that \textbf{3.7b} also showed a higher affinity for CB$_2$ receptor than diarylpyrazole inverse agonist SR144528 \textbf{1.13} ($K_i = 51.0 \pm 3.0$ nM at hCB$_2$) in both this study and when considering literature reported binding affinities for SR144528 \textbf{1.13}.\textsuperscript{277} \textbf{3.7b} also showed higher potency and efficacy than SR144528 \textbf{1.13} (Figure 38; Table 9), which is often used as a tool in pharmacological research on CB$_2$ receptor.\textsuperscript{142, 143} These promising results demonstrate the potential utility of \textbf{3.7b} as a tool for which to study CB$_2$ receptor.
Figure 38: Forskolin-stimulated cAMP concentration response curves for 3.7b and SR144528 1.13 at hCB2 receptor. Area under the curve values are normalised so that forskolin only response = 100% and basal response = 0%. Data shown is representative of a single experiment, conducted in duplicate and data points shown are the mean ± SEM.

The 7-O-ethanoic acid 3.6 showed significantly reduced potency and efficacy as an agonist compared to the 5-O-ethanoic acid analogue (2.25c; Table 5), despite the nearly three-fold higher affinity of 3.6 compared to 2.25c for CB2 receptor. This reiterates the differing requirements for a ligand to show high displacement of [3H]CP55,940 1.17 versus producing a signalling response in the absence of competition.

Compound 3.13 was the highest affinity pharmacophore-linker conjugate, and 3.24 was the longest linked derivative to show appreciable hCB2 receptor binding (Table 8). However, despite both 3.13 and 3.24, as well as 3.14 behaving as inverse agonists at hCB2 receptor, an IC50 could not be estimated as a maximal response plateau was not reached (Figure 39). An IC50 of >10 µM for 3.13, 3.14 and 3.24 was determined by use of a one-sample t-test (Table 9). Whilst inverse agonists induce the inactive R state of the receptor, they behave differently to neutral antagonists, in that they are able to reduce basal receptor signalling levels (section 1.1.3). The large loss of potency and efficacy in 3.13, 3.14 and 3.24 compared to 3.7b may be due to the long 7-substituents disrupting interactions necessary for inverse agonist-induced reductions in basal signalling.
Figure 39: Forskolin-stimulated cAMP concentration response curves for 3.13, 3.14 and 3.24 at hCB₂ receptor. cAMP values are the AUC. Data shown is representative of a single experiment, conducted in duplicate and data points shown are the mean ± SEM.

7-Hydroxyindole 3.4 and 7-O-propyl 3.7b were also analysed for CB₁ receptor potency and showed either agonism or no response respectively (Table 9). Compounds 3.6, 3.13, 3.14 and 3.24 were analysed at CB₁ receptor only at 10 µM and all showed agonist activity. These results continue the trend observed in Chapter Two, in which all compounds demonstrated either agonism or no response at CB₁ receptor. The CB₂ receptor inverse agonist AM630 1.14 also behaves as a partial agonist at CB₁ receptor, demonstrating that the structural requirements for an inverse agonist/agonist at CB₂ or CB₁ receptor can be quite distinct.

All the compounds analysed for function in the cAMP BRET assay were also tested at 10 µM and 1 µM in WT HEK293 cells to verify that the observed effects at hCB₂ and hCB₁ receptors were receptor-mediated (Table 10). 7-O-Ethanoic acid 3.6 showed no significant response in the WT cells, verifying that the agonist activity observed for this compound was indeed CB receptor mediated. 3.4, 3.7a, 3.7b, and 3.14 all invoked a small but significant response at 10 µM but not at 1 µM. As 3.4, 3.7b and 3.14 all demonstrated a response in HEK293-hCB₂ cells at 1 µM, it can be concluded that the WT HEK cell response is not large enough to have significantly affected the calculated CB₂ receptor potencies. 5-O-Propyl 3.7a showed no response in either receptor
subtype, so a WT response at 10 µM is surprising, but not concerning. It could be possible that the WT response observed for 3.7a (where cAMP is increased) masks an agonist effect (where cAMP would be decreased) in the CB2 receptor assay by cancelling each other out, resulting in an appearance of no response at CB2 receptor. However, the minimal effect of 3.7a at 1 µM in the CB2 receptor cAMP assay, combined with no significant response at 1 µM in the WT cells suggests that any masked agonist activity of 3.7a would be of very low efficacy. 7-Hydroxyindole 3.4 demonstrated a response in HEK293-hCB1 cells at 1 µM, so it can be concluded that the WT HEK cell response is not large enough to have significantly affected the calculated CB1 receptor potency of 3.4. 7-O-Propyl 3.7b demonstrated no response at CB1 receptor, whilst 3.14 showed agonism at CB1 receptor but was not tested at 1 µM, so it cannot be definitively concluded that the agonist activity observed for 3.14 at CB1 is receptor mediated. Linker-pharmacophores 3.13 and 3.24 showed a significant response in WT HEK cells at both 10 µM and 1 µM, but as both compounds demonstrated very weak potency at CB2 receptor (IC50 >10 µM) the potential effect of non-receptor mediated activity on the measured CB receptor response is of limited concern.
Table 10: Forskolin stimulated cAMP response in wild-type HEK293 cells.  

<table>
<thead>
<tr>
<th>Compound</th>
<th>10 µM (% ± SEM)</th>
<th>1 µM (% ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4</td>
<td>135.3 ± 4.0 *</td>
<td>104.3 ± 2.5</td>
</tr>
<tr>
<td>3.6</td>
<td>102.8 ± 1.8</td>
<td>104.5 ± 3.3</td>
</tr>
<tr>
<td>3.7a</td>
<td>127.6 ± 4.9 *</td>
<td>95.0 ± 3.7</td>
</tr>
<tr>
<td>3.7b</td>
<td>133.9 ± 4.7 *</td>
<td>105.4 ± 2.5</td>
</tr>
<tr>
<td>3.13</td>
<td>160.6 ± 2.6 *</td>
<td>124.3 ± 2.4 *</td>
</tr>
<tr>
<td>3.14</td>
<td>129.1 ± 3.5 *</td>
<td>105.6 ± 3.6</td>
</tr>
<tr>
<td>3.24</td>
<td>128.0 ± 2.7 *</td>
<td>114.8 ± 7.1 *</td>
</tr>
<tr>
<td>WIN 55,212-2 2.2</td>
<td>102.6 ± 2.1</td>
<td>96.1 ± 1.0</td>
</tr>
<tr>
<td>CP55,940 1.10</td>
<td>98.0 ± 4.0</td>
<td>99.2 ± 2.7</td>
</tr>
<tr>
<td>SR144528 1.13</td>
<td>94.5 ± 1.6</td>
<td>99.8 ± 2.2</td>
</tr>
</tbody>
</table>

* Assay carried out in wild-type HEK293 cells measuring forskolin-stimulated (5 µM) cAMP. Data is normalised so that forskolin only response is 100% and vehicle only response is 0%. Data are the mean ± SEM of three individual experiments performed in duplicate. A one-sample t-test was used to determine if values were significantly different from forskolin only response (100%), and where values are significantly different, they are marked with *. 

3.6.3 Off target activity

Compounds 3.3, 3.4 and 3.7a were accepted into the Eli Lilly and Company Open Innovation Drug Discovery Program and the Tres Cantos Open Lab Foundation and did not show significant proprotein convertase subtilisin kexin type 9 (PCSK9) inhibition, GPR120 agonism, disruption to IL-17 protein-protein interaction, Leishmania donovani growth inhibition, voltage-gated potassium channel KCNQ2/3 agonism or nicotinamide N-methyltransferase (NNMT) inhibition.
3.7 Homology modelling of AIs at CB$_2$ receptor

Whilst modelling of AIs binding to CB receptors has only been reported in a handful of studies,$^{240, 245}$ molecular modelling combined with mutational and ligand binding studies has contributed significantly to rational design of CB$_2$ receptor selective ligands across a range of scaffolds.$^{86}$ Homology modelling offers a useful method for rationalising the pharmacological results observed in this study. However, there are limitations to homology modelling when attempting to design ligands or rationalise SAR. Generation of a homology model requires the crystal structure of a closely related protein and small differences in sequence homology can lead to significant differences in secondary structure, reducing how true-to-life the homology model is. Also, a homology model is generated based on a static structure and so is itself static, failing to account for the flexible and dynamic nature of GPCRs.

3.7.1 Generation of hCB$_2$ receptor homology models

3.7.1.1 hCB$_2$ receptor homology model based on β$_2$-adrenoceptor crystal structure

The first step in generating a homology model is making a sequence alignment between the template receptor and CB$_2$ receptor. At the time this PhD project commenced, no crystal structure was available for CB$_1$ receptor and so there were no template receptors available with a high degree of sequence homology to CB$_2$ receptor. Therefore, to generate a more reliable alignment, a consensus sequence alignment was made between CB$_2$ receptor and four of the most closely related crystallised GPCRs (bovine rhodopsin, β$_1$-adrenoceptor, β$_2$-adrenoceptor, and D$_3$ receptor). The alignment was generated using T-Coffee$^{291}$ and then optimised manually to account for known structural features (Figure 40). For instance, unlike most other GPCRs (84%), CB$_2$ receptor lacks the highly conserved proline (P5.50) in TMH5 and in its place there is a leucine (L5.50) which has been shown to be critical to the function of CB$_2$ receptor.$^{292}$ The conserved cysteine in TMH3, which is required in most other GPCRs for the highly conserved disulphide bridge connecting THM3 to phenylalanine in ECL2, is
also not present. However, there is a disulphide bridge present in ECL2 between two cysteine residues (C174 & C179 in CB2 and C257 & C264 in CB1 receptor), which is highly conserved in CB receptors and key to ligand binding.\textsuperscript{293, 294} Across the GPCRs there is a huge variation in sequence, length and structure between extracellular loops, making them difficult to model. This is especially true of ECL2 as it is key to ligand binding and so has specific structural features to suit the endogenous ligands of its receptor subfamily.\textsuperscript{295} The N- and C-termini were deleted in the sequence alignment due to the lack of sequence homology and a template structure in these regions.
Figure 40: Consensus sequence alignment between hCB2 receptor (CRN2) bovine rhodopsin (OPSD), human β2-adrenoceptor (ADRB2), human D3 receptor (DRD) and human β1-adrenoceptor (ADRB1), generated by the T-coffee server and then manually optimised. Conserved residues are highlighted red. Highly conserved residues across GPCRs that are not present in CB2 are highlighted cyan. T-coffee highlights highly conserved regions pink, moderately conserved yellow and poorly conserved green. The ‘/’ symbol in the ICL3 sequence region indicates where there is a break in the chain, due to deleted sequence where stabilising moieties, such as antibodies, are present in the crystal structures. P34972, P02699, P07550, P35462-3 and P08588 are the Universal Protein Resource (UniProt) sequences used for hCB2, bovine rhodopsin, human β2-adrenoceptor, human D3 receptor, and human β1 adrenoceptor, respectively.

Using this consensus alignment (Figure 40), a series of 25 models was generated based on just one template to minimise errors. The human β2-adrenoceptor (protein database (PDB) ID: 2RH1) was selected as the template as it had the highest sequence homology
(18% identity, 35% similarity‡‡‡) to CB₂ receptor of all the available high resolution class A GPCR crystal structures. Models were evaluated by the DOPE (discrete optimised protein energy) method which is the most reliable method for detecting native-like models.²⁹⁶ The model with the best energy profile (most negative DOPE score; model A; Figure 41) was then chosen from the 25 models generated.

![Figure 41: Homology model of CB₂ receptor (model A), based on the crystal structure of inverse agonist bound β₂-adrenoceptor (PDB ID: 2RH1).](image)

3.7.1.2 CB₂ receptor homology models based on CB₁ receptor crystal structures

Homology model A was intended to be employed for ligand docking studies, to aid in the rationalisation of pharmacological results. However, after biological evaluation of the indole series was completed, two crystal structures of inactive state CB₁ receptor were reported (one inverse agonist-bound, one antagonist-bound),⁶⁶,⁶⁷ which were followed by two agonist-bound CB₁ crystal structures.⁶⁵ This was an exciting development as a CB₁ receptor template provided a much closer related structure and

‡‡‡ Sequence identity and similarity calculated by a similarity search on GPCRdb,²⁵ comparing the TMH and loop regions (not including the N-and C-terminals).
sequence to CB₂ receptor (44% sequence identity, 61% similarity\textsuperscript{88}), enabling generation of a more accurate homology model. Therefore, new homology models of CB₂ receptor were generated based on these CB₁ crystal structures.

A sequence alignment between hCB₂ and hCB₁ receptors was generated using T-coffee\textsuperscript{291} and manually optimised (Figure 42). CB₁ receptor, similarly to CB₂ receptor, does not possess the highly conserved cysteine residue in TMH3, has L5.50 in place of the highly conserved P5.50 and contains the cysteine disulphide bridge in ECL2.\textsuperscript{294, 297}

The sequence of hCB₁ receptor is much longer than hCB₂ receptor (472 versus 360

\textsuperscript{88} Sequence identity and similarity calculated by a similarity search on GPCRdb,\textsuperscript{25} comparing the TMH and loop regions (not including the N- and C-terminals).
amino acids), and contains much longer N- and C-terminals, as well as a longer ECL2 and ICL3. A key residue difference between CB2 and CB1 receptors is in the flexible hinge of TMH6 with the sequence CWFP, in which the phenylalanine of CB2 receptor is replaced with a glycine in CB1 receptor.

Using the CB2-CB1 sequence alignment (Figure 42), three sets of 25 models were generated using three of the CB1 crystal structures (PDB ID: 5U09; 5TGZ; 5XRA) as templates and then assessed by DOPE score. From each of the three sets, the model with the best energy profile was selected (Figure 44). These models will hereafter be referred to as model B, model C and model D, generated using the 5U09, 5TGZ and 5XRA CB1 crystal structures respectively. Models B and C have been used to model the inactive R state of CB2 receptor (as they are based on the inverse agonist- and antagonist-bound CB1 structures, respectively) and model D has been used to model the active R* state of CB2 receptor (as it is based on the agonist-bound CB1 structure). The conformation of residues within the ligand binding site of each of the models closely matched those of the respective crystal structures and the highly conserved disulphide bridge of ECL2 was present in all three models. Models B, C and D were used for docking studies of the indole series, as well as the naphthyridin-based series discussed in Chapters Four and Five.

### 3.7.2 Comparison of homology models A, B, C and D

Comparison of R state model A (generated based on β2-adrenoceptor) with R state models B and C (generated based on CB1 receptor) illustrates the considerable influence of the template on the secondary and tertiary structure of the homology model (Figure 43). Model A shows significant differences in the secondary structure of TMH2 as well as more subtle differences in the orientation of all other TMHs. Key residues, such as S285 show a significant shift in positioning and the ECLs (which are difficult to model accurately anyway due to inherent flexibility) are considerably different, in particular ECL2 and the disulphide bridge. It would be expected that these significant variations in structure would have a considerable impact on the results of ligand docking studies. Model A was not utilised in ligand docking studies and so it will not be discussed further.
The two R state models B and C show close overlap of residue conformation and secondary structure (Figure 44). One key point of difference is in the conformation of S285, however, they both match the S285 conformation of their respective CB₁ crystal structure templates. Comparison of the R* state model D with the R state models B and C shows some movement of TMHs between active and inactive states. For instance, in the active state (model D) the tops of TMH1 and TMH2 have moved in, whilst the bottom of TMH6 has splayed out at the point of the flexible CWFP hinge. This change in receptor conformation reflects the effect of agonist binding to the template CB₁ receptor and homology modelling relies on the assumption that a similar movement of TMHs will be observed upon agonist binding to CB₂ receptor. It is interesting to note that all three ECLs show very similar conformations (ECL2 in particular) between the three homology models. This is surprising as the ECLs are very flexible.²⁹⁸ The presence of the disulphide bridge in ECL2 may induce a fairly consistent conformation in this region. In addition, the overall similarity in the ECLs could be reflective of a favourable conformation of CB₁ receptor ECLs for crystallisation, rather than a favourable native state.
Figure 44: hCB$_2$ receptor homology models generated using a sequence alignment between hCB$_2$ and hCB$_1$ receptors and the crystal structures of hCB$_1$ receptor. Models B (green; based on PDB ID: 5U09) and C (red; based on PDB ID: 5TGZ) represent the inactive R state of CB$_2$ receptor, whereas model D (blue; based on PDB ID: 5XRA) represents the active R* state. (A) Viewed from the lipid membrane. (B) Viewed from the extracellular space.
There are two notable differences in residue orientation in the ligand binding pocket between the R* and R state models. In the R* state model, F117 has shifted considerably, whilst W258 of the flexible hinge has also moved (Figure 45). This shift in residue F117 is reflected in subtle differences in the conformation of TMH3. In the R state models B and C there are offset stacked π-interactions between residues F117 and W258, which may stabilise the receptor conformation. It is possible that bulky antagonist or inverse agonist binding causes F117 to be pushed down, locking its movement and ability to rotate up into the conformation seen in the R* model D. The flexible hinge region CWXP in TMH6 is largely accepted to act as a conformational switch in GPCRs and it is therefore not surprising to see considerable movement of the flexible hinge residues W258 and F259 between the R and R* state models. This variation in positioning of residues within the flexible hinge is likely responsible for TMH6 in R* state model D being splayed out below the flexible hinge (Figure 44).

Figure 45: Key ligand binding residues in CB2 receptor model B (green), C (red) and D (blue). Some extracellular loops are hidden for clarity. There is a clear shift in the orientation of residues F117 and W258 between the inactive and active states.

The shift in TMHs is also reflected in the surface of models of B, C and D, showing a change in openings between TMHs for potential ligand entrance into the receptor.
orthosteric binding site (Figure 46). In the R state models B and C there are openings between TMH1 and 2 and between TMH1 and 7. However, in the R* model D the openings have shifted to between TMH2 and 3 and between TMH3 and 4, indicating that ligand entry into CB$_2$ receptor would have to be achieved through a different region in the active state. Openings between the ECLs into the extracellular space have also moved between the R state and R* state models, however the flexibility of the ECLs makes it difficult to extrapolate meaningful observations in this change.

Figure 46: Surface of hCB$_2$ receptor homology models B (green), C (red) and D (blue), all viewed from the same angle, showing the entrance to the ligand binding pocket between TMH1 and 7 in models B and C, which is closed in model D.

A detailed comparison of these homology models with literature models has not been included here, as CB$_2$ receptor homology models reported prior to the determination of CB$_1$ crystal structures have utilised other, less closely related crystal structures and therefore will differ slightly to the models generated here.

### 3.7.3 Ligand docking studies

Ligand docking studies were carried out with the aim of rationalising the differences in CB$_2$ receptor affinity observed between ligands. Specifically, of interest was the affinity difference between 5-hydroxyl 2.23a and 6-hydroxyl 2.23b, as well as between the N1-methyl-THP derivatives 2.23e, 2.25c, 3.6 and 3.7a. In addition, the favourability of 7-
substitution over 5-substitution was of interest. Docking of longer linker-AI conjugates 3.13 and 3.24 was carried out with the aim of rationalising whether it was a promising lead for further development of fluorescent ligands.

### 3.7.3.1 Agonist docking studies

The agonists 2.23a, 2.23e, 2.25c and 3.6, as well as 3.7a (which showed no response in the cAMP assay) and 2.23b (which showed minimal CB$_2$ receptor affinity) were docked into the R* state model D. In order to validate the docking results, the CB$_2$ receptor-docked 5-hydroxyl 2.23e was compared to the orientation of agonist AM11542 which was bound in the CB$_1$ crystal structure (Figure 47A). The ligands overlapped, indicating the appropriate region of the binding site had been identified. The indole ring overlapped with the cyclohexane ring of AM11542 and the carbonyl overlapped with the oxygen atom of the middle ring of AM11542. The overlap fulfilled the expectation that the aromatic methoxyphenyl would be orientated towards the aromatic region of the ligand binding pocket between TMH3 and TMH6. As a control, the CB receptor agonist CP55,940 1.10 was also docked into model D and when overlaid with AM11542, it showed a close overlap of structural features, such as the cyclohexanol rings, aliphatic alkyl chains and cyclohexane rings, thereby validating the homology model and ligand docking protocol (Figure 47B).

![Figure 47: Overlay of CB$_1$ receptor-bound AM11542 (pink) with the most consistent, lowest energy poses of CB$_2$ receptor-docked (A) 2.23e (cyan) or (B) CP55,940 1.10 (blue). Receptor is hidden for clarity.](image)
The docking poses of 5-hydroxyl indole 2.23e, 5-O-ethanoic acid 2.25c, 5-O-propyl 3.7a and 7-O-ethanoic acid 3.6 were compared to try to rationalise the differences in CB\textsubscript{2} receptor affinity. The most consistent docking poses of 2.23e and 2.25c (three out of four and seven out of ten poses, respectively) showed the methoxyphenyl group orientated towards the aromatic TMH3-TMH6 region of the ligand binding pocket, the methyl-THP group pointing upwards, and the 5-O-substituent pointing between TMH2 and TMH3 (Figure 48A & B). The most consistent pose (three out of four) of 3.7a was similarly orientated (Figure 48C) to 2.23e and 2.25c, whereas the most consistent pose of 7-substituted 3.6 (three out of four) was shifted considerably (Figure 48D).

Figure 48: Consensus poses of (A) 2.23e, (B) 2.25c, (C) 3.7a and (D) 3.6 in R* state CB\textsubscript{2} receptor model D. Side chains of residues within 4 Å of the ligands are shown as sticks (pale yellow). Red atoms represent oxygen, blue atoms represent nitrogen and dark yellow atoms represent sulphur. Some ECLs are hidden for clarity.

The ligand docking studies did not produce an obvious explanation for the difference in CB\textsubscript{2} receptor affinity between the high affinity 5-hydroxyl 2.23e and the analogous, moderate affinity 5-O-ethanoic acid 2.25c and 5-O-propyl 3.7a, as the docking poses of
these ligands are very similar (Figure 49A). 5-<i>O</i>-propyl 3.7a and 5-<i>O</i>-ethanoic acid 2.25c show similar affinity, so it does not appear to be principally related to the ionised carboxylic acid of 2.25c. The 5-hydroxyl of 2.23e may be engaged in favourable hydrogen bonding not demonstrated in the docking results, which could be disrupted by extension with a propyl or ethanoic acid. Comparing the docking poses of 2.25c and 3.6, there is a clear downward shift in the positioning of the indole ring of 3.6, allowing the methoxyphenyl group to extend a bit further into the aromatic region of the ligand binding pocket than 2.25c. This may allow greater aromatic interactions and van der Waals interactions with the hydrophobic pocket, improving the affinity of 3.6 for CB<sub>2</sub> receptor. In addition, the different positioning of hydrogen bond acceptors such as the carbonyl or ether moieties may allow for formation of more favourable hydrogen bond interactions between 3.6 and CB<sub>2</sub> receptor.

![Figure 49: Overlay of the most consistent, lowest energy docking poses of (A) 5-hydroxyl 2.23e (cyan), 5-<i>O</i>-ethanoic acid 2.25c (green) and 5-<i>O</i>-propyl 3.7a (magenta) and (B) 5-<i>O</i>-ethanoic acid 2.25c (green) and the analogous 7-<i>O</i>-ethanoic acid 3.6 (orange) docked into R* state CB<sub>2</sub> receptor model D. Side chains of residues within 4 Å of the ligands are shown as sticks (pale yellow). Some ECLs are hidden for clarity.](image)

Docking studies with 5-hydroxyl 2.23a and 6-hydroxyl 2.23b into R* state model D failed to identify a reason for the poor affinity of 2.23b, as they both docked in almost identical positions.
3.7.3.2 Inverse agonist docking studies

Docking of inverse agonists 3.4, 3.7b, 3.13 and 3.24 was initially attempted in both model B and C, however docking with model C produced more consistent docking poses and so was used for all AI inverse agonists. The aim of the docking studies was to rationalise the high affinity of 3.7b and investigate why 7-hydroxyl 3.4 and 7-O-propyl 3.7b had higher CB2 receptor affinity than the analogous 5-hydroxyl 2.23e and 5-O-propyl 3.7a. The binding position of the linker-AI conjugates 3.13 and 3.24 was also of interest, as the lead compounds for further development of AI-based fluorescent ligands for CB2 receptor.

The docked poses of 7-O-propyl 3.7b and 7-hydroxyl 3.4 show overlap in the methoxyphenyl and ethylmorpholino moieties, but the indole ring of 3.7b is positioned at an angle of about 40° along the C3 and C8 edge of the 3.4 indole ring (Figure 50). This shift in positioning of the core indole, as well as the lipophilic O-propyl being able to engage in van der Waals interactions with hydrophobic side chains, may be responsible for the 9-fold increase in CB2 receptor affinity for 3.7b compared to 3.4.
Figure 50: Overlay of the most consistent, lowest energy docking poses of 7-hydroxyl 3.4 (yellow) with 7-O-propyl 3.7b (orange) docked into R state CB$_2$ receptor model C. Side chains of residues within 4 Å of the ligands are shown as sticks (pale green). Some ECLs are hidden for clarity.

The most evident SAR identified in this chapter was the higher CB$_2$ receptor affinity conferred by 7-substituents compared to 5-substituents. Comparing the poses of 7-hydroxyl 3.4 and 7-O-propyl 3.7b docked into R state model C, with the analogous 5-hydroxyl 2.23e or 5-O-propyl 3.7a docked into R* state model D, there is a clear difference in the ligand orientation. Overall, 7-hydroxyl 3.4 is positioned deeper into the ligand binding pocket than 5-hydroxyl 2.23e and the methoxyphenyl of 3.4 protrudes deeper into the hydrophobic pocket between TMH3-6 (Figure 51A). Similarly, 7-O-propyl 3.7b is also buried further into the ligand binding pocket, with the methoxyphenyl deeper into the hydrophobic pocket, than the analogous 5-O-propyl 3.7a (Figure 51B). This pattern is similar to that shown between the docking of 7-O-ethanoic acid 3.6 and 5-O-ethanoic acid 2.25c in the active R* model D (Figure 49).

The ability to engage in greater aromatic interactions, which are key to CB$_2$ receptor affinity, may be a significant factor in the higher affinity of the 7-substituted AIs in this series.
Figure 51: Overlay of the most consistent, lowest energy docking poses of (A) 5-hydroxyl 2.23e (cyan) and 7-hydroxyl 3.4 (yellow) and (B) 5-O-propyl 3.7a (magenta) and 7-O-propyl 3.7b (orange). 7-Hydroxyindole 3.4 and 7-O-propyl 3.7b are docked into R state CB2 receptor model C (shown), whereas 2.23e and 3.7a are docked into R* state model D (not shown). Side chains of residues within 4 Å of the ligands are shown as sticks (pale green). Some ECLs are hidden for clarity.

The inverse agonist linker-AI conjugates 3.13 and 3.24 were also docked into R state model C to gain further understanding of how these two ligands were able to retain affinity for CB2 receptor and how they might be successfully developed into fluorescent ligands. The docking poses of 3.13 were clustered into two groups, with the most consistent pose showing the linker oriented out of the binding pocket, into the lipid bilayer (Figure 52A). In this pose, the 7-ether demonstrates hydrogen bonding with S285, whilst THP hydroxyl and the ester carbonyl form hydrogen bonds with residue N20 of the amino terminal. The other, smaller cluster of poses (three out of ten) of 3.13 showed the linker exiting through the extracellular loops, however the flexibility of extracellular loops makes docking in this region difficult. The most consistent docking pose of acetylated 7-O-hexylamine 3.24 showed close overlap to the most consistent pose of 3.13, with the 7-ether and THP hydroxyl engaging in hydrogen bonding with the same residues as 3.13 (Figure 52A). The phenyl groups of 3.13 and 3.24 are positioned in a hydrophobic pocket between TMH3-TMH6 and the indole core and phenyl are able to engage in van der Waals interactions with multiple nearby aromatic
residues (F183, W194, W258 and F281). The linker terminals of the docked ligands 3.13 and 3.24 protrude slightly out of the ligand binding pocket, orientating towards the gaps between TMH1 and TMH7 and between TMH1 and TMH2 (Figure 52B). Molecular dynamics simulations conducted by Jakowiecki et al have revealed a likely entry route between TMH1 and TMH7 for (-)-Δ⁹THC 1.5 and AEA 1.1 binding to CB₁ receptor. Similarly, an exit pathway from the orthosteric binding site of CB₂ receptor between TMH1 and TMH7 has been proposed for the biotin-containing CB₂ receptor probe 1.20. In addition, the taranabant-bound CB₁ receptor crystal structure indicated a ligand entry channel between TMH1 and TMH7. The linker poses of 3.13 and 3.24 reinforce that the indole C7 position holds promise for linker and fluorescent dye conjugation.

Figure 52: The most consistent, lowest energy docking pose of inverse agonists 3.13 (cyan) and 3.24 (orange) docked into R state hCB₂ receptor homology model C. (A) Hydrogen bonding (yellow dashed line) is shown between the 7-ether and S285 as well as the THP hydroxyl and the ester carbonyl (of 3.13) with N20 of the N-terminal. Side chains of residues within 4 Å of the ligands are shown as sticks (pale green). (B) Model C shown with surface, indicating potential entry points between TMH1 and 7 or TMH1 and 2 for 3.13 and 3.24 into CB₂ receptor.

Examining how far the linker terminals of 3.13 and 3.24 reach out of the ligand binding pocket, may help to determine why further extension of these ligands was not ultimately successful. Linker-pharmacophore conjugate 3.24 was conjugated directly to
the fluorophore to give 3.25, however the docking pose of 3.24 (Figure 52B) suggests that the linker would not have been long enough to allow favourable positioning of both the fluorophore and pharmacophore of 3.25. Conversely, 3.13 was extended by 10 atoms with a PEG linker prior to conjugation of the fluorophore and it is possible that the linker in the resulting fluorescent ligand was too long. This hypothesis is reinforced by examining the docking results of the more successful CB2 receptor fluorescent ligands identified in Chapter Five (section 5.5) which suggest that a favourable linker length reaches slightly further out of the ligand binding pocket than 3.24.
3.8 Conclusions

The work discussed in this chapter was initiated by a desire to explore whether the CB$_2$ receptor affinity of the 5-substituted linker-AI conjugates evaluated in Chapter Two could be improved by reducing the polarity of the region of the linker immediately adjacent to the indole core. However, contrary to expectations, it was demonstrated that replacement of the ethanoic acid moiety of 2.25c with the same length propyl group (3.7a) led to no significant improvement in affinity. The finding that substitution with a simple, small propyl group did not retain CB$_2$ receptor affinity indicated that extension of the lead compound from Chapter Two, 5-hydroxyl 2.23e, would likely lead to only low affinity compounds.

Simultaneously to the exploration of varying polarity at the C5 position, 7-substituted indoles were also investigated as CB$_2$ receptor ligands. A clear preference for 7- over 5-substituted indoles was demonstrated and a new, high affinity CB$_2$ receptor selective inverse agonist 3.7b was identified ($K_i = 5.7 \pm 1.4$ nM at hCB$_2$, $K_i$hCB$_1$/$K_i$hCB$_2 = 88$). 7-O-Propyl 3.7b is an especially high affinity CB$_2$ receptor inverse agonist with high potency and efficacy both within the AI scaffold and other classes of CB$_2$ receptor inverse agonists. A general trend of agonism for 5-substituted indoles and inverse agonism for 7-substituted indoles was observed in the cAMP assays.

Extension of the new lead compound 3.7b resulted in the pharmacophore-linker conjugate 3.13 which retained CB$_2$ receptor affinity and demonstrated that a linker at the C7 position of indole is tolerated. Modelling suggests that 3.13 would be amenable to further extension and fluorophore conjugation. Unfortunately, further linker extension and conjugation of a fluorescent dye to the linker-indole derivatives 3.13 and 3.24 failed to produce a high affinity CB$_2$ receptor fluorescent ligand.

3.8.1 Future directions

3.13 remains a promising pharmacophore-linker lead and future studies could explore extension of 3.13 to identify more appropriate linker lengths and types in addition to
conjugation with different fluorescent dyes. Ligand docking studies with 3.13 and 3.24 suggest that the linker lengths of the final fluorescent compounds 3.18 and 3.25 were unfavourable. In light of the results from Chapter Five, it may be worthwhile exploring conjugation of a short linker, between three to eight atoms (rather than the longer 10 atom linker 2.28b), onto 3.13, before conjugation of BODIPY 630/650-X OSu 2.15. In addition, conjugation of a linker possessing multiple groups capable of hydrogen bonding with the polar residues located around the exit of the orthosteric site may help improve affinity for CB₂ receptor.

In the context of the C5-C7 indole work presented in this chapter and Chapter Two and in light of previous efforts of linker conjugation via the C3 acyl group²⁵⁴ or extension of the N1 substituent,²⁴³ the search for an AI-based fluorescent ligand for CB₂ receptor remains elusive.
Chapter Four: Fluorescent ligands for CB$_2$ receptor based on N-substituted 1,8-naphthyridin

4.1 Scaffold selection

Biological evaluation of the indole-based compounds (Chapters Two and Three) indicated very few positions on the indole scaffold tolerated a linker and then attachment of fluorescent dye. Therefore, a literature review of cannabinoid structural classes was undertaken to select a second scaffold for development into CB$_2$ selective fluorescent ligands. The criteria for selecting this scaffold was similar to the indole scaffold, in that it should have high affinity and selectivity for CB$_2$ receptor, have existing SAR indicating positions amenable to change and demonstrate antagonist or inverse agonist activity. In addition, a relatively polar cannabinoid was sought in order to reduce the overall hydrophobicity of the final fluorescent compound, thereby in theory reducing non-specific membrane binding. It was with this criteria in mind that the 1,8-naphthyridin-2(1$H$)-one-3-carboxamide scaffold$^{145}$ was chosen for development of a series of fluorescent CB$_2$ receptor ligands. The following section (4.1.1) outlines the literature SAR of this scaffold as a CB receptor ligand, which then informed the decision of linker placement (as discussed in section 4.2).

4.1.1 1,8-Naphthyridin-3-carboxamide-based cannabinoids

1,8-Naphthyridin-4(1$H$)-one-3-carboxamide (Figure 53) based cannabinoids were initially designed by including key structural motifs of other classes of cannabinoids,$^{300}$ such as the N1-alkyl or aryl alkyl substituents of AAIs (e.g. WIN 55,212-2 2.2) and the C3-aliphatic or aromatic carboxamide substituents of quinolines (e.g. JTE-907 1.15). This strategy led to identification of several nanomolar affinity (e.g. 4.1 and 4.2, Figure 53A) CB$_2$ receptor ligands with moderate selectivity ($K_{i}$CB$_1$/$K_{i}$CB$_2$ approx. 20–40).
Refinement of the series shown in Figure 53A guided by molecular modelling and ligand docking of nonaromatic substituents at the C3 carboxamide group (R$^2$) and different N1 (R$^1$) lipophilic substituents, resulted in ligands with moderately improved affinity and selectivity for CB$_2$ receptor (e.g. 4.3 and 4.4, Figure 53B). Varying the C7 (R$^3$) substituent with methyl, chloro, methoxy or hydrogen had only a minimal impact on affinity and selectivity, indicating that C7 (R$^3$) substituents were not essential (unsubstituted C7-H derivatives are detailed in Table 11). In addition, it was demonstrated that the presence of a carbonyl group on the naphthyridin ring (which was hypothesised to induce planarity with the carboxamide via intramolecular bonding with the amidic NH) was necessary for CB$_2$ receptor affinity.

Exploration of how to further improve the CB$_2$ receptor affinity and selectivity of this scaffold, led to considering the position of the carbonyl group. Superimposition of a 1,8-naphthyridin-4(1H)-one-3-carboxamide derivative with the corresponding 1,8-naphthyridin-2(1H)-one-3-carboxamide derivative showed adequate overlap of the key substituents. Therefore, a series of 1,8-naphthyridin-2(1H)-one-3-carboxamide derivatives (Figure 54) was synthesised.

| Figure 53: Examples of the first 1,8-naphthyridin-4(1H)-one-3-carboxamide based cannabinoids, (A) 4.1 and 4.2$^{300}$ and (B) 4.3 and 4.4$^{301}$ |  |
|---|---|---|
| A | 4.1 | R$^1$ = ethylimorpholino, <br>R$^2$ = cycloheptyl, R$^3$ = CH$_3$ <br>$K_f = 22$ nM mCB$_2$ <br>$K_i = 560$ nM mCB$_1$ | B | 4.3 | R$^1$ = benzyl, <br>R$^2$ = cycloheptyl, R$^3$ = CH$_3$ <br>$K_i = 5.1$ nM mCB$_2$ <br>$K_i = 143$ nM mCB$_1$ |
|  | 4.2 | R$^1$ = ethylimorpholino, <br>R$^2$ = cyclohexyl, R$^3$ = Cl <br>$K_f = 25$ nM mCB$_2$ <br>$K_i = 1000$ nM mCB$_1$ |  | 4.4 | R$^1$ = phenethyl, <br>R$^2$ = cyclohexyl, R$^3$ = CH$_3$ <br>$K_i = 16.3$ nM mCB$_2$ <br>$K_i = >1000$ nM mCB$_1$ |
Figure 54: General structure of 1,8-naphthyridin-2(1H)-one-3-carboxamide based cannabinoids. Refer to Table 12 for example R groups. The grey dashed line between the C2 carbonyl and NH indicate the position of a potential intramolecular hydrogen bond.

Manera et al concluded that the 2-one (Figure 54) derivatives\textsuperscript{303} showed a significant improvement in CB\textsubscript{2} receptor affinity (and in selectivity for the R\textsuperscript{1} ethylmorpholino containing derivative), when compared with the 4-one (Figure 53) derivatives\textsuperscript{301} (see Table 11 for comparison). Therefore, 2-one derivatives were pursued exclusively in the development of this scaffold. However, it is important to note that the two studies used different species of CB receptor to determine $K_i$ values: mouse brain membranes (CB\textsubscript{1}) and mouse spleen homogenate (CB\textsubscript{2}) for the 4-one derivatives\textsuperscript{301} versus HEK293 hCB\textsubscript{1} and hCB\textsubscript{2} membranes for the 2-one derivatives.\textsuperscript{303} The $K_i$ value for a reference compound, JWH-133 (a classical cannabinoid) was determined in both studies, with significantly different selectivity found. At mouse receptors, JWH-133 showed a $K_i$ of 65 nM at CB\textsubscript{2} and 458 nM at CB\textsubscript{1} receptor (seven fold selectivity for CB\textsubscript{2} receptor),\textsuperscript{301} whereas at human receptors JWH-133 showed a $K_i$ of 3 nM at CB\textsubscript{2} and 677 nM at CB\textsubscript{1} receptor (226 fold selectivity for CB\textsubscript{2} receptor; Table 11).\textsuperscript{303} This difference in affinity and selectivity broadly echoes the differences found between the 4-one and 2-one series and therefore, without repeating binding assays of the 4-one derivatives at human CB receptor, it is questionable how significant the difference in affinity and selectivity really is between these two series. Any real improvement in affinity and selectivity may be due to the 3-carboxamide group not having to rotate to form an intramolecular hydrogen bond between the amidic NH and the 2-one, therefore altering the spatial arrangement of the carboxamide R\textsuperscript{2} substituent (Figure 54).
Table 11: A comparison of literature\textsuperscript{301,303} reported radioligand binding data for 1,8-napthyridin-4(1\textit{H})-one-3-carboxamide and 1,8-napthyridin-2(1\textit{H})-one-3-carboxamide derivatives. \textsuperscript{a}

![Chemical structure](image)

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\textsuperscript{a} Data represent mean values of \(K_i\) (nM) from experiments performed at least three times in duplicate.

\textsuperscript{b} Data from Manera \textit{et al}, 2006.\textsuperscript{301} A dash indicates that a direct comparison compound to the 2\textit{-}one derivative is not available, as the 4\textit{-}one derivatives with matching R\textsuperscript{1} and R\textsuperscript{2} groups contained either chloro or methyl substituents at C7. Affinity of compounds determined using mouse spleen for CB\textsubscript{2}, mouse brain membranes for CB\textsubscript{1} receptor and \([\text{H}]\text{CP55,940}\).

\textsuperscript{c} Data from Manera \textit{et al}, 2009.\textsuperscript{303} Affinity of compounds determined using membranes from HEK293 cells transfected with either hCB\textsubscript{2} or hCB\textsubscript{1} receptor and \([\text{H}]\text{CP55,940}\) (\(K_d = 0.18\) nM at CB\textsubscript{1} or \(K_d = 0.31\) nM at CB\textsubscript{2}).

\textsuperscript{d} SI = \(K_i(\text{CB}_1)/K_i(\text{CB}_2)\).
Several high affinity, selective ligands were identified amongst the 1,8-naphthyridin-2(1H)-one-carboxamide series, with two derivatives possessing subnanomolar affinity for CB$_2$ receptor and >200 fold selectivity over CB$_1$ receptor (Table 11, entry 8 and 10). In addition, this series revealed some interesting SAR, including that 4-methylcyclohexyl substitution at the carboxamide (R$^2$) confers better affinity and selectivity for CB$_2$ receptor than with a cyclohexyl (Table 11, cf. entry 4 with 1, 5 with 2, or 8 with 3). Some derivatives were tested with the 4-methylcyclohexyl substituent (R$^2$) as a mixture of both the cis and trans stereoisomers (i.e. the orientation of the methyl para to the carboxamide; Table 11, entries 4, 5 and 8). The SAR shows that it is the cis stereoisomer that is responsible for the high CB$_2$ receptor affinity and selectivity of these compounds, as the isolated trans isomers show significantly worse affinity and selectivity (Table 11, cf. entry 6 with 7, or 9 with 10). Other substituents trialled at the carboxamide (R$^2$) included cycloheptyl, which led to improved CB$_1$ receptor affinity and therefore worse CB$_2$ receptor selectivity, and 4-fluorophenethyl which resulted in a decrease in affinity at both receptors. At the N1 position, the highest CB$_2$ receptor affinity R$^1$ substituent was p-fluorobenzyl, followed by ethylmorpholino, then benzyl (Table 11, cf. entries 8, 4 and 5, or (2-one) 3, 1 and 2). In contrast, the substituent conferring the highest CB$_2$ receptor selectivity was ethylmorpholino, followed by p-fluorobenzyl then benzyl (cf. entries 5, 8 and 6 or (2-one) 1, 3 and 2).

Functional characterisation of N-(4-methylcyclohexyl)-1-benzyl-1,8-naphthyridin-2(1H)-one-3-carboxamide (CB74) 4.5 (Table 11, entry 5; Figure 55) indicated this compound acts as an agonist, exerting a CB$_2$ receptor-mediated inhibition of human basophil activation. Additionally, in a cytotoxicity assay 4.5 elicited a concentration dependent decrease in the viability of the Jurkat human T cell leukaemia line. The potential immunomodulatory activity of 4.5 was further investigated in a study on activated lymphocytes isolated from MS patients. CB74 (4.5) was shown to inhibit cell activation markers linked to MS progression and to block cell proliferation. In particular, 4.5 reduced cyclooxygenase-2 (a key enzyme in inflammatory reaction) levels in MS lymphocytes, but not in healthy control lymphocytes. The promising activity of this 1,8-naphthyridin-2(1H)-one-3-carboxamide derivative in autoimmune pathologies has increased interest in this class of cannabinoids. Intermediates synthesised in the course of fluorescent ligand development in this chapter may
therefore also be of interest themselves as novel analogues of this class of cannabinoids.

The 1,8-naphthyridin-2(1H)-one-3-carboxamide scaffold has already shown promise as a scaffold for the development of chemical tools. Radiofluorination of derivative N-(4-methylcyclohexyl)-1-(p-fluorobenzyl)-1,8-naphthyridin-2(1H)-one-3-carboxamide (CB91, Table 11, entry 8) yielded [18F]CB91 4.6 (Figure 55), which was evaluated as a tool for PET imaging CB2 receptor. Whilst uptake of [18F]CB91 4.6 was demonstrated in CB2 receptor rich spleen, it showed widespread distribution. The authors cite ongoing studies to demonstrate specific CB2 receptor binding.

Substitution of a hydroxyl group at C4 of the 1,8-naphthyridin-2(1H)-one-3-carboxamide core has been explored, with mixed SAR in terms of CB2 receptor affinity and selectivity. However, one compound of note was the agonist N-cycloheptyl-4-hydroxy-1-(2-morpholin-4-ylethyl-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (VL15) 4.7 (Figure 55), which showed high affinity (Ki = 1.8 nM at hCB2) and selectivity (Ki(CB1)/Ki(CB2) = 130). Compound 4.7 was investigated for immunomodulatory properties and found to reduce peripheral blood mononuclear cell proliferation, block cell cycle progression and downregulate T cell activation markers usually enhanced in autoimmune disease. Additionally, 4.7 was found to have promising physicochemical properties for MS treatment, with high intestinal absorption and blood-brain barrier penetration.

Figure 55: CB2 receptor agonist CB74 (4.5), radioligand [18F]CB91 (4.6) and agonist VL15 (4.7).
Variation of the N1 and C6 substituents (R₁ and R³ respectively in Figure 56) of this scaffold have been explored while keeping the C3 carboxamide 4-methylcyclohexyl constant.¹⁴⁵ Differing lengths of alkyls, alcohols, esters, carboxylic acids and alkyl fluorides were substituted at N1 (R¹), which demonstrated a tolerance for chain length variation at this position (Table 12, entries 11-23). Exploration of the C6 position with R³ = bromine, 2-thienyl, 2-furyl, p-methoxyphenyl or p-fluorophenyl while holding N1 (R¹) as p-fluorobenzyl or ethylmorpholino resulted in all high affinity CB₂ receptor compounds (entries 25-35 and 37-41). The combination of R¹ = ethylmorpholino and R² = p-methoxyphenyl (as a mixture of cis and trans isomers) offered the best CB₂ receptor selectivity, with a SI of >6802 (entry 38). The cis isomers again had higher CB₂ receptor affinity and selectivity than the corresponding trans isomers (cf. entries 27, 32 and 35 with 26, 31 and 34).

Figure 56: General structure of 4-methylcyclohexyl-1,8-napthyridin-2(1H)-one-3-carboxamide derivatives.
Table 12: Literature reported radioligand binding data for 4-methylcyclohexyl-1,8-naphthyridin-2(1H)-one-3-carboxamide derivatives (as shown in Figure 56) from Lucchesi et al.\textsuperscript{145}.\textsuperscript{a}

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\textsuperscript{a} Data represent mean values of $K_i$ (nM) from experiments performed at least three times in duplicate.

\textsuperscript{b} Affinity of compounds determined using HEK293-hCB\textsubscript{1} or -hCB\textsubscript{2} membranes and $[^3\text{H}]$CP55,940 ($K_d = 0.18$ nM at hCB\textsubscript{1} or $K_d = 0.31$ nM at hCB\textsubscript{2}).

\textsuperscript{c} cis- or trans-4-methylcyclohexylcarboxamide.

\textsuperscript{d} $SI = K_i$ (CB\textsubscript{1})/$K_i$ (CB\textsubscript{2}).
Lucchesi et al also carried out functional assays on three 6-substituted derivatives (Table 12: R\(^1\) = \(p\)-fluorobenzyl, R\(^3\) = bromine, entry 25; R\(^1\) = ethylmorpholino, R\(^3\) = bromine, entry 37; and R\(^1\) = ethylmorpholino, R\(^3\) = \(p\)-methoxyphenyl, entry 38) and 4 unsubstituted derivatives (R\(^1\) = \(p\)-fluorobenzyl, entry 24; R\(^1\) = ethylmorpholino, entry 36; R\(^1\) = butan-1-ol, entry 13; and R\(^1\) = 4-fluorobutane, entry 21). β-Arrestin 2 recruitment and forskolin-stimulated cAMP assays revealed that derivatives with a C6-substituent (R\(^3\)) behaved as an antagonist and inverse agonist at CB\(_2\) receptor, whereas derivatives with only hydrogen at C6 behaved as CB\(_2\) receptor agonists.\(^{145}\) The authors used molecular modelling and docking studies to hypothesise that this change in functionality was due to a ‘molecular toggle switch’, whereby C6 (R\(^3\)) substituents could penetrate deep into the ligand binding pocket and block movement of residue W6.48 (258), part of the CB\(_2\) receptor TMH6 flexible hinge (CWXP).

Several 1,8-naphthyridin-2(1\(H\))-one-3-carboxamide ligands with high affinity for CB\(_2\) receptor possess relatively low cLogP values when compared with the typically lipophilic cannabinoids. For example, 6-bromo-N-(4-methylcyclohexyl)-1-(2-morpholinoethyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (Table 12; entry 37) has a cLogP of 2.07, compared with 7.77 for SR144528 \(^{1.13}\), 6.16 for CP55,940 \(^{1.10}\) and 4.19 for AM630 \(^{1.14} \).\(^{****}\) Conjugation of a fluorescent dye such as BODIPY 630/650-X to a pharmacophore will make the overall ligand more lipophilic and therefore prone to high non-specific membrane binding. Selection of a less lipophilic pharmacophore such as the 1,8-naphthyridin-2(1\(H\))-one-3-carboxamides should help to reduce this non-specific membrane binding in the final fluorescent compounds.

Within the broad category of 1,8-naphthyridin derivatives, biological activities reported include anti-viral, anti-microbial, anti-tuberculosis, anti-cancer and anti-inflammatory.\(^{3.09}\) A structure search of the 1,8-naphthyridin-2(1\(H\))-one-3-carboxamide scaffold revealed that the number of biological targets other than CB receptors was limited and included hypoxia inducible factor prolyl hydroxylases,\(^{3.10}\) cytomegalovirus\(^{3.11}\) and human immunodeficiency virus (HIV)-1 integrase strand transfer.\(^{3.12}\) The 1,8-naphthyridin-4(1\(H\))-one-3-carboxamide scaffold has been used in

\(^{****}\) cLogP values calculated using Crippen's fragmentation,\(^{2.65}\) in ChemDraw Prime 15.1, PerkinElmer Informatics, Inc.
development of ligands for another GPCR, adenosine A\textsubscript{2A} receptor.\textsuperscript{313} However, the reported adenosine A\textsubscript{2A} receptor ligands differ quite significantly from CB\textsubscript{2} receptor 1,8-naphthyridin ligands, lacking N1 substituents and with very different carboxamide substituents. The only report of CB\textsubscript{1} receptor selective 1,8-naphthyridinones presents structures with quite different N1 and C3 substituents and simultaneous substitution of \textit{p}-chlorophenyl at C6 and \textit{o,p}-dichlorophenyl at C7.\textsuperscript{314}

\textbf{4.1.2 Conclusions on literature SAR of the 1,8-naphthyridin-2(1H)-one-3-carboxamide scaffold}

In conclusion, the 1,8-naphthyridin-2(1H)-one-3-carboxamide scaffold possesses many of the desirable characteristics for development into fluorescent ligands. There are many high affinity CB\textsubscript{2} receptor ligands identified within this class, several with subnanomolar affinity. Compounds are generally CB\textsubscript{2} receptor selective, with many exhibiting very high selectivity (\textit{K}_i\textsubscript{CB\textsubscript{1}}/\textit{K}_i\textsubscript{CB\textsubscript{2}} > 500) and SAR is well characterised as to which groups confer this high selectivity. The presence of a C6 substituent appears to influence ligand function for CB\textsubscript{2} receptor in a predictable way therefore, either agonist or antagonist fluorescent ligands may be intentionally developed. There are areas of the scaffold that are tolerant to change and steric bulk, providing guidance as to where conjugation of a linker and fluorescent dye might be tolerated, and the scaffold itself possesses desirable physicochemical properties.
4.2 Fluorescent ligand design

4.2.1 Linker position and ‘R’ group choice

The first goal was to develop a fluorescent antagonist and therefore inclusion of bulky substituents at C6 of naphthyridin was planned (R³, Figure 57). Docking studies by Lucchesi et al suggested that C6 substituents are positioned deep into the orthosteric CB₂ receptor-binding pocket. Therefore C6 is an unsuitable location for conjugation of a fluorescent dye. Variation of substituents at the C3 carboxamide appears to have a significant impact on CB₂ receptor affinity and selectivity, as demonstrated by the cis trans isomers of 4-methylcyclohexyl derivatives (Table 12, entries 26-27, 31-32, 34-35). In contrast, variation in length of substituents at the N1 position appears to be well tolerated (Table 12, entries 11-23). Therefore, it is possible that conjugation of a linker and fluorescent dye will be tolerated at the N1 (R¹) position of the naphthyridin scaffold.

The 4-methylcyclohexyl substituent at the C3 carboxamide has well characterised SAR and offers high affinity and selectivity for CB₂ receptor, therefore this group was preserved in the ligand design (Figure 57). The significant difference in affinity and selectivity between the cis and trans isomers made pursuing the cis isomers preferable, however the expense of sourcing the starting material, 4-methylcyclohexylamine, as the single cis isomer made this prohibitive. Therefore, the compounds were synthesised and pharmacologically evaluated as a mixture of cis and trans isomers, with the intention of re-synthesising or isolating the cis isomers of any promising candidates.

From amongst the existing SAR for C6 (R³) substituents, p-methoxyphenyl, 2-furyl and bromine were considered in the ligand design. p-Methoxyphenyl stood out as consistently providing a good combination of high CB₂ receptor affinity and selectivity in conjunction with varying N1 substituents (Table 12, entries 28 and 38). However, bromine-containing derivatives also possess high CB₂ receptor affinity and selectivity (entries 25 and 37) and require one less synthetic step. Derivatising C6 with (R³) 2-
furyl would result in a ligand with reduced lipophilicity, however high selectivity is not as consistently conserved with N1 variation, when compared with p-methoxyphenyl (entries 33 and 41). Due to time constraints, it was not possible to synthesise large numbers of derivatives, so it was determined that a bromine atom at C6 (R3) would be conserved throughout the series (Figure 57). However, to inform a later, refined series, one example with p-methoxyphenyl at R3 was planned for comparison.

![Chemical Structure](image)

4.8 R1 = ethylmorpholino, R3 = Br
4.9 R1 = methyl valerate, R3 = Br
4.10 R1 = Me-4-MeOBz, R3 = Br
4.11 R1 = methyl valerate, R3 = p-MeOPh

Figure 57: Core ligand design of 1,8-naphthyridin-2(1H)-one-3-carboxamide based fluorescent ligands.

### 4.2.2 Linker

Literature C6-substituted antagonists contain N1 (R1) substituents unsuitable for direct conjugation of a fluorescent dye (e.g. ethylmorpholino, p-fluorobenzyl). However, there are a number of literature agonists, which are unsubstituted at C6, that contain varying lengths of alkyls, alcohols and esters at N1 (Table 12, entries 11-23). Attachment of an ester R1 group at N1 could be followed by ester hydrolysis, revealing a carboxylic acid suitable for amide coupling to a linker. Therefore, methyl valerate was selected as an R1 group (Figure 57). As aromatic substituents are well tolerated at N1 (Table 11, entries 5-10; Table 12, entries 24-35), linker extension from an

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†††† cLogP values calculated for bromo (2.99), p-methoxyphenyl (3.71) and 2-furyl (2.45) C6-derivatives with methyl valerate substitution at N1. Calculated using Crippen's fragmentation, in ChemDraw Prime 15.1, PerkinElmer Informatics, Inc.
aromatic group was explored. Alkylation at N1 with methyl-4-methylbenzoate would provide an intermediate that could be extended in parallel with the methyl valerate derivatives (Figure 57). In addition, a literature derivative containing ethylmorpholino was planned for use as a reference in pharmacological testing (Table 12; entry 37).

As previously stated in section 1.5.4, including a linker between the pharmacophore and fluorophore creates space, hopefully reducing the impact of the large fluorophore on the binding properties of the pharmacophore. For this series, two of the diamine linkers previously synthesised for the indole series, N-Boc-1,8-octanediamine 2.28a and N-Boc-2,2’-(ethylenedioxy)diethylamine 2.28b (‘PEG’ linker; Scheme 3) were used. It was hypothesised that the polar dipeptide linkers used in the indole scaffold may be too hydrophilic and attenuate binding to the lipid CB receptor, therefore they were not utilised. Ligand entry into CB receptors is thought to occur via the lipophilic membrane (section 1.2.2.1),90 which the peptide linkers may inhibit.
4.3 Synthesis

Synthesis up to the intermediate 4.15 followed procedures reported by Lucchesi et al (Scheme 10).\textsuperscript{145} Bromination of the commercially available 2-amino-3-pyridinecarboxaldehyde 4.12 yielded 4.13 (step i), introducing a bromine group and a handle for derivatisation at what would become the C6 position of the pharmacophore. An aldol condensation between 4.13 and diethyl malonate using piperidine formed the 1,8-naphthyridin-2-one core 4.14 in high yield (step ii). Preparation of 4.15, ready for derivatisation at N1 and C6, was achieved by reacting 4.14 with 4-methylcyclohexylamine (50:50 mixture of cis and trans isomers) at high temperature, forming a mixture of the cis and trans isomers of the carboxamide 4.15 (step iii).

Analysis of the \textsuperscript{1}H NMR spectra of 4.15 showed splitting of the carboxamide NH into two doublets (9.51 & 9.96 ppm) and the adjacent tertiary CH into two multiplets (3.90 & 4.25) as a result of the isomeric mix. Subsequently, all compounds in this chapter were synthesised/carried through as a mixture of cis and trans isomers, giving rise to the same proton splitting at the carboxamide NH and adjacent CH as well as carbon splitting throughout the scaffold in \textsuperscript{13}C NMR spectra.

Alkylation of 4.15 at N1 was achieved using the corresponding chloro or bromo alkyl halide to yield 4.8, 4.9 and 4.10 in typically low yields (22-37%; Scheme 10; step iv). This was due to incomplete conversion of 4.15 and challenging separation of product from unreacted starting material (4.15), which was achieved by either flash silica gel chromatography or recrystallisation. The difficulty in purifying sufficient quantities of 4.10, along with a strict synthesis schedule, meant that further development of the methyl-4-methylbenzoate derivative into a fluorescent ligand was abandoned. However, pharmacological evaluation of the intermediate 4.10 would inform whether it was worth a repeated attempt at this route in later synthesis. Literature compound 4.8 was not developed further beyond this point, as it was only being synthesised as a reference compound for pharmacological testing. Lucchesi et al reported a microwave assisted Suzuki coupling to derivatise the bromo at C6 of 1,8-naphthyridin compounds (yields: 41-94%).\textsuperscript{145} In the absence of a microwave reactor, Suzuki coupling of 4.9 with 4-methoxyphenylboronic acid was achieved under modified conditions, at 110°C and using a mixture of water and DMF as solvent, to give 4.11, with moderate and
comparable yield (55%). Purification of all compounds shown in Scheme 10 was achieved by either recrystallisation or silica column chromatography.

\[
\text{4.12} \xrightarrow{i.} \text{4.13} \xrightarrow{ii.} \text{4.14}
\]

\[
\text{iii.} \quad \text{4.15} \xrightarrow{iv.} \text{4.8 } R = \text{ ethylmorpholine} \\
\text{4.9 } R = \text{ methyl valerate} \\
\text{4.10 } R = \text{ methyl-4-methylbenzoyl}
\]

Saponification of 4.9 with 10% NaOH gave the carboxylic acid 4.16, with an unexpectedly low yield (43%), likely due to loss of product during the work up (Scheme 11, step i). HATU-mediated amide coupling of 4.16 with either \(N\)-Boc-1,8-octanediamine 2.28a or \(N\)-Boc-2,2'-ethylenedioxy)diethylamine 2.28b (Scheme 3) yielded the Boc-protected pharmacophore-linker conjugates 4.17a and 4.17b, which were purified by silica column chromatography (step ii). Boc deprotection with TFA
gave the amines 4.18a and 4.18b (step iii) which were purified by semi-preparative RP-HPLC prior to fluorescent dye conjugation to ensure optimal purity. The amines 4.18a and 4.18b were then either acylated to give 4.19a and 4.19b or used in 3-fold excess for conjugation to BODIPY 630/650-X-OSu 2.15, yielding the final fluorescent compounds 4.20a and 4.20b, all of which were purified by semi-preparative RP-HPLC for pharmacological testing. As expected, the mixtures of cis trans isomers eluted as two very close peaks in RP-HPLC, which were collected together and pharmacologically evaluated as a mixture. All compounds that underwent pharmacological testing (4.8-4.11, 4.19a-b and 4.20a-b) had >95% purity (as the mixture of cis trans isomers) as determined by analytical RP-HPLC.

Scheme 11: Reagents and conditions: (i) 10% NaOH, EtOH, 110°C, 5 h, 43%; (ii) N-Boc-1,8-octanediame 2.28a or N-Boc-2,2’-(ethylenedioxy)diethylamine 2.28b, DIPEA, HATU, DMF, rt, 14 h, 71–77%; (iii) TFA, DCM, rt, 1 h, 86-91%; (iv) Ac₂O, DIPEA, DCM, rt, 1 h, 95-96%; (v) BODIPY 630/650-X-OSu 2.15, DIPEA, DMF, rt, 15 h, 88-92%.
4.4 Pharmacological evaluation

Four pharmacophore intermediates, 4.8, 4.9, 4.10 and 4.11, two pharmacophore-linker conjugates, 4.19a and 4.19b, and two final fluorescent compounds, 4.20a and 4.20b were subjected to pharmacological evaluation. Radioligand binding assays were carried out to determine affinity at hCB2 and hCB1 receptors and compounds with high affinity at CB2 receptor were tested in cAMP assays to determine function (refer to section 1.6).

4.4.1 Radioligand binding assays

A 10 µM competition binding screen was used to identify which compounds could displace more than 50% of bound \[^3\text{H}]\text{CP55,940}\) at hCB2 and hCB1 receptors (Figure 58). As expected, literature compound 4.8 displayed high displacement (95%) at hCB2 receptor, along with compounds 4.9 and 4.11 (73% and 87%, respectively). Only compounds 4.8 and 4.11 reached close to 50% displacement of \[^3\text{H}]\text{CP55,940}\) at hCB1 receptor (50% and 43%, respectively).

![Figure 58: Combined results of competition binding screens showing displacement of \[^3\text{H}]\text{CP55,940}\) at HEK293 hCB2 (10 µg/point) (black) and HEK293 hCB1 (7.5 µg/point) (grey) membranes by a fixed concentration (10 µM) of test compound. Raw data was expressed in ccpm and normalised to unlabelled CP55,940 (10 µM) (100%) and vehicle control (0%). Data shown is the mean value ± SEM of three independent experiments conducted in triplicate, except for 4.8, 4.9 and 4.11 at hCB2, where n = 2. Dashed line indicates 50% displacement criteria for progression to concentration response assay.](image-url)
Compounds 4.10, 4.19a, 4.19b, 4.20a and 4.20b displaced less than 50% of bound [³H]CP55,940 1.17 at hCB₂ receptor (Figure 58) and so were unlikely to have affinity high enough to be able to accurately determine a $K_i$ value. Therefore, statistical analysis was used to determine whether binding occurred. The percentage displacement values (n = 3, in triplicate) of 4.10, 4.19a, 4.19b, 4.20a and 4.20b at hCB₂ receptor all passed the D’Agostino & Pearson normality test, showed significant difference from the vehicle control (0%) in a one sample $t$-test and were thus determined to have a $K_i$ value >10 μM at hCB₂ receptor (Table 13). Similarly, 4.9, 4.10, 4.19a, 4.19b, 4.20a and 4.20b were also determined to have a $K_i$ value >10 μM at hCB₁ receptor.

Compounds that displaced 50% or more of bound [³H]CP55,940 1.17 at hCB₂ or hCB₁ receptor were subjected to a concentration response binding assay to generate $K_i$ values (Figure 59, Table 13). A concentration response assay was also carried out for 4.11 at hCB₁ receptor, as the SEM of radioligand displacement reached the 50% target.

![Figure 59: Competition binding curves for 4.8, 4.9 and 4.11 at hCB₂ receptor, determined using [³H]CP55,940 (2.5 nM) and HEK293-hCB₂ membranes. Data points are the mean ± SEM of one independent experiment conducted in triplicate points. Assay performed by Jamie Manning.](image)

†‡‡ Concentration response assays (n = 4) at hCB₂ receptor for 4.8, 4.9 and 4.11 were carried out by Jamie Manning.
The highest affinity compound at hCB<sub>2</sub> receptor was the literature compound 4.8 which showed a \( K_i \) of 45.2 nM (Figure 59), compared to the literature reported value of 1.26 nM.\textsuperscript{145} This difference in values is augmented due to the different \( [\textsuperscript{3}H]CP55,940 \) \( K_d \) values used to determine \( K_i \) (refer to section 1.6.1 for equation explanation). In this study a \( K_d \) of 3 nM at hCB<sub>2</sub> receptor was used, versus a \( K_d \) of 0.31 nM in the literature study. Utilising the literature \( [\textsuperscript{3}H]CP55,940 \) \( 1.17 \) \( K_d \) value (0.31 nM) with the raw data in this study brings the \( K_i \) value (9.1 nM) of 4.8 at CB<sub>2</sub> receptor into an acceptable range of the literature \( K_i \) value (1.26 nM). In this study, compound 4.8 showed an average \( K_i \) of 16.7 µM at hCB<sub>1</sub> (calculated using a \( [\textsuperscript{3}H]CP55,940 \) \( 1.17 \) \( K_d \) value of 2 nM) and was therefore designated as >10 µM due to the inaccuracy of determining \( K_i \) in this low affinity range. Utilising the literature \( [\textsuperscript{3}H]CP55,940 \) \( 1.17 \) \( K_d \) value (0.18 nM) at CB<sub>1</sub> receptor, with the raw data in this study, 4.8 shows a \( K_i \) of 2.52 µM, closer to the literature value of 750 nM. The selectivity of 4.8 in this study (\( K_i \) CB<sub>1</sub>/\( K_i \) CB<sub>2</sub> = 369, using \( K_i \) = 16.7 µM at CB<sub>1</sub>) was found to be quite similar to the literature selectivity difference (\( K_i \) CB<sub>1</sub>/\( K_i \) CB<sub>2</sub> = 595).

Amongst the C6-bromine derivatives, ethylmorpholino at N1 conferred the highest affinity at CB<sub>2</sub> receptor (4.8), followed by methyl valerate (4.9; Figure 59; Table 13). The N1-methyl-4-methylbenzoate derivative 4.10 showed minimal binding (\( K_i \) >10 µM; Figure 58) and is therefore unsuitable for further development into a fluorescent compound. This latter result was quite surprising, as aromatic groups such as benzyl (in C6-H derivatives) and \( p \)-fluorobenzyl (in both C6-H and C6-substituted derivatives) have been shown to be tolerable at the N1 position (Table 11, entries 5 and 8; Table 12, entries 25-35).\textsuperscript{145,303} Additionally, the hydrophilic terminal methyl ester is tolerated in the methyl valerate derivative (4.9; Table 13) as well as the corresponding C6-unsubstituted literature analogue (Table 12; entry 20). It is possible that the unique combination of the aromatic benzyl and the methyl ester in the N1 group of 4.10 significantly alters the ligand orientation, hindering CB<sub>2</sub> receptor binding.

Substituting the C6-bromine for \( p \)-methoxyphenyl in the N1-methyl valerate derivatives led to a three-fold improvement in hCB<sub>2</sub> receptor affinity from \( K_i \) = 906 nM (4.9) to \( K_i \) = 265 nM (4.11; Figure 59; Table 13) and therefore represents a potential means for refinement in a subsequent fluorescent compound series. This increase in affinity could
be due to the hydrophobicity of the $p$-methoxyphenyl group. The larger $p$-methoxyphenyl group may also be able to form more favourable interactions with the ligand binding pocket than the smaller bromine.

The cLogP values of the four pharmacophore intermediates were calculated to explore whether there was any correlation between overall compound lipophilicity and CB$_2$ receptor binding affinity. The rank order of lipophilicity (by cLogP value) in descending order was: 4.10 (4.03), 4.11 (3.71), 4.9 (2.99), and 4.8 (2.07).\textsuperscript{§§§§} This contrasts to the order of CB$_2$ receptor affinity (high to low: 4.8, 4.11, 4.9 and 4.10; Table 13), which indicates that the varying, but similarly sized N1 substituents are likely having subtle effects on binding interactions, perhaps by altering the ring core orientation, rather than lipophilicity being the determining factor.

Comparing the binding affinities of the C6-substituted, N1-methyl valerate derivatives 4.9 and 4.11 with the published data of the literature C6-unsubstituted analogue (Table 12, entry 20, $K_i = 27.1$ nM at hCB$_2$ receptor\textsuperscript{145}), it initially appears as if C6-substitution leads to a significant decrease in CB$_2$ receptor affinity. However, recalculating the binding affinities of 4.9 and 4.11 using the literature $K_d$ value (0.31 nM at hCB$_2$ receptor), generates much lower $K_i$ values of 183 nM and 53.5 nM at CB$_2$ receptor, respectively which, for 4.11 in particular, shows a much less dramatic effect of C6-substitution on affinity.

\textsuperscript{§§§§} cLogP values calculated using Crippen's fragmentation,\textsuperscript{265} in ChemDraw Prime 15.1, PerkinElmer Informatics, Inc.
<table>
<thead>
<tr>
<th>Compound</th>
<th>C6 Subst.</th>
<th>N1 Substitution</th>
<th>10 µM displ.</th>
<th>Kᵢ (nM ± SEM)</th>
<th>10 µM displ.</th>
<th>Kᵢ (nM ± SEM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4.8</strong></td>
<td>Me</td>
<td>Ethylmorpholino</td>
<td>94.7 ± 2.4</td>
<td>45.2 ± 10.8</td>
<td>50.0 ± 23</td>
<td>&gt;10000</td>
<td>221</td>
</tr>
<tr>
<td><strong>4.9</strong></td>
<td>Me</td>
<td>Me-4-MeOβz</td>
<td>19.2 ± 3.4</td>
<td>&gt;10000</td>
<td>16.7 ± 4.8</td>
<td>&gt;10000</td>
<td>-</td>
</tr>
<tr>
<td><strong>4.10</strong></td>
<td>Me</td>
<td>(CH₂)₄CO₂Me</td>
<td>73.1 ± 1.0</td>
<td>906 ± 223</td>
<td>27.4 ± 3.1</td>
<td>&gt;10000</td>
<td>11</td>
</tr>
<tr>
<td><strong>4.11</strong></td>
<td>Me</td>
<td>MeOPh</td>
<td>87.2 ± 1.3</td>
<td>265 ± 31.9</td>
<td>43.4 ± 6.1</td>
<td>8708 ± 617</td>
<td>33</td>
</tr>
<tr>
<td><strong>4.19a</strong></td>
<td>Me</td>
<td>(CH₂)₂CONH(CH₂)₂NHAc</td>
<td>28.0 ± 2.9</td>
<td>&gt;10000</td>
<td>25.2 ± 5.0</td>
<td>&gt;10000</td>
<td>-</td>
</tr>
<tr>
<td><strong>4.19b</strong></td>
<td>Me</td>
<td>(CH₂)₂CONH(CH₂)₂NHAc</td>
<td>17.8 ± 1.2</td>
<td>&gt;10000</td>
<td>25.2 ±4.0</td>
<td>&gt;10000</td>
<td>-</td>
</tr>
<tr>
<td><strong>4.20a</strong></td>
<td>Br</td>
<td>(CH₂)₂CONH(CH₂)₂NH-BODIPY-630/650-X</td>
<td>22.5 ± 3.2</td>
<td>&gt;10000</td>
<td>18.6 ± 5.3</td>
<td>&gt;10000</td>
<td>-</td>
</tr>
<tr>
<td><strong>4.20b</strong></td>
<td>Br</td>
<td>(CH₂)₂CONH(CH₂)₂NH-BODIPY-630/650-X</td>
<td>34.6 ± 1.3</td>
<td>&gt;10000</td>
<td>25.0 ± 3.6</td>
<td>&gt;10000</td>
<td>-</td>
</tr>
<tr>
<td>SR144528</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>51.0 ± 3.0</td>
<td>-</td>
<td>5628 ± 2890</td>
<td>110</td>
</tr>
</tbody>
</table>

*a* Radioligand binding assays performed with [³H]CP55,940 (2.5 nM) and HEK293-hCB₂ or -hCB₁ membranes. Data is the mean of at least three or *c* two individual experiments performed in triplicate.

*b* Percentage displacement of [³H]CP55,940 by test compound (10 µM). Raw data was normalised to the specific binding window (unlabelled CP55,940 (10 µM), 100%; vehicle control, 0%).

c* Kᵢ* calculated using [³H]CP55,940 *K*d = 3 nM at hCB₂ or 2 nM at hCB₁ receptor. For compounds showing <50% displacement of [³H]CP55,940 at 10µM, the D’Agostino & Pearson normality test and a one sample *t*-test was used to determine if significant competition with [³H]CP55,940 had occurred (*Kᵢ* >10 µM).

c* SI = KᵢCB₁/KᵢCB₂.*

* Compounds were tested as approximately a 50:50 mixture of cis and trans isomers

* Compounds were tested as a 57:43 or 40:60 mixture of cis and trans isomers, which are unassigned. See experimental Chapter 7.

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Table 13: Affinity of N-substituted 1,8-naphthyridin based compounds for hCB₂ and hCB₁ receptors.
Unfortunately, none of the longer pharmacophore linkers (4.19a and 4.19b) or final fluorescent compounds (4.20a and 4.20b) showed good affinity for hCB$_2$ receptor. The two linkers of compounds 4.19a and 4.19b were of identical length and it is possible that this linker length was either too long or too short to allow favourable positioning of the fluorescent dye whilst still allowing the pharmacophore to bind to the receptor. It is also possible, in light of these results, that the N1 position is in fact a poor choice for linker and fluorophore conjugation regardless of linker length or type. It was pleasing to see that all compounds tested in this series showed minimal affinity at hCB$_1$ receptor, in agreement with literature results for 4.8 and other similar scaffolds.

### 4.4.2 Forskolin-stimulated cAMP assays

High affinity hCB$_2$ receptor compounds (4.8, 4.9 and 4.11) were evaluated in a concentration response BRET assay measuring forskolin-stimulated cAMP (section 1.6.2) to determine potency (IC$_{50}$; Table 14). The response evoked at the highest concentration (10 µM) was used to determine efficacy (E$_{\text{max}}$).

The HEK293 hCB$_2$ S4 low cell line has high receptor expression and is therefore ideal for producing membranes for radioligand binding assays and was also used for the cAMP assays carried out on the indole derivatives (sections 2.4.2 and 3.6.2). However, due to the high receptor expression levels, often only a small window between vehicle and forskolin-stimulated cAMP levels was observed. Therefore, it was decided that the lower expressing HEK293-Flp cell line overexpressing hCB$_2$ receptor would be better suited for cAMP assays. Pharmacological evaluation of 4.8, 4.9 and 4.11 was carried out in both cell lines (Table 14). There are significant differences in the IC$_{50}$ values calculated for the two hCB$_2$ receptor cell lines. However, they both generate the same pattern in terms of potency, with the most potent being 4.8, followed by 4.11, then SR144528 1.13, and least potent 4.9. There appeared to be only a small variation in E$_{\text{max}}$ values between compounds, therefore a one way ANOVA (analysis of variance; Tukey’s multiple comparisons test) was performed on the E$_{\text{max}}$ values at hCB$_2$ receptor in each cell line to determine if there was any significance to the different efficacy values observed. There was a significant difference between the E$_{\text{max}}$ of 4.8 and 4.11 in the HEK-Flp hCB$_2$ cell line (p = 0.0277), therefore it can be concluded that the efficacy
of 4.8 is greater than 4.11 in the HEK-Flp hCB2 cells. In the hCB2 S4 low cell line, both 4.9 (p = 0.0064) and 4.11 (p = 0.0008) were found to have significantly higher efficacy than SR144528 1.13. There is no significant difference between any of the other compound E\textsubscript{max} values in either cell line.

Table 14: Potency (IC\textsubscript{50}) and efficacy (E\textsubscript{max}) of N-substituted 1,8-naphthyridin based compounds determined using a forskolin-stimulated cAMP assay.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>hCB2, S4 low line \textsuperscript{b}</th>
<th>hCB2, Flp line \textsuperscript{c}</th>
<th>hCB1 \textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} nM ± SEM</td>
<td>E\textsubscript{max} % ± SEM \textsuperscript{e}</td>
<td>IC\textsubscript{50} nM ± SEM</td>
</tr>
<tr>
<td>4.8</td>
<td>114 ± 12.2 /</td>
<td>237 ± 6.0 /</td>
<td>46.5 ± 20.2</td>
</tr>
<tr>
<td>4.9</td>
<td>1764 ± 491 /</td>
<td>257 ± 12.9 /</td>
<td>584 ± 62.9</td>
</tr>
<tr>
<td>4.11</td>
<td>463 ± 41.3 /</td>
<td>269 ± 11.3 /</td>
<td>91.8 ± 49.0</td>
</tr>
<tr>
<td>SR144528</td>
<td>760 ± 61.7 /</td>
<td>227 ± 2.2</td>
<td>132 ± 24.1</td>
</tr>
<tr>
<td>1.13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} cAMP levels measured in a BRET assay using a CAMYEL sensor, performed in either \textsuperscript{b} HEK293-hCB2 S4 low cells, \textsuperscript{c} HEK-Flp hCB2 cells or \textsuperscript{d} HEK293 hCB1 pEF4A cells.

\textsuperscript{e} E\textsubscript{max} is the response at 10 µM, normalised to basal (0%) and forskolin only (100%) levels of cAMP.

All values represent the mean ± SEM of at least three independent experiments conducted in duplicate, except / are two independent experiments performed in duplicate.

A one sample t-test was used to determine if E\textsubscript{max} values were significantly different from forskolin only values (100%) and for / no significant difference was found, therefore the compounds were determined to show no response under the assay conditions.

All three compounds evaluated were found to be inverse agonists for hCB2 receptor, in agreement with the literature for 4.8 and as speculated for the C6-substituted compounds 4.9 and 4.11. The literature compound, 4.8 showed the highest potency of the series. However, the lead compound for further development of this series, 4.11, showed potential in the functional assay, demonstrating greater potency at hCB2 receptor than the inverse agonist SR144528 1.13 (Figure 60).
Comparing 4.9 and 4.11, it is interesting to note that the substitution of bromine for p-methoxyphenyl at C6, leads to a large increase in potency (Table 14). At hCB₁ receptor, 4.9 and 4.11 were found to have low potency, whilst 4.8 and SR144528 showed Eₘₐₓ values close to the forskolin only response (100%) and using a one sample t-test, were determined as showing no response.

The hCB₂ receptor IC₅₀ of 4.8 in the HEK-Flp cell line compares well with the literature value of 59.6 nM. However, it is worth noting that the literature study uses a different cell line (U2OS cell line), and therefore a comparison is of limited significance, due to variables in expression of both CB₂ and endogenously expressed receptors.

All compounds were screened in a BRET forskolin-stimulated cAMP assay in WT HEK293 cells to verify that the response observed at hCB₂ and hCB₁ receptor was receptor mediated (Table 15). The compound cAMP responses were normalised to basal (0%) and forskolin only (100%) response, and then analysed in a one sample t-test to determine if the compound responses were significantly different from the forskolin only response. Compounds 4.8 and 4.11 showed no significant response in
both the WT S4 low line and WT HEK-Flp line, verifying that the inverse agonist activity observed for these compounds is indeed receptor mediated. However, 4.9 showed a significant response in the S4 low line at 10 µM, but when screened at 1 µM, showed no significant response. As 4.9 shows a significant response at 1 µM in the S4 low hCB2 line, it can be concluded that the inverse agonist activity observed is hCB2 receptor mediated. However, as 4.9 was not tested at 1 µM in HEK293 cells overexpressing hCB1 receptor, it cannot be concluded whether the inverse agonist activity observed at hCB1 is receptor mediated. SR144528 1.13, CP55,940 1.10 and WIN 55,212-2 2.2 were also screened for activity in WT cells. SR144528 1.13 and CP55,940 1.10 demonstrated no significant response in either cell line. WIN 55,212-2 2.2 showed a response at 10 µM in the HEK-Flp line but not the S4 low line. It is interesting to note that the response of WIN 55,212-2 2.2 is observed in a different cell line to that of 4.9, indicating that the responses are likely to be mediated by different pathways in the WT cells.

Table 15: Forskolin stimulated cAMP response in WT HEK293 cells. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>% response ± SEM, S4 low line</th>
<th>% response ± SEM, Flp line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>4.8</td>
<td>114.9 ± 8.2</td>
<td>103.0 ± 2.0</td>
</tr>
<tr>
<td>4.9</td>
<td>121.5 ± 3.5 *</td>
<td>95.7 ± 3.6</td>
</tr>
<tr>
<td>4.11</td>
<td>107.2 ± 2.8</td>
<td>97.9 ± 2.6</td>
</tr>
<tr>
<td>CP55,940 1.10</td>
<td>98.0 ± 4.0</td>
<td>99.2 ± 2.7</td>
</tr>
<tr>
<td>SR144528 1.13</td>
<td>94.5 ± 1.6</td>
<td>99.8 ± 2.2</td>
</tr>
<tr>
<td>WIN 55,212-2 2.2</td>
<td>102.6 ± 2.1</td>
<td>96.1 ± 1.0</td>
</tr>
</tbody>
</table>

* cAMP levels measured in a BRET assay using a CAMYEL sensor. Data represent mean values ± SEM for at least three independent experiments conducted in duplicate. Values are normalised to basal (0%) and forskolin (100%) response. Data was analysed for normality in a D’Agostino & Pearson normality test and then analysed using a one sample t-test for significant difference to forskolin only (100%) response and values marked with * demonstrated significant difference.
4.5 Molecular modelling

Ligand docking studies were carried out using the CB$_2$ receptor homology model B, based on the crystal structure of inverse agonist-bound CB$_1$ receptor (PDB ID: 5U09),\textsuperscript{67} generated as previously described in Chapter 3 (section 3.7.1.2). Ligands 4.8, 4.9, 4.10 and 4.11 were all docked into the CB$_2$ receptor homology model B in an attempt to understand the differences in CB$_2$ receptor affinity observed during pharmacological testing.

The orientation of the docked cis-4.8 was compared with that of the antagonist and inverse agonist present in the two published inactive CB$_1$ receptor crystal structures.\textsuperscript{66,67} The naphthyridin core rings of cis-4.8 partially overlaps with aromatic rings in both taranabant and AM6538, whilst the C6-bromine overlaps with the chlorine atom in taranabant and the C3-carboxamide also overlaps with the carboxamide in taranabant (Figure 61).

![Figure 61](image)

Figure 61: Orientation of docked cis-4.8 (pink) in CB$_2$ receptor homology model B overlapped with the inverse agonist taranabant (orange) and antagonist AM6538 (purple) from CB$_1$ receptor crystal structures. Image viewed from above the receptor, down from the extracellular space. Receptors have been hidden for clarity.

The arrangement of the residues F87, K109, F117, W194, W258 and S285 in the CB$_2$ receptor homology model generated by Lucchesi et al (built using an inactive Rhodopsin crystal structure)\textsuperscript{145} matches up well with the arrangement of these residues in the CB$_1$ receptor-based homology model generated in this study. Docking poses of
*cis*-4.8 and *cis*-4.9 were clustered into two groups, with the most consistent poses (three out of five and six out of ten, respectively; Figure 62) showing the C6-bromine buried deep in the ligand binding pocket, orientated close to residue W258, which is in agreement with the findings of Lucchesi *et al* for the 1,8-naphthyridin scaffold.\textsuperscript{145}

![Clusters of the most consistent docking poses for (A) *cis*-4.8 and (B) *cis*-4.9 in hCB\textsubscript{2} receptor homology model B. The C6-bromine of both atoms comes into close proximity with residue W258. Side chains of residues within 4 Å of *cis*-4.8 and *cis*-4.9 are shown as sticks (pale blue). The N-terminal is hidden for clarity.](image)

Docking poses of *trans*-4.8 were also clustered into two groups and the most consistent pose (four out of five) showed significant shift in orientation from the *cis* isomer (Figure 63), which could explain the significant difference in affinity typically observed\textsuperscript{303} between the *cis* and *trans* isomers of the *N*-(4-methylcyclohexyl)-1,8-naphthyridin-2(1*H*)-one-3-carboxamide scaffold.
Figure 63: Overlap of the most consistent, lowest energy docking poses for cis-4.8 (raspberry) and trans-4.8 (pale pink), showing significant shift in the positioning of the core naphthyridin rings and C6-bromine substituent. Side chains of residues within 4 Å of cis- or trans-4.8 are shown as sticks (pale blue). The N-terminal is hidden for clarity.

The docking poses of cis-4.8 and cis-4.9 showed almost an exact overlap with each other (Figure 64). However, all four clusters of docking poses for cis-4.10, showed a significant alteration in the orientation of the core rings when compared to cis-4.8 and cis-4.9. In the most consistent pose (seven out of ten), the 4-methylcyclohexyl group of cis-4.10 still points in the same direction, but the C6 and N1 groups have effectively swapped positions, with the N1-methylbenzoate pointing towards residue W258 (Figure 65). The presence of the bulky methylbenzoate group at N1 may force a less favourable orientation of the core rings, resulting in the loss of CB2 receptor affinity observed in the pharmacological results.
Figure 64: Overlap of the most consistent, lowest energy docking poses of cis-4.8 (raspberry) and cis-4.9 (green). Side chains of residues within 4 Å of cis-4.8 or cis-4.9 are shown as sticks (pale blue). The N-terminal is hidden for clarity.

Figure 65: The most consistent docking poses of cis-4.10, showing a significant shift in orientation from other derivatives. Side chains of residues within 4 Å of cis-4.10 are shown as sticks (pale blue). The N-terminal is hidden for clarity.

It was not possible to achieve consistent docking poses with cis-4.11, which produced nine different poses. However, the highest ranked solution showed the p-MeOPh group at C6 pushed down into the receptor pocket, but pointing in a significantly different
position to the C6-bromine of cis-4.9 (Figure 66). It is somewhat expected that such a large group as p-MeOPh would lead to a significant shift in ligand orientation, however, it is difficult to draw conclusions from the docking of cis-4.11 due to the inconsistency in poses. Docking of cis-4.11 was also attempted in CB2 receptor homology model C, built from the other CB1 receptor crystal structure (PDB ID: 5TGZ, see section 3.7.1.2 for description of model), however, docking poses were just as inconsistent.

Figure 66: The lowest energy docking poses of cis-4.9 (green) and cis-4.11 (brown). Side chains of residues within 4 Å of cis-4.9 and are shown as sticks (pale blue). The N-terminal is hidden for clarity.

Consistently, across the ligand docking studies of 4.8, 4.9 and 4.11, the N1-alkyl group has protruded sideways towards TMH2 and TMH3, whereas in docking studies conducted by Lucchesi et al.,145 N1 substituents of analogues appeared to point upwards. If the N1 substituents in 4.8, 4.9 and 4.11 do indeed point sideways in the ligand binding site, that might explain why linker and dye conjugation was not tolerated at the N1 position. Interestingly, in the docking studies described here, the 4-methylcyclohexyl substituent appears to slightly protrude through a gap between TMH1 and TMH7 and therefore may be a potential site for linker extension.
4.6 Conclusions

1,8-Naphthyridin-2(1H)-one-3-carboxamide was chosen as a scaffold for development of fluorescent ligands because of the many high affinity, CB$_2$ receptor selective compounds already identified within this compound class, as well as the well-defined functional switch with C6 substitution. The relatively low lipophilicity of this scaffold also made it an attractive target for development into a fluorescent ligand with potentially reduced hydrophobicity and therefore reduced non-specific membrane binding. A series of 1,8-naphthyridin-2(1H)-one-3-carboxamide based compounds were synthesised, exploring variation of the N1 and C6 groups, as well as linker and fluorescent dye conjugation via an N1-alkyl group. Aliphatic methyl valerate was identified as a promising N1 substituent for extension, whilst the aromatic methyl-4-methylbenzoate substituent (4.10) had poor CB$_2$ receptor affinity. Substitution of a p-methoxyphenyl group at C6 (4.11) showed improved CB$_2$ receptor affinity over the bromine substituted analogue (4.9). However, extension of the methyl valerate by conjugation of a linker and fluorophore only produced ligands with CB$_2$ receptor $K_i > 10 \mu\text{M}$. Docking studies of the pharmacophore intermediates suggested that the poor affinity of the fluorescent ligands could be due to the N1-alkyl group orientating into the ligand binding pocket, rather than extending out as initially thought based on literature biological activity. Considering both the biological results and modelling results, it was decided to abandon linker extension at the N1 position of the scaffold and instead explore extension from the 4-methylcyclohexyl substituent (Chapter Five).
Chapter Five: Fluorescent ligands for CB$_2$ receptor based on C3-carboxamide cyclohexyl-substituted 1,8-naphthyridin

Evaluation of the N-substituted 1,8-naphthyridin based compounds synthesised in Chapter Four indicated that N1 was not an appropriate position for linker and fluorophore substitution (Figure 67). However, subsequent ligand docking studies on these compounds suggested that the 4-methylcyclohexyl substituent may protrude out of the orthosteric binding pocket though a gap between TMH1 and TMH7 and therefore may be a suitable alternative position for conjugation of a fluorescent dye. This chapter explores that hypothesis in the design, synthesis and pharmacological evaluation of fluorescent C3-carboxamide cyclohexyl-substituted fluorescent ligands.

C6:
- Ligand docking shows substituents buried deep in binding pocket
- methoxyphenyl favoured R$^2$ group

Cyclohexyl:
- Ligand docking shows methyl protruding through gap between TMH1 and 7

N1:
- SAR suggested promising position, but linker and fluorophore conjugation yielded low affinity ligands ($K_i > 10 \mu M$)
- ethylmorpholine favoured R$^1$ group

Figure 67: Summary of Chapter Four findings for the 1,8-naphthyridin-2(1$H$)-one-3-carboxamide scaffold.
5.1 Ligand docking studies

Ligand docking studies using R state CB$_2$ receptor homology model B (section 3.7.1.2) were carried out to explore how linker extension from the 4-methylcyclohexyl group would affect the docked ligand orientation. Ligands were built in Avogadro$^{315}$ with an ethylmorpholino at N1 and a $p$-methoxyphenyl group at C6. Whilst these two groups were not used in combination in any of the Chapter Four ligands, they were the favoured groups at their relative positions and they have been used in combination in 4-methylcyclohexyl literature derivatives (e.g. Table 12, entry 38, 1.16).$^{145}$ Replacement of the 4-methylcyclohexyl with a 4-cyclohexanol was explored along with linker extension from the hydroxyl (Figure 68-71).

*Cis-* and *trans*-cyclohexanol derivatives 5.1a and 5.1b (Figure 68A) were docked into the CB$_2$ receptor homology model (Figure 68B & C) and the most consistent poses were in a similar orientation to the 4-methylcyclohexyl and C6-methoxyphenyl derivative 4.11 in Chapter Four (Figure 66). The presence of a hydroxyl group at the cyclohexyl instead of a methyl did not appear to significantly alter the preferred docking position of the core naphthyridin rings, indicating that this chemical change may be tolerated. The cyclohexyl hydroxyl groups of 5.1a and 5.1b appeared to protrude through a gap in the surface of the homology model between TMH1 and 2 (Figure 68C), whereas the methylcyclohexyl derivatives in Chapter Four, protruded through the gap between TMH1 and TMH7. It is unclear whether this change is due to the replacement of the methyl with a hydroxyl, or the presence of N1-ethylmorpholino and C6-methoxyphenyl in combination. The *trans-*hydroxyl of 5.1a appeared to point directly out of the opening between the TMHs, whereas the *cis-*hydroxyl pointed towards receptor residues, suggesting that *trans* derivatives may be more amenable to extension (Figure 68C).
Docking of ligands with linkers (such as an ethanoic acid 5.2, a methyl valerate 5.3 or a hexylamide 5.4) extended from the trans-cyclohexanol groups resulted in the same orientation as the smaller trans-5.1a, with the linker moiety still protruding out of the orthosteric binding pocket between TMH1 and TMH7 (Figure 69). Extension of the trans-ethanoic acid 5.2 by conjugation of a PEG (NH(C_2H_4O)_2C_2H_4NHAc) linker (5.5) was also tolerated, with the ligand docking into the same orientation, with just the ethylmorpholino group slightly rotated (Figure 69). However, extension of the trans-ethanoic acid by conjugation of an alkyl (NHC_8H_16NH) linker disrupted the docked orientation of the ligand, with the methoxyphenyl group pointing up towards the ECLs.
whilst the alkyl linker exited the orthosteric binding pocket between TMH1 and TMH2 further down into the lipid bilayer. It would be expected that the hydrophobic alkyl linker would be strongly favoured to bind in the lipid bilayer (and the hydrophobic portion of the lipid-buried receptor surface) and this presumably caused the disruption to docking orientation of the core ligand.

Figure 69: Ligand docking exploring linker extension of trans derivatives with an ethanoic acid 5.2 (cyan), a methyl valerate 5.3 (salmon), a hexylamide 5.4 (white) or an acylated PEG linker conjugated to the ethanamide (5.5, yellow), using CB₂ receptor homology model B. Docking poses shown are representative of the lowest energy, most consistent poses generated. Side chains of residues within 4 Å of the ligands are shown as sticks (pale blue).
It was promising to see the linkers protrude into the lipid bilayer, as this may allow for favourable lipid-mediated ligand entry (Figure 70).

![Figure 70: Top view of CB$_2$ receptor homology model B with *trans* derivatives 5.2-5.5 docked, showing the linkers protruding into the lipid bilayer (viewed from the extracellular space).](image)

Unexpectedly, docking of the analogous ethanoic acid 5.6, methyl valerate 5.7 or hexylamide 5.8 *cis*-linked derivatives resulted in significantly different positioning of the naphthyridin core, with the linkers buried in the orthosteric binding pocket, whilst the C6 methoxyphenyl and N1-ethylmorpholino both protruded out of the orthosteric binding pocket (Figure 71). Docking of *cis* derivatives with an acylated PEG or alkyl chain conjugated to the ethanamide moiety consistently produced poses in which the linkers were within the orthosteric binding pocket, whilst the core ligand and N1 and C6 groups were almost entirely in the extracellular space or lipid bilayer. Docking of these ligands in CB$_2$ receptor homology model C did not show any improvement in the docking poses generated.
Figure 71: Ligand docking exploring linker extension of cis derivatives with an ethanoic acid 5.6 (cyan), a methyl valerate 5.7 (salmon) or a hexylamide 5.8 (green), using CB2 receptor homology model B. Docking poses shown are representative of the lowest energy, most consistent poses generated. Side chains of residues within 4 Å of the ligands are shown as sticks (pale blue).
5.2 Ligand design

It was fortunate that between the large body of published SAR and the work carried out in Chapter Four, a favourable design of the core ligand had already been elucidated. Therefore the fluorescent ligand design focused on exploring linker type and length.

5.2.1 Core ligand

The most favourable N1 group amongst the compounds synthesised in Chapter Four was the ethylmorpholino (4.8), which conferred high affinity and selectivity and has also been successfully utilised in published CB2 receptor ligands.\(^{145}\) At C6, methoxyphenyl was favoured over bromine (in N1-methylvalerate derivatives 4.9 and 4.11). Therefore, the core ligand was designed to possess N1-ethylmorpholino and C6-methoxyphenyl (Figure 72).

![Figure 72: Fluorescent ligand design for cyclohexylcarboxamide substituted series. Ligands were designed to possess various combinations of cis- or trans-cyclohexyl moieties with either ethanamide or pentanamide and alkyl or PEG chains.](image)

5.2.2 Linker attachment

The significant differences observed between docking poses of cis and trans linker derivatives raised an interesting design question regarding which isomers should be synthesised. The cis isomers of methylcyclohexyl N-substituted 1,8-naphthyridin
derivatives are consistently favoured in the literature over the analogous trans isomer,\textsuperscript{145,303} but the docking studies (Figure 68 - 71) appeared to indicate that trans derivatives may be favoured for linker extension. Whilst published studies provide indisputable evidence in favour of cis-methylcyclohexyl isomers, there is no biological data reported for compounds possessing any groups other than methyl at the 4-cyclohexyl position. It is reasonable to expect that extension and substitutions of the methyl would have a significant effect on CB\textsubscript{2} receptor binding. However, it is also important to consider the limitations of the docking studies, which uses a CB\textsubscript{2} receptor homology model based on CB\textsubscript{1} receptor, which has considerable differences in sequence identity and presumable secondary structure. In addition, the type of docking studies carried out here, whether with a crystal structure or homology model, utilise a static structure, whereas in reality GPCRs are highly flexible and dynamic. Therefore, it seemed prudent to design a ligand library containing both cis and trans isomers.

Modification of the 4-methylcyclohexyl substituent was necessary to allow for linker conjugation and was guided by the commercial availability of 4-substituted cyclohexylamino based reagents and the ligand docking studies carried out in section 5.1. The methyl of methylcyclohexyl was replaced with an oxygen because this was tolerated in ligand docking studies, the requisite reagents (cis- or trans-4-aminocyclohexanol) were commercially available and the oxygen would allow for conjugation of linkers (Figure 72).

5.2.2.1 Planned linkers

As a way of exploring varying linker length, an ethanamide and a pentanamide moiety were planned for attachment at the 4-cyclohexanol, which would utilise reagents already available in the lab and was tolerated in ligand docking studies on trans derivatives (section 5.1). The ethanamide and pentanamide could be easily extended with linkers of varying polarity such as an alkyl (2.28a) or PEG (2.28b) linkers (Figure 72). However, a problematic synthesis meant that this intended design was not achieved (see section 5.3.2).
5.2.2.2 Linkers prepared

An alternative design was proposed whereby a glycine or an aminoheptyl moiety was attached, followed by conjugation of the fluorophore (Figure 73). At the time of this redesign there was only a short period remaining in which synthesis could be completed. This strict schedule along with the availability of reagents in the lab were the primary influences on the designs selected, which is discussed further in section 5.3.2.

![Diagram](attachment:image.png)

Figure 73: Alternative design of fluorescent ligands 5.9a-d.
5.3 Synthesis

5.3.1 Core ligand

The N1 and C6 substituents were kept constant in this series and the first point at which the compounds diverged was in attachment of a cis- or trans-cyclohexanol group. Therefore, the synthetic route for the core ligand was reordered from that carried out in Chapter Four (where the cyclohexyl group was attached first, followed by N1 and C6 substituents), so that divergence was delayed until the last possible step, to reduce the number of reactions carried out.

Synthesis proceeded from 4.14 (Scheme 10), which was N-alkylated on a small scale (20 mg of 4.14) with 4-(2-chloroethyl)morpholinhydrochloride and recrystallised in acetonitrile (ACN) to give the ethylformate 5.10a (41%). However, when this reaction was repeated at a larger scale (6.08 g of 4.14) the reaction mixture got wet, which combined with the basic conditions led to partial ester hydrolysis of the ethylformate 5.10a. The crude residue precipitated in ACN therefore yielding 5.10a and 5.10b as a 1:3 mixture (Scheme 12, step i). It was anticipated that Suzuki coupling of 5.10a with 4-methoxyphenylboronic acid would lead to complete hydrolysis of the ester anyway due to the use of aqueous basic conditions therefore the mixture of 5.10a and 5.10b was not purified. The 1:3 mixture of 5.10a/5.10b carried into the Suzuki coupling reaction, however afforded a 1:15 mixture of the ethylformate (5.11a) and the carboxylic acid (5.11b) (Scheme 12, step ii). The 5.11a/5.11b 1:15 mixture was subjected to hydrolysis conditions using 0.2 M aq. lithium hydroxide monohydrate to give the carboxylic acid 5.11b in pure form (Scheme 12, step iii). Coupling of the carboxylic acid 5.11b with either trans- or cis-4-aminocyclohexanol gave the carboxamides 5.1a or 5.1b respectively, which were purified by precipitation in ACN in good yield (79-82%; Scheme 12, step iv).
Scheme 12: Synthesis of 5.1a and 5.1b. Reagents and conditions: (i) 4-(2-chloroethyl)morpholinhydrochloride, Cs$_2$CO$_3$, DMF, 50°C, 12 h, 41% (for ethylformate 5.10a only formation); (ii) 4-methoxyphenylboronic acid, Na$_2$CO$_3$, Pd(OAc)$_2$, H$_2$O, DMF, 110°C, 3 h; (iii) 0.2 M aq. LiOH.H$_2$O, THF, 0°C, 1 h; (iv) trans- or cis-4-aminocyclohexanol, DIPEA, HATU, DMF, rt, 14 h, 79-82%.

***** Where yields are not quoted it is because of a mixture of products (see experimental in Chapter Seven).
5.3.2 Linker and fluorophore

The next step was attempted installation of a mini-linker, via reaction with tert-butylbromoacetate or methyl-5-bromovalerate. Alkylation of the cyclohexanol of 5.1a and 5.1b with tert-butylbromoacetate was attempted using Williamson ether conditions using 2 eq. of NaH in DMF at 60°C, however this yielded only minimal product 5.12a or 5.12b (Scheme 13, reaction i.). Most of the starting material (5.1a, 5.1b) appeared to have broken down, possibly as a result of the harsh NaH base. The same reaction on 5.1a was attempted again using 3 eq. of CsCO₃ as the base, however, this also yielded insufficient product 5.12a (reaction ii.). The high pKₐ of the cyclohexanol likely required a very strong base for deprotonation, so the reaction was attempted with NaH again, but with batch wise addition of the NaH (1 eq., then 1 eq. again) to try to reduce breakdown of starting material (reaction iii.), however, this attempt was no more successful than the first attempt with NaH. Changing the solvent was not considered as the polar, aprotic DMF should have favoured the alkoxide nucleophile over the hydroxyl. Bulky alkoxides such as the deprotonated cyclohexanol can sometimes favour E2 elimination over SN2 reactions.³¹⁶ Therefore, the reaction of 5.1a and 5.1b with methyl-5-bromovalerate using batch wise addition of NaH (1 eq., then 1 eq. again) was carried out at room temperature to favour the SN2 reaction (reaction iv.). Monitoring of the reaction by MS showed a high conversion rate of starting material to a product with the desired molecular mass. However, NMR spectra of the isolated product indicated that the methyl valerate had added to the aromatic ring at the C4 position yielding products (suspected to be 5.13a and 5.13b) with the exact same mass as the intended product 5.3 and 5.7. If other potential linker positions were explored in future work, it would be interesting to pharmacologically evaluate 5.13a and 5.13b.
Scheme 13: Attempted alkylation of cyclohexanol. Reagents and conditions: (i) tert-butylbromoacetate, NaH, DMF, 60°C, 3 h; (ii) CsCO₃, DMF, 60°C, 14 h; (iii) tert-butylbromoacetate, batch wise addition of NaH, DMF, 60°C, 14 h; (iv) tert-butylbromoacetate, batch wise addition of NaH, DMF, rt, 14 h.

It seems that the $pK_a$ (calculated to be 15.43)††††† of the hydroxyl of 5.1a and 5.1b was perhaps too high for deprotonation and the conditions utilised were too harsh for the rest of the molecule. An alternative synthetic route to install small linkers onto the cyclohexanol could be to first alkylate the cis- or trans-4-aminocyclohexanol and then couple to the carboxylic acid 5.11b to form 5.12a-b, 5.3 and 5.7. This proposed route is illustrated for the synthesis of 5.12a (Scheme 14). Harsh conditions would still have to be used for the alkylation of the hydroxyl as the $pK_a$ (15.26)‡‡‡‡‡ of the trans-4-aminocyclohexanol 5.14 hydroxyl is not so dissimilar to that of the hydroxyl of 5.1a. Therefore, it is probable that the amino group of 5.14 would first need to be protected.

††††† $pK_a$ values for the hydroxyl of 5.1a and 5.1b and
‡‡‡‡‡ 4-aminocyclohexanol 5.14 were calculated in Marvin Sketch 17.2.27, ChemAxon Ltd.
The amino protecting group would need to be non-labile under the strong basic conditions needed for alkylation and it also could not be deprotected using acidic conditions, as that would cleave the tert-butyl group of the tert-butylacetate mini-linker. Therefore, a potential protecting agent could be benzylchlorofomate, to form the carboxybenzyl (Cbz) protected 5.15. Alkylation of 5.15 with tert-butylbromoacetate to form 5.16 could be followed by hydrogenation to cleave the Cbz group, revealing the amine 5.17, ready for coupling with the carboxylic acid 5.11b to form 5.12a. The major drawback to this route is the number of synthetic steps required and the early divergence of the cis and trans isomers, meaning that synthesis of 5.12a-b, 5.3 and 5.7 from 5.11b would require 14 reactions versus the six reactions required in the original attempted route. This synthetic route was considered, however time constraints and the length of the synthetic route, combined with insufficient supplies of 5.11b, meant that it was not feasible to attempt this synthesis. It would be interesting to attempt this proposed synthetic route to 5.12a-b, 5.3 and 5.7 in future work.

Scheme 14: Proposed alternative route for alkylation of trans-4-aminocyclohexanol to form 5.12a.
As synthesis of the original ligand design (5.12a-b, 5.3 and 5.7) was not possible, an alternative design was sought that would enable evaluation of the cyclohexanol as a position for linker and fluorophore conjugation. Derivatising of the cyclohexanol required a reaction where it did not matter that the hydroxyl was a poor nucleophile. Converting the hydroxyl to either a bromine or a mesylate was considered, as these groups could then be reacted with a hydroxyl functionalised linker. However, this reaction would then be relying upon the likely poor nucleophilic character of the linker hydroxyl.

Conjugating an activated carboxylic acid to the hydroxyl of 5.1a and 5.1b to form an ester would be a relatively straightforward reaction, which could be carried out quickly. The major drawback of this approach is that an ester would not usually be desirable in a fluorescent ligand due to the potential for hydrolysis. However, methyl esters had been demonstrated to be stable under the assay conditions (section 3.6.1, page 96) and this design would at least enable evaluation of some compounds with linkers and dyes conjugated to the cyclohexyl group as a proof of concept. Boc-glycine and 7-aminoheptanoic acid 5.18 were readily available in-house and coupling of these as linkers to the cyclohexanol would allow for sampling of varying lengths of linker. 7-Aminoheptanoic acid 5.18 was Boc protected to yield 5.19 (60%; Scheme 15, step i). Carbodiimides such as N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide (EDC) are commonly used in ester coupling reactions, but can lead to formation of N-acylurea side products, reducing product yield, which is especially likely when the alcohol is a very poor nucleophile, such as 5.1a or 5.1b. Therefore, tetramethylfluoroformamidinium hexafluorophosphate (TFFH) was chosen to activate the carboxylic acid, as it would be less likely to form side products. Boc-glycine or Boc-protected 7-aminoheptanoic acid 5.19 were reacted with TFFH and triethylamine to form the activated acyl fluoride prior to 4-dimethylaminopyridine (DMAP) catalysed coupling to 5.1a or 5.1b, yielding 5.20a-5.20d (Scheme 15, step ii). The isolated yield of 5.20b was particularly low (9%) due to poor conversion of 5.1b to 5.20b. The MS-ESI of the crude 5.20b showed a large M+H* peak corresponding to unreacted starting material 5.1b. It is interesting to note that the longer, linear, less bulky (at the point of reaction) linker (5.19) gave higher yields in the formation of 5.20c and 5.20d (66-80%) than reaction with the glycine linker (9-22%).
Scheme 15: Synthesis of linker conjugates and fluorescent ligands. Reagents and conditions: (i) Boc₂O, dioxane, NaOH, H₂O, rt, 14 h, 60%; (ii) Boc-glycine or 5.19, TFFH, Et₃N, DMAP, DCM, rt, 14 h, 9-80%; (iii) TFA, DCM, rt, 1 h, 45-55%; (iv) Ac₂O, DIPEA, DCM, rt, 1 h, 96-98%; (v) BODIPY 630/650-X-OSu 2.15, DIPEA, DMF, rt, 14 h, 72-97%.

Boc deprotection of 5.20a-d using TFA revealed the amines 5.21a-d (Scheme 15, step iii) which were purified by semi-preparative RP-HPLC to obtain maximum purity before further reaction. The amines 5.21a-d were then either acetylated (Scheme 15, step iv) to yield 5.22a-d or conjugated to BODIPY 630/650-X-OSu 2.15 to yield fluorescent ligands 5.9a-d (step v). The acetylated 5.22a-d and fluorescent ligands 5.9a-d were purified by semi-preparative RP-HPLC.
5.4 Pharmacological evaluation

Two pharmacophores (5.1a, 5.1b) four pharmacophore-linker conjugates (5.22a-d) and four final fluorescent compounds (5.9a-d) were subjected to pharmacological evaluation. Radioligand binding assays were carried out to determine affinity at hCB$_2$ and hCB$_1$ receptors and compounds with high affinity at CB$_2$ receptor were tested in cAMP assays to determine function (refer to section 1.6).

5.4.1 Radioligand binding assays

A competition binding screen was used to identify which compounds at a fixed concentration of 10 µM could displace more than 50% of bound [$^3$H]CP55,940 1.17 at hCB$_2$ and hCB$_1$ receptors (Figure 74). All compounds, apart from 5.22a and 5.9c displaced more than 50% of radioligand at hCB$_2$ receptor. It was gratifying to observe that all compounds showed low displacement of [$^3$H]CP55,940 1.17 at hCB$_1$ receptor, indicating CB$_2$ receptor subtype selectivity. Statistical analysis using the D’Agostino and Pearson normality test and a one sample $t$-test was carried out to determine whether compounds which displaced less than 50% of the radioligand showed significant binding at CB$_1$ and CB$_2$ receptors. All compounds demonstrated significant binding at CB$_1$ receptor ($K_i = >10$ µM), apart from 5.1a and 5.22a which both failed the normality test (Table 16). Linker-pharmacophore 5.22a showed significant binding at CB$_2$ receptor, whereas 5.9c also failed the normality test.
Figure 74: Combined results of competition binding screens showing displacement of $[^3]H$CP55,940 (1 nM) at HEK293-hCB$_2$ (7.5 µg/point; black) and -hCB$_1$ (5 µg/point; grey) membranes by a fixed concentration (10 µM) of test compound. Raw data was expressed in cpm and normalised to unlabelled CP55,940 (10 µM) (100%) and vehicle control (0%). Data shown is the mean value ± SEM of three independent experiments conducted in triplicate, except for 5.1a, 5.22c, 5.9a and 5.9d at hCB$_2$, where $n$ = 2 and 5.1b, 5.22b, 5.22d and 5.9b at hCB$_2$, where $n$ = 1. The dashed line indicates the 50% displacement criteria for progression to concentration response assays.

All of the compounds that showed greater than 50% displacement of radioligand at CB$_2$ receptor were subjected to concentration response assays to determine $K_i$ values (Table 16). Amongst these compounds were three fluorescent ligands 5.9a, 5.9b and 5.9d, of which the fluorescent glycine conjugate 5.9b showed particularly high displacement (90.2 ± 0.7%) of $[^3]H$CP55,940. Concentration response assays determined the highest affinity compound amongst the series was fluorescent ligand 5.9b which had a $K_i = 467 ± 20.0$ nM at hCB$_2$ receptor and >21-fold selectivity over CB$_1$ receptor (Table 16). Fluorescent ligand 5.9b represents a high affinity, selective fluorescent ligand for CB$_2$ receptor and fulfils the central aim of this thesis and the significance of this result will be discussed in more depth on page 179.

Cis isomers of the pharmacophore, pharmacophore-linkers and fluorescent ligands consistently showed higher affinity for CB$_2$ receptor than the analogous trans isomers, sometimes as much as ten-fold (5.22b vs 5.22a and 5.9b vs 5.9a). This pattern was contrary to what was predicted by ligand docking studies, which showed trans linkers
positioned more favourably for fluorescent dye conjugation. However, cis-4-methylcyclohexyl derivatives have been shown to be favoured in literature reports.\textsuperscript{145} It was interesting that substitution of the cis-4-methylcyclohexyl for a cis-4-cyclohexanol or even an extended cis-4-cyclohexyl ester linker still resulted in cis derivatives being favoured (section 4.1.1, pg 131). The identification of linker conjugates and fluorescent ligands with affinity for CB\textsubscript{2} receptor showed that the ligand docking carried out with a CB\textsubscript{2} receptor homology model was a reliable method for selecting an appropriate position for linker extension. However, the favoured cis isomers demonstrate that the ligand docking studies were perhaps not accurate at predicting precise ligand orientations, but nevertheless were a very useful exercise for ligand design in this instance.
Table 16: Affinity of C3-carboxamide cyclohexyl-substituted 1,8-naphthyridin based compounds for hCB\textsubscript{2} and hCB\textsubscript{1} receptors. \textsuperscript{a}

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cis or Trans</th>
<th>R</th>
<th>hCB\textsubscript{2} receptor</th>
<th>hCB\textsubscript{1} receptor</th>
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<tbody>
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<td></td>
<td></td>
<td>10 \mu M displ.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>[^{3}H]CP 1.17</td>
<td>51.0 ± 3.0</td>
<td>5628 ± 2890</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Radioligand binding assays performed with \[^{3}H\]CP55,940 (1 nM) and HEK293-hCB\textsubscript{2} or -hCB\textsubscript{1} membranes. Data is the mean of at least three experiments performed in triplicate.

\textsuperscript{b} Percentage displacement of \[^{3}H\]CP55,940 by test compound (10 \mu M). Raw data was normalised to the specific binding window (unlabelled CP55,940 (10 \mu M), 100%; vehicle control, 0%).

\textsuperscript{c} \(K\text{\textsubscript{i}}\) calculated using \[^{3}H\]CP55,940 \(K\text{\textsubscript{d}} = 3\) nM at hCB\textsubscript{2} or 2 nM at hCB\textsubscript{1} receptor. For compounds showing <50% displacement of \[^{3}H\]CP55,940 at 10 \mu M, the D’Agostino & Pearson normality test and a one sample \textit{t}-test were used to determine if significant competition with \[^{3}H\]CP55,940 had occurred.

\textsuperscript{d} SI = \(K\text{\textsubscript{i}}(CB\textsubscript{1})/K\text{\textsubscript{i}}(CB\textsubscript{2})\).
The glycine linker was clearly favoured for CB₂ receptor affinity over the aminoheptanoate linker in both the linker conjugates and fluorescent ligands (Table 16). For instance, the cis-glycine conjugate 5.22b showed a three-fold improvement in CB₂ receptor affinity over the analogous cis-aminoheptanoate conjugate 5.22d and the cis-glycine fluorescent ligand 5.9b showed a 17-fold improvement over the analogous cis-aminoheptanoate fluorescent ligand 5.9d. It was somewhat surprising that the glycine linker was long enough to allow favourable positioning of both the ligand and the fluorescent dye, however the pentyl chain of BODIPY 630/650-X does increase linker length between fluorophore and core ligand. The ligand docking studies with the original ligand designs (section 5.1) suggested that cyclohexanol substituted linkers may exit into the lipid membrane and therefore a linker as short as glycine may allow for favourable positioning of the lipophilic pentyl chain and fluorophore in the lipid membrane. In addition, the glycine linker conjugates 5.22b and 5.9b possess an ester and an amide in close proximity to each other which may both be able to form hydrogen bonds with residues located around the ligand binding pocket exit between TMH1 and TMH7 and this was explored in later ligand docking studies (see section 5.5). In contrast the amide of the aminoheptanoate conjugates 5.22d and 5.9d would likely be positioned in the membrane or extracellular space and thus may not be able to form hydrogen bonds with receptor residues.

A trend of increasing molecular weight correlating to increased CB₂ receptor affinity was observed - conjugation of a glycine linker (5.22b) to the cis isomer of the pharmacophore (5.1b) improved affinity for CB₂ receptor by two-fold, whilst conjugation of a fluorophore (5.9b) improved affinity even further (by almost five fold cf. to 5.1b; Table 16; Figure 75). Similar observations that a fluorescent ligand has a higher affinity for the receptor than the parent pharmacophore have been reported in the literature.198, 207, 318 This pattern indicates that the linker and fluorophore are making favourable interactions with receptor residues and the membrane and are not latent bystanders to ligand binding.
Identification of the high affinity fluorescent ligand 5.9b ($K_i = 467 \pm 20.0$ nM at hCB$_2$, $K_i > 10$ µM at hCB$_1$ receptor) was an exciting development in this project and validated the ligand design of conjugating fluorophores and linkers to the cyclohexyl moiety. It is difficult to compare the affinity of 5.9b to existing CB$_2$ fluorescent ligands (section 1.5.3.1), as many of these only have affinities reported for mCB$_2$ receptor,$^{184, 199, 200, 202, 204}$ and ligand receptor affinities are known to show dramatic variation between species,$^{247}$ making comparison meaningless. Whilst NIR760-Q 1.26 has an affinity reported for hCB$_2$ receptor ($K_i = 75.5 \pm 28.0$ nM),$^{203}$ it was determined using a fluorescence saturation binding assay, so cannot be reliably compared to the $K_i$ value of 5.9b determined using a [$^3$H]CP55,940 1.17 competition binding assay. NMP6 1.19 has a reported affinity for hCB$_2$ receptor ($K_i = 387$ nM) which was determined using a radioligand binding assay, but in CHO-K1 cells (versus HEK293 cells used here) and does not report the $K_d$ value used for [$^3$H]CP55,940 1.17,$^{201}$ also prohibiting comparison. Several fluorescent ligands for CB$_2$ receptor do not have an affinity value reported for CB$_1$ receptor and therefore have undetermined selectivity. The selectivity of 5.9b compares favourably to fluorescent ligands which have reported selectivity. For example, 5.9b ($K_i = 467 \pm 20.0$ nM at hCB$_2$; 22.5% displacement at 10 µM at hCB$_1$ receptor) has equal (or higher) selectivity for CB$_2$ receptor than NMP6 1.19 ($K_i = 387$ nM at hCB$_2$).
nM at hCB₂, <40% displacement at 10 µM at hCB₁ receptor)²⁰¹. The biotin tagged HU-210 1.20 showed high affinity for CB₂ receptor, but no selectivity ($K_i = 1.6 \pm 0.4$ nM at hCB₂, $K_i = 2.4 \pm 0.4$ nM at hCB₁ receptor; determined for biotin-HU-210 1.20, not the final fluorescent ligand conjugate).³⁸⁷ A fluorescent ligand needs high CB₂ receptor selectivity for use in mixed receptor population, native cell types. Therefore the good selectivity of 5.9b for CB₂ receptor over CB₁ receptor is a promising indicator for its potential as a pharmacological tool.

5.4.2 Forskolin-stimulated cAMP

Compounds that showed greater than 50% displacement of $[^{3}H]$CP55,940 1.17 at CB₂ receptor were analysed for functional activity using a BRET sensor to measure modulation of forskolin-stimulated cAMP in whole HEK293 Flp cells overexpressing hCB₂ or hCB₁ receptors (refer to section 1.6.2). Compounds were initially analysed at 10 µM in the absence of forskolin to assess effect on basal cAMP, and in the presence and absence of an EC₉₀ concentration of CP55,940 1.10 to test for antagonism, inverse agonism and agonism (Figure 76). All compounds behaved as inverse agonists at hCB₂ receptor, reducing basal signalling levels, whilst 5.1b, 5.22b, 5.22d and 5.9b also blocked receptor access of the agonist CP55,940 1.10. Compounds all behaved as inverse agonists at CB₁ receptor, but were not able to block CP55,940 1.10.
Figure 76: BRET assay screen measuring effect of compound (10 µM) on forskolin-stimulated (5 µM) cellular cAMP levels in HEK293 cells overexpressing (A) hCB₂ receptor or (B) hCB₁ receptor. Compounds were checked for effect on basal cAMP (compound/V/V; light grey) and in the presence (compound/Fsk/CP; dark grey) and absence of CP55,940 (compound/Fsk/V; mid grey). Data is the mean ± SEM of at least three independent experiments conducted in duplicate, except for those marked by * are two independent experiments conducted in duplicate or # are one experiment conducted in duplicate. Data has been normalised to a percentage of forskolin only response (V/Fsk/V; 100%) and basal response (V/V/V; 0%).

The four compounds with the highest affinity for CB₂ receptor (5.1b, 5.22b, 5.22d and 5.9b) were then subjected to functional concentration response assays at CB₂ receptor to determine potency as inverse agonists and 5.9b was also tested at CB₁ receptor.
Compounds 5.1b, 5.22b, 5.9b and the literature control SR144528 \textsuperscript{1.13} all showed similar IC\textsubscript{50} values at CB\textsubscript{2} receptor and statistical analysis by a one way ANOVA (Tukey’s multiple comparisons test) showed no significant difference between them. In contrast, 5.22d showed a much lower potency (in the micromolar range), which is interesting considering it is the only compound with the aminohexanoate linker. It is possible that the aminohexanoate linker has much less favourable interactions with receptor residues for cAMP signalling than the glycine linker. Fluorescent ligand 5.9b showed a significantly reduced potency at CB\textsubscript{1} receptor (IC\textsubscript{50} = 4252 ± 530 nM) and all compounds showed reduced efficacy at CB\textsubscript{1} receptor, demonstrating CB\textsubscript{2} receptor selectivity.

Table 17: Potency (IC\textsubscript{50}) and efficacy (E\textsubscript{max}) of C3-carboxamide cyclohexyl-substituted 1,8-naphthyridin based compounds determined using a forskolin-stimulated cAMP assay. \textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>hCB\textsubscript{2} receptor \textsuperscript{b}</th>
<th>hCB\textsubscript{1} receptor \textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} nM ± SEM</td>
<td>E\textsubscript{max} % ± SEM \textsuperscript{d}</td>
</tr>
<tr>
<td>5.1a</td>
<td>-</td>
<td>160 ± 4.6</td>
</tr>
<tr>
<td>5.1b</td>
<td>211 ± 83.4</td>
<td>156 ± 5.5</td>
</tr>
<tr>
<td>5.22b</td>
<td>109 ± 28.4</td>
<td>154 ± 5.4</td>
</tr>
<tr>
<td>5.22c</td>
<td>-</td>
<td>165 ± 10.1</td>
</tr>
<tr>
<td>5.22d</td>
<td>1738 ± 489</td>
<td>179 ± 12.1</td>
</tr>
<tr>
<td>5.9a</td>
<td>-</td>
<td>172 ± 11.1</td>
</tr>
<tr>
<td>5.9b</td>
<td>221 ± 75.1</td>
<td>210 ± 15.7</td>
</tr>
<tr>
<td>5.9d</td>
<td>-</td>
<td>201 ± 3.6</td>
</tr>
<tr>
<td>SR144528 \textsuperscript{1.13}</td>
<td>132 ± 24.1</td>
<td>153 ± 3.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} cAMP levels measured in a BRET assay using a CAMYEL sensor, performed in either \textsuperscript{b} HEK293-hCB\textsubscript{2} Flp cells or \textsuperscript{c} HEK293 hCB\textsubscript{1} S4 low cells.

\textsuperscript{d} E\textsubscript{max} is the response at 10 µM, normalised to basal (0%) and forskolin only (100%) levels of cAMP.

All values represent the mean ± SEM of at least three independent experiments conducted in duplicate, except \textsuperscript{e} which are two independent experiments performed in duplicate.

A one sample t-test was used to determine if E\textsubscript{max} values were significantly different from forskolin only values (100%) and for \textsuperscript{f} no significant difference was found, therefore the compounds were determined to show no response under the assay conditions.
Comparison of the efficacy ($E_{\text{max}}$) of compounds at CB$_2$ receptor (Table 17) by a one way ANOVA (Tukey’s multiple comparisons test) showed few significant differences between them. However, 5.9b showed statistically significant higher efficacy than 5.1a ($p = 0.0196$), 5.1b ($p = 0.0021$), 5.22b ($p = 0.0013$) while SR144528 1.13 ($p = 0.0020$) and 5.9d also showed statistically significant higher efficacy than 5.1b ($p = 0.0293$), 5.22b ($p = 0.0195$) and SR144528 1.13 ($p = 0.0176$). It is interesting that the fluorescent ligand 5.9b should show higher efficacy than the analogous linker-pharmacophore 5.22b and pharmacophore 5.1b, as this demonstrates that the steric bulk of the fluorescent dye and linker is not having a negative impact on the efficacy of the ligand. It appears that ligand entry into receptor is not impeded by this bulk either.

The observation that lead fluorescent ligand 5.9b showed a similar IC$_{50}$ value (221 ± 75.1 nM) and a higher efficacy at hCB$_2$ receptor than SR144528 1.13 was an excellent indicator for its potential use as a pharmacological tool. Published fluorescent ligands for CB$_2$ receptor have not reported characterisation of the ligand function through signalling assays. However, a selection of fluorescent ligands with varying functions would allow for a diversity of applications. An inverse agonist fluorescent ligand such as 5.9b could potentially be used in studies of CB$_2$ receptor expression and localisation and binding assays.

The inverse agonist function observed for compounds 5.1a, 5.1b, 5.22b-d, 5.9a, 5.9b and 5.9d is in line with literature reports for C6-substituted 1,8-naphthyridin-2(1H)-one-3-carboxamide derivatives, as well as the results observed in Chapter Four.

All of the compounds (5.1a, 5.1b, 5.22b-d, 5.9a, 5.9b and 5.9d) analysed for function were also screened in a cAMP BRET assay in WT HEK293 Flp and WT HEK293 S4 low cells to verify that the observed effects at CB$_2$ and CB$_1$ receptors were receptor mediated (Table 18). The compound cAMP responses were normalised to basal (0%) and forskolin only (100%) response, and then analysed in a one sample $t$-test to determine if the compound responses were significantly different from the forskolin only response. Compounds 5.1b, 5.22b and 5.22c showed no significant response in the WT Flp cells, verifying that the inverse agonist activity observed for these compounds at CB$_2$ receptor was indeed CB$_2$ receptor mediated. Compounds 5.1a, 5.22d and 5.9b
invoked a small but significant response at 10 μM but not at 1 μM. As 5.22d and 5.9b demonstrated a response in HEK293-hCB2 cells at 1 μM, it can be concluded that the WT HEK293 Flp cell response is not large enough to have significantly affected the calculated CB2 receptor potency. However, as 5.1a was not tested at 1 μM in HEK293-hCB2 cells, it cannot be definitively concluded that the inverse agonist efficacy observed at CB2 is receptor mediated. Fluorescent ligands 5.9a and 5.9d also invoked a small but significant response at 10 μM, but were not tested at 1 μM in either WT HEK293 Flp or HEK293-hCB2 Flp cells, so it similarly cannot be concluded whether the inverse agonist efficacy observed at CB2 is receptor mediated. Fluorescent ligand 5.9b showed a significant response at 10 μM in the WT HEK293 S4 low cells, but not at 1 μM and as 5.9b invoked a response at 1 μM in HEK293-hCB1 S4 low cells, it can be concluded that the response observed is CB1 receptor mediated. Linker-pharmacophore conjugates 5.22b and 5.22d, and fluorescent ligands 5.9a and 5.9d also showed significant responses at WT HEK293 S4 cells. However, as 5.22b and 5.9a did not show significant responses at HEK293-hCB1 cells, their response in WT cells is of limited concern. Whilst 5.22d and 5.9d did not show a significant response at 1 μM in WT S4 low cells, neither compound was tested at 1 μM in HEK293-CB1 S4 low cells, so it cannot be absolutely demonstrated that the inverse agonist responses observed in cells overexpressing CB1 receptor for these compounds was CB1 receptor mediated.
Table 18: Forskolin stimulated cAMP response in WT HEK293 cells. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>% response ± SEM, Flp line</th>
<th>% response ± SEM, S4 low line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>SR144528 1.13</td>
<td>97.4 ± 5.9</td>
<td>98.5 ± 3.8</td>
</tr>
<tr>
<td>WIN 55.212-2 2.2</td>
<td>87.2 ± 2.9 *</td>
<td>90.1 ± 4.6</td>
</tr>
<tr>
<td>CP55,940 1.10</td>
<td>90.1 ± 5.5</td>
<td>91.4 ± 3.1</td>
</tr>
<tr>
<td>5.1a</td>
<td>118.0 ± 5.1 *</td>
<td>96.5 ± 6.4 c</td>
</tr>
<tr>
<td>5.1b</td>
<td>111.9 ± 5.4</td>
<td>95.3 ± 1.4</td>
</tr>
<tr>
<td>5.22b</td>
<td>109.8 ± 5.2</td>
<td>92.2 ± 1.1</td>
</tr>
<tr>
<td>5.22c</td>
<td>110.1 ± 5.2</td>
<td>-</td>
</tr>
<tr>
<td>5.22d</td>
<td>122.9 ± 6.2 *</td>
<td>98.3 ± 2.5</td>
</tr>
<tr>
<td>5.9a</td>
<td>120.9 ± 7.6 *</td>
<td>-</td>
</tr>
<tr>
<td>5.9b</td>
<td>139.5 ±10.0 *</td>
<td>100.5 ± 2.5</td>
</tr>
<tr>
<td>5.9d</td>
<td>127.3 ± 4.4 *</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* cAMP levels measured in a BRET assay using a CAMYEL sensor. Data represent mean values ± SEM for at least three independent experiments conducted in duplicate, except which b are two independent experiments conducted in duplicate, or c which is one experiment conducted in duplicate. Values are normalised to basal (0%) and forskolin (100%) response. Data was analysed using a one sample t-test for significant difference to forskolin only (100%) response and values marked with * demonstrated significant difference.
5.5 Molecular modelling

The cis-glycine linked 5.22b was docked into homology model C of CB2 receptor, to investigate its receptor binding interactions. The docking poses generated were clustered into two groups, with the most consistent pose (six out of ten) showing the methoxyphenyl group positioned down in the aromatic region between TMH3 and 6 and the glycine linker pointing out of the orthosteric binding pocket (Figure 77). The core naphthyridin rings and the C6 and N1 substituents appear to fill a large portion of the orthosteric binding site (Figure 78C). A fluorophore conjugated to 5.22b, such as in the lead fluorescent ligand 5.9b, may potentially exit between either TMH1 and 7 or TMH1 and 2 (Figure 78B). The methoxyphenyl is able to form a hydrogen bond with S292, whilst the phenyl is within 3.0 Å of W258 with which it can engage in aromatic stacking or van der Waals interactions (Figure 78A). The glycine linker is able to form multiple hydrogen bonds with Q32 of TMH1 and D24 of the amino terminus (Figure 77). This supports the hypothesis previously postulated - that glycine linkers may be favourable over the heptylamide linkers due to the close proximity of the ester and the amide groups in glycine, which are both capable of forming hydrogen bonds with residues around the exit of the orthosteric binding pocket. In contrast, the amide of the heptylamide linked derivatives may be too distant to position favourably for hydrogen bonding with receptor residues.
Figure 77: The lowest energy, most consistent docking pose of the glycine linker-pharmacophore 5.22b (cyan) docked into CB$_2$ receptor homology model C. The glycine linker is able to form hydrogen bonds (yellow dashed lines) with two amino acid residues (D24 and Q32) located by the exit from the orthosteric binding site. Side chains of residues within 4 Å of 5.22b are shown as sticks (pale green).
Figure 78: The lowest energy, most consistent docking pose of the glycine linker-pharmacophore 5.22b (cyan) docked into CB₂ receptor homology model C. (A) The ether of the methoxyphenyl is able to hydrogen bond (yellow dashed line) with S292 on TMH7, whilst the phenyl is in close range (3.0 Å) of W258 on TMH6 for potential π stacking or van der Waals interactions. Side chains of residues within 4 Å of 5.22b are shown as sticks (pale green). (B) Fluorophore conjugated to the glycine linker (as in 5.9b) may exit between TMH1 and TMH7 or TMH1 and TMH2. (C) Glycine linker-pharmacophore 5.22b fills the orthosteric pocket of CB₂ receptor.
5.6 Design and synthesis of a non-ester analogue of 5.9b

A major concern for the use of 5.9b as a pharmacological imaging tool is the potential for hydrolysis of the ester bond between the cyclohexyl group and the glycine. Whilst the stability of methyl esters have been demonstrated under the radioligand assay conditions, it remains to be seen whether the ester present in 5.9b would be stable across the range of temperatures and experiment lengths it could be subjected to during CB₂ receptor studies.

5.6.1 Design

Since 5.9b showed such good biological activity it is now of interest to try to refine the design of 5.9b by replacing the ester with a more stable group. An amide was selected as a promising substitution for the ester, as it would maintain the carbonyl group in the same position, retain similar molecular geometry and the ability to engage in hydrogen bonding with receptor residues. However, the reduced flexibility of the amide bond compared to the ester, as well as the slight increase in hydrophilicity may affect CB₂ receptor binding.

5.6.2 Synthesis

Coupling of the commercially available mono-Boc protected cis-1,4-cyclohexyldiamine with 5.11b yielded 5.23 (Scheme 16, step i). Subsequent TFA cleavage of the Boc group of 5.23 (step ii) revealed the amine 5.24 which was coupled to Boc-glycine to give 5.25 (step iii). Cleavage of the Boc of 5.25 gave the amine 5.26 (step iv) which was purified by semi-preparative RP-HPLC to ensure maximum purity. Conjugation of BODIPY 630/650-X-OSu 2.15 to 5.26 yielded the fluorescent ligand 5.27 (step v), which was also purified by semi-preparative RP-HPLC.
Scheme 16: Synthesis of fluorescent ligand 5.27. Reagents and conditions: (i) 1-N-Boc-cis-1,4-cyclohexyldiamine, HATU, DIPEA, DMF, rt, 14 h, 71%; (ii) TFA, DCM, rt, 1 h, quantitative; (iii) Boc-glycine, HATU, DIPEA, DMF, rt, 14 h, 31%; (iv) TFA, DCM, rt, 1 h, 57%; (v) BODIPY 630/650-X-OSu 2.15, DIPEA, DMF, rt, 14 h, quantitative yield. See experimental for details of yield.

§§§§§
5.7 Conclusions

In this chapter, a series of fluorescent ligands was synthesised by substitution of the C3-carboxamide cyclohexyl group with linkers and fluorophore. Pharmacological evaluation of this series identified a high affinity, CB₂ receptor selective fluorescent ligand 5.9b ($K_i = 467 \pm 20.0$ nM at hCB₂ receptor, $K_i$(CB₁)/$K_i$(CB₂) = >21; Figure 79) with inverse agonist activity at CB₂ receptor.

![Figure 79: The lead fluorescent ligand 5.9b.](image)

The identification of 5.9b fulfils the major aim of this PhD project. As well as being a promising potential imaging tool, it has validated the linker and fluorophore substitution position selected in this chapter. Two additional fluorescent ligands (5.9a and 5.9d) were also identified with micromolar affinity for CB₂ receptor. Furthermore, pharmacological evaluation of the precursor linker-ligand conjugates 5.22a-d and ligands 5.1a and 5.1b revealed a clear preference for the cis over trans isomers.

5.7.1 Future directions

Confocal imaging studies of 5.9b with whole live cells expressing CB₂ receptor will be carried out by collaborators at the University of Auckland. Non-specific membrane binding is a typical stumbling block in the pursuit of fluorescent ligands for GPCRs, so it is important to properly validate a fluorescent ligand as a practical tool for imaging studies. Experiments are planned in which HEK293 cells overexpressing hCB₂ receptor
will be incubated with 5.9b in the presence or absence of a high concentration of pre-incubated high affinity, CB$_2$ receptor selective non-fluorescent ligand SR144528 1.13. This will enable receptor-specific binding versus membrane binding of 5.9b to be examined. If successful and 5.9b demonstrates selective CB$_2$ receptor binding with low level of non-specific binding, it can then begin rigorous evaluation using, for example, cells overexpressing CB$_1$ receptor (in the hope of there being little binding) and in other cell types and native cells with mixed receptor populations.

The ester bond present in 5.9b could potentially be hydrolysed, therefore, a key priority is to carry out stability studies on 5.9b. In addition, 5.9b will be functionally characterised at other signalling pathways, such as β arrestin recruitment, $[^{35}S]$-GTP$_\gamma$S binding and membrane potential assays.

Pharmacological evaluation of the fluorescent ligand 5.27 (which contains an amide bond in place of the ester bond of 5.9b) will be undertaken in future studies by collaborators to evaluate its affinity, selectivity and function at CB$_2$ receptor. It will be interesting to see if 5.27 retains the affinity of the analogous 5.9b, or whether substitution of the ester for an amide leads to a loss (or gain) in affinity for CB$_2$ receptor. These pharmacological results will inform further efforts to refine the design of 5.9b. For instance, it is possible that presence of an amide might induce too much rigidity for favourable CB$_2$ receptor binding, especially in the narrow region between TMH1 and 7 or TMH1 and 2, where ligand docking studies suggest the amide bond may be positioned. If this proves to be the case, it would be interesting to replace the ester of 5.9b with a flexible ethyl ether, maintaining the same linker length as 5.9b, as illustrated in 5.28 (Figure 80).
It was extremely gratifying to identify a high affinity fluorescent ligand for CB₂ receptor in this study. The validation of C3-carboxamide cyclohexyl as an appropriate position for linker and fluorophore conjugation has opened up new opportunities for design and synthesis of further fluorescent ligands for CB₂ receptor. Future studies could explore variations on this design to improve upon the affinity for CB₂ receptor.

It could be worthwhile exploring replacement of the BODIPY 630/650-X fluorophore used in the lead fluorescent ligand 5.9b with other fluorophores, such as BODIPY FL-X, BODIPY TMR-X, BODIPY TR-X, or a cyanine or Alexa Fluor dye. Replacing the fluorophore could have a significant effect on ligand affinity and physicochemical properties, such as non-specific membrane binding. In addition, generating a range of fluorescent ligands with different wavelengths would enable application in varying techniques, such as FRET or BRET assays.

This study has clearly demonstrated that linker length can have a huge impact on CB₂ receptor affinity. Therefore, synthesis of derivatives with varying linker length, perhaps exploring the range between the three and eight atoms already trialled might be a potential avenue to improving CB₂ receptor affinity. In addition, published studies have shown that the individual amino acid residues present in a peptide linker hugely impact GPCR affinity of fluorescent ligands. Substitution of the glycine in 5.9b (or non-ester analogues) with other single amino acid residues is likely to significantly affect CB₂ receptor affinity.
A pattern of inverse agonism and/or antagonism for C6-substituted and agonism for C6-unsubstituted 1,8-napthyridin-2(1H)-one-3-carboxamide derivatives was identified by Lucchesi et al. In accordance with this pattern, the inverse agonist derivatives identified in this chapter all contained methoxyphenyl substitutions at C6. It would be interesting to synthesise analogues of 5.9b (or any other fluorescent ligands later identified in this series) without C6 substituents, to explore whether agonism in C6-unsubstituted ligands is preserved upon extension with a fluorophore. A fluorescent agonist would have substantially different applications to an inverse agonist or antagonist in pharmacological studies (section 1.5.3) and so expanding the available toolbox in this way would be a highly worthwhile endeavour.
Chapter Six: Executive Conclusions

The aim of this PhD research was to develop a high affinity, selective fluorescent ligand for CB2 receptor. Libraries of pharmacophores, linker-pharmacophore conjugates and fluorescent ligands were developed from two classes of cannabinoids: indole and 1,8-naphthyridin.

Literature SAR of the indole cannabinoids was utilised to guide selection of the C5-7 positions for linker and fluorophore substitution (Chapters Two and Three). The C7 position was found to be strongly favoured for linker substitution over the C5 and C6 positions. A very high affinity, selective inverse agonist ligand 3.7b (Figure 81) for CB2 receptor was identified, which showed higher affinity, potency and efficacy for CB2 receptor than the inverse agonist SR144528 1.13, which is commonly used in pharmacological research. The indole-based fluorescent ligands did not have high affinity for CB2 receptor, however, a linker-pharmacophore lead 3.13 (Figure 81) could potentially be optimised in future work to develop fluorescent ligands for CB2 receptor.

![Figure 81: Indole based CB2 ligands: high affinity, selective inverse agonist 3.7b and lead linker-pharmacophore 3.13.](image)

A library of 1,8-naphthyridin-2(1H)-one-3-carboxamide based compounds with linker and fluorophore substitution at the N1 position was designed based on literature SAR
for this scaffold (Chapter Four). However, pharmacological evaluation of this series indicated that the N1 position was unsuitable for extension. Ligand docking studies aided design of a series of C3-carboxamide cyclohexyl substituted fluorescent ligands, which resulted in a high affinity, selective inverse agonist fluorescent ligand 5.9b (Figure 82) for CB$_2$ receptor. These results demonstrated that the cyclohexyl group is a suitable position for linker and fluorophore conjugation and provides opportunities for developing a range of fluorescent ligands with varied functionality at CB$_2$ receptor, expanding the variety of tools available for studying CB$_2$ receptor. Future studies will evaluate 5.9b as an imaging tool for CB$_2$ receptor.

![Chemical structure of 5.9b](image)

**5.9b**

$K_i = 467 \pm 20.0 \text{ nM at hCB}_2$

$K_i = >10 \text{ \mu M at hCB}_1$

Figure 82: CB$_2$ receptor fluorescent ligand 5.9b
Chapter Seven: Experimental

7.1 Chemistry

Chemicals and solvents were purchased from Sigma Aldrich, Merck or AK Scientific and were used without further purification. BODIPY 630/650-X-OSu 2.15 was purchased from Life Technologies. Anhydrous grade solvents were used when a dry atmosphere was required. Unless stated, all reactions were carried out at room temperature (rt) and under atmospheric pressure.

TLC was carried out on 0.2 mm aluminium-backed silica gel plates 60 F\textsubscript{254} and visualised under UV light at \(\lambda = 254\) nm and 365 nm and then with potassium permanganate dip. Flash column chromatography was carried out using 40-63 \(\mu\)m silica. All columns were performed by a gradient method and the starting conditions are given in each instance. RP-HPLC was carried out on an Agilent 1260 Infinity system, using an YMC C8 5 \(\mu\)m (150 \(\times\) 10 mm) column for semi-preparative RP-HPLC and an YMC C8 5 \(\mu\)m (150 \(\times\) 4.6 mm) column for analytical RP-HPLC. The mobile phases used were A: H\textsubscript{2}O (0.05\% TFA) and B: 9:1 ACN/H\textsubscript{2}O (0.05\% TFA). Analytical RP-HPLC retention times quoted below were determined with a standard method - 5\% A for 1 min, then a linear gradient of 5-95\% B from 1-27 min (followed by 1 min at 95\% B, 2 min linear gradient 95-5\% B and then 4 min re-equilibration at 5\% A). All compounds analysed for biological activity were >95\% purity by UV detection at 254 and 380 nm (and 550 nm for fluorescent compounds) by analytical RP-HPLC. All compounds HPLC purified as the TFA salt were neutralised using an Amberlyst A21 ion exchange resin before biological testing.

High resolution electrospray ionisation mass spectra (HRMS-ESI) were obtained on a microTOF\textsubscript{Q} mass spectrometer. Proton and carbon NMR spectra were obtained on either a 400 MHz or a 500 MHz Varian NMR spectrometer. Two-dimensional NMR experiments, including COSY, HSQC, HMBC and NOESY were used to assign spectra. Chemical shifts are listed on the \(\delta\) scale in part(s) per million (ppm), referenced
to CDCl₃, MeOD-d₄ or DMSO-d₆ with residual solvent as the internal standard and coupling constants (J) recorded in hertz (Hz). Signal multiplicities are assigned as: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; dd, doublet of doublets; dt, doublet of triplets; td, triplet of doublets; br, broad; or m, multiplet.

¹H and/or ¹³C NMR spectra are not reported for some compounds near the end of multistep syntheses. This is because these compounds were synthesised and/or purified on a small scale and had poor solubility, so NMR characterisation would require a large fraction of the product to be dissolved in deuterated dimethyl sulfoxide (DMSO), which would be difficult to recover. However, HRMS-ESI was obtained for all compounds and >95% purity demonstrated by analytical RP-HPLC. In addition, NMR characterisation was carried out for the precursors of products without NMR spectra. For instance, Boc-protected linker-indole conjugates were characterised by NMR and as Boc-cleavage is a straightforward procedure, it is extremely unlikely any other changes would occur to these molecules. Therefore, there can be confidence in the structures of the resulting deprotected amines, especially when considering the matching HRMS-ESI data. Similarly, the dye coupling reactions are straightforward and the HRMS-ESI corroborates the correct product was obtained.
2-(Morpholin-4-yl)ethyl methanesulfonate (2.17a)

A stirred solution of 4-(2-hydroxyethyl)-morpholine 2.16a (2.5 mL, 20.7 mmol) and Et₃N (8.6 mL, 62.0 mmol) in anhydrous DCM (48 mL) under N₂ was cooled to 0°C and then methanesulfonyl chloride (2.4 mL, 31.0 mmol) was added dropwise. The mixture was stirred at rt for 2 h, filtered, the solid washed with minimal DCM and this filtrate combined with the original filtrate was evaporated under reduced pressure to give a yellow oil (10.24 g) as a mixture of the desired product 2.17a and a salt (Et₃NH⁺Cl⁻). This material was used without further purification.

(Oxan-4-yl)methyl methanesulfonate (2.17b)

4-(Hydroxymethyl) tetrahydropyran 2.16b (2.9 mL, 25.0 mmol), Et₃N (10.4 mL, 74.9 mmol) and methanesulfonyl chloride (2.9 mL, 37.5 mmol) in DCM (50 mL) were reacted as described in the procedure for 2.17a to give an orange oil (7.77 g) as a mixture of the desired 2.17b and a salt. This material was used without further purification.

(Oxolan-3-yl)methyl methanesulfonate (2.17c)

Tetrahydro-3-furanmethanol 2.16c (0.86 mL, 9.0 mmol), Et₃N (3.7 mL, 26.9 mmol) and methanesulfonyl chloride (1.0 mL, 13.4 mmol) in DCM (21 mL) were reacted as described in the procedure for 2.17a to give a yellow oil (3.29 g) as a mixture of the desired 2.17c and a salt. This material was used without further purification.
5-(Benzyloxy)-1-[2-(morpholin-4-yl)ethyl]-1H-indole (2.19a)

A stirred solution of 5-benzyloxyindole 2.18a (600 mg, 2.7 mmol) in anhydrous DMF (11 mL) under N₂ was cooled to 0°C, and then NaH (60% by mass dispersion in mineral oil; 358 mg, 9.0 mmol) was added. The mixture was stirred at 0°C for 10 min and then warmed to rt and stirred for 30 min. The reaction mixture was cooled to 0°C and a solution of 2.17a (1.13 g, 5.4 mmol) in anhydrous DMF (5 mL) was added. The mixture was warmed to 45°C and stirred for 2 h, cooled to rt, diluted with EA (20 mL) and quenched with sat. aq. NH₄Cl (20 mL) and H₂O (10 mL). The layers were separated and the aqueous layer extracted with EA (3 × 20 mL). The combined organics were washed with H₂O (4 × 70 mL), dried over MgSO₄, filtered and evaporated under reduced pressure. The crude product was purified using flash silica column chromatography (1:2 PE/Ea) to yield the desired 2.19a (690 mg, 2.1 mmol, 76%) as a light brown wax (Rf 0.17, 1:2 PE/Ea).

1H NMR (400 MHz, CDCl₃): δ 7.52 – 7.46 (m, 2H, aromatic(Ar)H Bn), 7.43 – 7.37 (m, 2H, ArH Bn), 7.37 – 7.30 (m, 1H, ArH Bn), 7.26 (d, J = 8.8 Hz, 1H, ArH indole), 7.18 (d, J = 2.3 Hz, 1H, ArH indole), 7.13 (d, J = 3.1 Hz, 1H, ArH indole), 6.98 (dd, J = 8.9, 2.2 Hz, 1H ArH indole), 6.41 (dd, J = 3.2, 1.1 Hz, 1H, ArH indole), 5.12 (s, 2H, CH₂ Bn), 4.22 (t, J = 6.9 Hz, 2H, N1-CH₂), 3.72 (t, J = 4.6 Hz, 4H, O-CH₂ morpholino), 2.75 (t, J = 6.9 Hz, 2H, N1-CH₂CH₂), 2.49 (t, J = 4.6 Hz, 4H, N-CH₂ morpholino).

13C NMR (101 MHz, CDCl₃): δ 153.33, 137.86, 131.56, 128.98, 128.69, 128.61, 127.85, 127.64, 112.69, 110.03, 104.38, 101.03, 71.01, 67.03, 58.36, 53.99, 44.29.
HRMS-ESI calculated for C₂₁H₂₅N₂O₂ [M+H]⁺ 337.1911, found m/z 337.1884.

6-(Benzyloxy)-1-[2-(morpholin-4-yl)ethyl]-1H indole (2.19b)

6-Benzyloxyindole 2.18b (1.34 g, 6.0 mmol), NaH (732 mg, 18.3 mmol), and 2.17a (2.51 g, 12.0 mmol) in DMF (48 mL) were reacted as described in the procedure for 2.19a. The crude product was purified using flash silica.
column chromatography (1:2 hexane/EA) to yield the desired 2.19b (1.10 g, 3.3 mmol, 54%) as a brown solid (Rf 0.13, 2:1 PE/EA).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.55 – 7.44 (m, 3H, ArH Bn & ArH indole), 7.44 – 7.36 (m, 2H, ArH Bn), 7.36 – 7.29 (m, 1H, ArH Bn), 7.04 (d, J = 3.2 Hz, 1H, ArH indole), 6.95 – 6.83 (m, 2H, ArH indole), 6.42 (dd, J = 3.2, 0.7 Hz, 1H, ArH indole), 5.14 (s, 2H, CH$_2$ Bn), 4.32 – 4.04 (br m, 2H, N1-CH$_2$), 3.82 – 3.57 (br m, 4H, O-CH$_2$ morpholino), 2.83 – 2.62 (br m, 2H, N1-CH$_2$C$_2$H$_5$), 2.60 – 2.29 (br m, 4H, N-CH$_2$ morpholino).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 155.44, 137.60, 136.67, 128.67, 127.97, 127.63, 123.27, 121.68, 109.95, 101.38, 94.83, 70.94, 67.02, 58.08, 53.99, 44.10.

HRMS-ESI calculated for C$_{21}$H$_{25}$N$_2$O$_2$ [M+H]$^+$ 337.1911, found m/z 337.1880.

5-(Benzyloxy)-1-[(oxan-4-yl) methyl]-1H-indole (2.19c)

5-Benzoylindole 2.18a (600 mg, 2.7 mmol), NaH (481 mg, 12.0 mmol) and 2.17b (1.04 g, 5.4 mmol) in DMF (17 mL) were reacted as described in the procedure for 2.19a. The crude product was purified using flash silica column chromatography (2:1 PE/EA) to yield the desired 2.19c (570 mg, 1.8 mmol, 66%) as a light brown oil (Rf 0.44, 2:1 PE/EA).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.52 – 7.44 (m, 2H, ArH Bn), 7.43 – 7.36 (m, 2H, ArH Bn), 7.35 – 7.29 (m, 1H, ArH Bn), 7.23 (d, J = 8.9 Hz, 1H, ArH indole), 7.17 (d, J = 2.4 Hz, 1H, ArH indole), 7.03 (d, J = 2.4 Hz, 1H, ArH indole), 6.96 (dd, J = 8.9, 2.4 Hz, 1H, ArH indole), 6.40 (d, J = 3.0 Hz, 1H, ArH indole), 5.11 (s, 2H, CH$_2$ Bn), 3.99 – 3.91 (m, 4H, N-CH$_2$ & O-CH$_2$ tetrahydropyran (THP)), 3.31 (td, J = 11.7, 2.3 Hz, 2H, O-CH$_2$ THP), 2.16 – 2.02 (m, 1H, CH THP), 1.53 – 1.32 (m, 4H, O-CH$_2$CH$_2$ THP).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 153.29, 137.85, 131.77, 129.02, 128.96, 128.61, 127.85, 127.64, 112.68, 110.25, 104.24, 100.72, 70.97, 67.58, 52.65, 36.40, 30.98.

HRMS-ESI calculated for C$_{21}$H$_{24}$N$_2$O$_2$ [M+H]$^+$ 322.1802, found m/z 322.1778.
5-(Benzyloxy)-1-[(oxolan-3-yl) methyl]-1H-indole (2.19d)

5-Benzylxoyindole 2.18a (600 mg, 2.7 mmol), NaH (322 mg, 8.1 mmol) and 2.17c (969 mg, 5.4 mmol) in DMF (16 mL) were reacted as described in the procedure for 2.19a. The crude product was purified using flash silica column chromatography (2:1 PE/EA) to yield the desired 2.19d (582 mg, 1.9 mmol, 70%), as a red-orange oil (Rf 0.63, 2:1 EA/PE).

1H NMR (400 MHz, CDCl3) δ 7.55 – 7.46 (m, 2H, ArH Bn), 7.44 – 7.37 (m, 2H, ArH Bn), 7.37 – 7.30 (m, 1H, ArH Bn), 7.27 (d, J = 8.9 Hz, 1H, ArH indole), 7.19 (d, J = 2.5 Hz, 1H, ArH indole), 7.08 (d, J = 3.1 Hz, 1H, ArH indole), 6.98 (dd, J = 8.9, 2.4 Hz, 1H, ArH indole), 6.43 (dd, J = 3.1, 0.8 Hz, 1H, ArH indole), 5.12 (s, 2H, CH2 Bn), 4.06 (d, J = 7.8 Hz, 2H, N-CH2), 3.97 (td, J = 8.3, 5.5 Hz, 1H, O-CH2 tetrahydrofuran (THF)), 3.82 – 3.67 (m, 2H, O-CH2 THF), 3.61 (dd, J = 8.9, 4.7 Hz, 1H, O-CH2 THF), 2.90 – 2.75 (m, 1H, CH THF), 2.10 – 1.94 (m, 1H, CH2 THF), 1.73 – 1.57 (m, 1H, CH2 THF).

13C NMR (101 MHz, CDCl3) δ 153.39, 137.84, 131.69, 129.02, 128.63, 128.43, 127.87, 127.65, 112.84, 101.14, 101.14, 71.13, 71.01, 67.68, 49.22, 40.16, 29.97.

HRMS-ESI calculated for C20H21NNaO2 [M+Na]+ 330.1464, found m/z 330.1441.

Formation of [(Phen)Pd(OAc)2] complex (2.21)

Palladium (II) acetate 2.20 (324 mg, 1.5 mmol) was dissolved in acetone (28 mL) and left unstirred for 30 min at rt, after which, undissolved solid Pd(OAc)2 was removed by filtration with fine filter paper. 1,10-Phenanthroline (313 mg, 1.7 mmol) was added to the reddish-brown filtrate, the solution swirled for 1 min in which time a precipitate started to form. The solution was left to sit unstirred for 30 min, the precipitate was filtered, washed with cold acetone and vacuum dried, yielding 2.21 (336 mg, 0.83 mmol, 57%) as a canary yellow solid, as described in the literature.273
5-(Benzyloxy)-3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indole (2.22a)

A stirred solution of 2.19a (104 mg, 0.31 mmol), 4-methoxybenzonitrile (119 mg, 0.89 mmol) and 2.21 (13 mg, 31.0 μmol) dissolved in H₂O (0.12 mL), glacial AcOH (0.18 mL) and 1,4-dioxane (0.6 mL) was heated to 140°C in a sealed pressure tube for 42 h. The reaction mixture was cooled to rt, diluted with DCM (18 mL), filtered through a celite pad and the celite washed with DCM (6 mL). The filtrate was evaporated under reduced pressure. The crude residue was purified by flash silica column chromatography (99:1 DCM/MeOH with 0.3% Et₃N to load silica), and recrystallised using MeOH to yield 2.22a (74 mg, 0.16 mmol, 51%) as dark brown crystals (Rᶠ 0.53, 95:5 DCM/MeOH).

¹H NMR (400 MHz, CDCl₃) δ 8.06 (d, J = 2.4 Hz, 1H, ArH indole), 7.90 – 7.81 (m, 2H, ArH MeOPh), 7.65 (s, 1H, ArH indole), 7.53 – 7.47 (m, 2H, ArH Bn), 7.43 – 7.36 (m, 2H, ArH Bn), 7.35 – 7.31 (m, 1H, ArH Bn), 7.29 (dd, J = 8.9, 0.5 Hz, 1H, ArH, indole), 7.05 (dd, J = 8.9, 2.5 Hz, 1H, ArH indole), 7.02 – 6.96 (m, 2H, ArH MeOPh), 5.17 (s, 2H, CH₂ Bn), 4.22 (t, J = 6.3 Hz, 2H, N1-CH₂), 3.90 (s, 3H, O-CH₃), 3.70 (t, J = 4.6 Hz, 4H, O-CH₂ morpholino), 2.77 (t, J = 6.4 Hz, 2H, N1-CH₂CH₂), 2.48 (t, J = 4.7 Hz, 4H, N-CH₂ morpholino).

¹³C NMR (101 MHz, CDCl₃) δ 189.85, 162.30, 155.70, 137.49, 137.18, 133.59, 131.90, 130.98, 128.64, 128.31, 127.95, 127.81, 115.46, 114.71, 113.63, 110.48, 105.36, 70.69, 67.02, 57.84, 55.58, 53.82, 44.45.

HRMS-ESI calculated for C₂₉H₃₁N₂O₄ [M+H]+ 471.2278, found m/z 471.2239.

6-(Benzyloxy)-3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indole (2.22b)

A solution of 2.19b (123 mg, 0.37 mmol), 4-methoxybenzonitrile (144 mg, 1.1 mmol) and 2.21 (14 mg, 37.0 μmol) in H₂O (0.14 mL), glacial AcOH (0.21 mL) and 1,4-dioxane (0.7 mL) was reacted as described in the procedure for 2.22a. The crude
residue was purified by flash silica column chromatography (99:1 DCM/MeOH, with 0.3% of 25% aq. ammonia solution to load silica) to yield **2.22b** (129 mg, 0.27 mmol, 75%) as a brown solid (Rf 0.47, 95:5 DCM/MeOH).

**1H NMR (500 MHz, CDCl₃)** δ 8.29 (d, J = 8.7 Hz, 1H, ArH indole), 7.87 – 7.80 (m, 2H, ArH MeOPh), 7.56 (s, 1H, ArH indole), 7.50 – 7.44 (m, 2H, ArH Bn), 7.42 – 7.36 (m, 2H, ArH Bn), 7.36 – 7.30 (m, 1H, ArH indole), 7.05 (dd, J = 8.7, 2.2 Hz, 1H, ArH indole), 7.00 – 6.93 (m, 2H, ArH MeOPh), 6.90 (d, J = 2.2 Hz, 1H, ArH indole), 5.14 (s, 2H, CH₂ Bn), 4.15 (t, J = 6.4 Hz, 2H, N1-CH₂), 3.87 (s, 3H, O-CH₃), 3.68 (t, J = 4.6 Hz, 4H, O-CH₂ morpholino), 2.72 (t, J = 6.4 Hz, 2H, N1-CH₂CH₂), 2.46 (t, J = 4.7 Hz, 4H, N-CH₂ morpholino).

**13C NMR (126 MHz, CDCl₃)** δ 189.64, 162.25, 156.39, 137.55, 137.15, 136.38, 133.43, 130.93, 128.65, 128.02, 127.57, 123.54, 121.79, 115.74, 113.53, 112.23, 95.19, 70.75, 66.94, 57.51, 55.50, 53.74, 44.12.

HRMS-ESI calculated for C₂₉H₃₁N₂O₄ [M+H]+ 471.2276, found m/z 471.2278.

**5-(Benzyloxy)-1-[2-(morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indole (2.22c)**

A solution of **2.19a** (525 mg, 1.6 mmol), 1-naphthonitrile (717 mg, 4.7 mmol) and **2.21** (76 mg, 0.19 mmol) in H₂O (0.63 mL), glacial AcOH (0.93 mL) and 1,4-dioxane (3.1 mL) was reacted as described in the procedure for **2.22a**.

The crude residue was purified by flash silica column chromatography twice (1st 99:1 DCM/MeOH, with 0.3% Et₃N to load silica, 2nd 10:1 EA/hexane with 0.5% of 25% aq. ammonia solution to load silica) to yield **2.22c** (138 mg, 0.28 mmol, 18%) as a brown solid (Rf 0.34, 4:1 EA/hexane).

**1H NMR (400 MHz, CDCl₃)** δ 8.22 – 8.15 (m, 2H, ArH indole & naphthalene), 8.00 – 7.89 (m, 2H, ArH naphthalene), 7.65 (dd, J = 7.0, 1.2 Hz, 1H, ArH naphthalene), 7.57 – 7.45 (m, 5H, ArH Bn & naphthalene), 7.45 – 7.37 (m, 3H, ArH Bn & indole), 7.37 – 7.27 (m, 2H, ArH Bn & indole), 7.08 (dd, J = 8.9, 2.5 Hz, 1H, ArH indole), 5.20 (s, 2H,
CH$_2$Bn), 4.29 – 3.97 (br m, 2H, N1-CH$_2$), 3.76 – 3.36 (br m, 4H, O-CH$_2$ morpholino), 2.85 – 2.58 (br m, 2H, N1-CH$_2$CH$_3$), 2.57 – 2.19 (br m, 4H, N-CH$_2$ morpholino).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 192.18, 156.04, 139.21, 138.98, 137.42, 133.83, 132.12, 130.87, 130.04, 128.66, 128.32, 128.00, 127.86, 127.76, 126.89, 126.43, 126.05, 125.80, 124.60, 117.54, 114.86, 110.71, 105.55, 70.72, 66.87, 57.57, 53.65, 44.40.

HRMS-ESI calculated for C$_{32}$H$_{31}$N$_2$O$_3$ [M+H]$^+$ 491.2329, found m/z 491.2303.

5-(Benzyloxy)-3-cyclohexanecarbonyl-1-[2-(morpholin-4-yl)ethyl]-1H-indol (2.22d)

A solution of 2.19a (100 mg, 0.3 mmol), cyclohexanecarbonitrile (106 µL, 0.89 mmol) and 2.21 (12 mg, 30.0 µmol) in H$_2$O (0.12 mL), glacial AcOH (0.18 mL) and 1,4-dioxane (0.6 mL) was reacted as described in the procedure for 2.22a. The crude residue was purified by flash silica column chromatography (99:1 DCM/MeOH, with 0.3% Et$_3$N to load silica) to yield a red oil as a 4:1 mixture of 2.22d and 2.19a (100 mg) (R$_f$ 0.17, 99:1 DCM/MeOH). This mixture could not be separated further and was therefore used without further purification in subsequent reactions.

HRMS-ESI calculated for 2.22d C$_{28}$H$_{35}$N$_2$O$_3$ [M+H]$^+$ 447.2642, found m/z 447.2605 (from HRMS-ESI of the 4:1 mixture).

5-(Benzyloxy)-3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indole (2.22e)

A solution of 2.19c (103 mg, 0.32 mmol), 4-methoxybenzonitrile (127 mg, 0.95 mmol) and 2.21 (14 mg, 32.0 µmol) in H$_2$O (0.12 mL), glacial AcOH (0.18 mL) and 1,4-dioxane (0.6 mL) was reacted as described in the procedure for 2.22a. The crude residue was purified by flash silica column chromatography (2:1 PE/EA) to yield 2.22e (118 mg, 0.26 mmol, 81%) as a brown oil (R$_f$ 0.32, 1:1 PE/EA).
$^1$H NMR (400 MHz, CDCl$_3$) δ 8.04 (d, $J = 2.5$ Hz, 1H, ArH indole), 7.87 – 7.80 (m, 2H, ArH MeOPh), 7.53 – 7.47 (m, 3H, ArH Bn & ArH indole), 7.43 – 7.36 (m, 2H, ArH Bn), 7.36 – 7.30 (m, 1H, ArH Bn), 7.28 (dd, $J = 8.9$, 0.5 Hz, 1H, ArH indole), 7.06 (dd, $J = 8.9$, 2.5 Hz, 1H, ArH indole), 7.03 – 6.97 (m, 2H, ArH MeOPh), 5.17 (s, 2H, CH$_2$ Bn), 4.01 (d, $J = 7.3$ Hz, 2H, N-CH$_2$), 3.99 – 3.93 (m, 2H, O-CH$_2$ THP), 3.90 (s, 3H, O-CH$_3$), 3.31 (td, $J = 11.8$, 2.2 Hz, 2H, O-CH$_2$ THP), 2.21 – 2.05 (m, 1H, CH THP), 1.56 – 1.28 (m, 4H, O-CH$_2$CH$_2$ THP).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 189.89, 162.34, 155.71, 137.47, 136.76, 133.54, 132.11, 130.96, 128.65, 128.39, 127.97, 127.82, 115.43, 114.88, 113.71, 110.83, 105.31, 70.69, 67.42, 55.58, 53.33, 36.06, 30.86.

HRMS-ESI calculated for C$_{29}$H$_{30}$NO$_4$ [M+H]$^+$ 456.2169, found m/z 456.2128.

5-(Benzyloxy)-3-(naphthalene-1-carbonyl)-1-[(oxan-4-yl)methyl]-1H-indole (2.22f)

A solution of 2.19c (103 mg, 0.32 mmol), 1-naphthonitrile (147 mg, 0.96 mmol) and 2.21 (14 mg, 32.0 μmol) in H$_2$O (0.12 mL), glacial AcOH (0.18 mL) and 1,4-dioxane (0.6 mL) was reacted as described in the procedure for 2.22a. The crude residue was purified by flash silica column chromatography (2:1 PE/EA) to yield 2.22f (87 mg, 0.18 mmol, 59%) as a brown oil (R$_f$ 0.5, 1:1 PE/EA).

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.19 (dd, $J = 8.5$, 1.2 Hz, 1H, ArH naphthalene), 8.13 (d, $J = 2.5$ Hz, 1H, ArH indole), 7.98 (dd, $J = 8.3$, 1.1 Hz, 1H, ArH naphthalene), 7.95 – 7.89 (m, 1H, ArH naphthalene), 7.66 (dd, $J = 7.0$, 1.3 Hz, 1H, ArH naphthalene), 7.60 – 7.44 (m, 5H, ArH Bn & naphthalene), 7.44 – 7.37 (m, 2H, ArH Bn), 7.37 – 7.31 (m, 1H, ArH indole), 7.28 (d, $J = 8.9$ Hz, 1H, ArH Bn), 7.27 (s, 1H, ArH indole), 7.08 (dd, $J = 8.9$, 2.5 Hz, 1H, ArH indole), 5.18 (s, 2H, CH$_2$ Bn), 4.00 – 3.85 (m, 4H, N-CH$_2$ & O-CH$_2$ THP), 3.28 (td, $J = 11.8$, 2.1 Hz, 2H, O-CH$_2$ THP), 2.13 – 1.96 (m, 1H, CH THP), 1.48 – 1.24 (m, 4H, O-CH$_2$CH$_2$ THP).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 192.14, 156.02, 139.12, 138.36, 137.40, 133.88, 132.31, 130.90, 130.16, 128.67, 128.33, 128.01, 127.88, 126.86, 126.45, 126.11, 125.97.
5-(Benzyloxy)-3-cyclohexanecarbonyl-1-[(oxan-4-yl)methyl]-1H-indole (2.22g)

A solution of 2.19c (100 mg, 0.31 mmol), cyclohexanecarbonitrile (0.11 mL, 0.93 mmol) and 2.21 (13 mg, 28.0 μmol) in H₂O (0.12 mL), glacial AcOH (0.18 mL) and 1,4-dioxane (0.6 mL) was reacted as described in the procedure for 2.22a. The crude residue was purified by flash silica column chromatography (2:1 PE/EA) to yield 2.22g (112 mg, 0.26 mmol, 83%) (Rf 0.36 2:1 PE/EA) as a red brown oil.

1H NMR (400 MHz, CDCl₃) δ 8.07 (d, J = 2.5 Hz, 1H, ArH indole), 7.68 (s, 1H, ArH indole), 7.49 (d, J = 7.2 Hz, 2H, ArH Bn), 7.43 – 7.36 (m, 2H, ArH Bn), 7.35 – 7.29 (m, 1H, ArH Bn), 7.24 (d, J = 8.9 Hz, 1H, ArH indole), 7.02 (dd, J = 8.9, 2.5 Hz, 1H, ArH indole), 5.15 (s, 2H, CH₂ Bn), 4.11 – 3.84 (m, 4H, N-CH₂ & O-CH₂ THP), 3.33 (td, J = 11.7, 2.3 Hz, 2H, O-CH₂ THP), 2.99 (tt, J = 11.8, 3.3 Hz, 1H, CH cyclohexane), 2.23 – 2.05 (m, 1H, CH THP), 1.96 – 1.82 (m, 4H, CH₂ cyclohexane), 1.79 – 1.33 (m, 10H, CH₂ cyclohexane & O-CH₂CH₂ THP).

13C NMR (101 MHz, CDCl₃) δ 199.77, 155.74, 137.49, 134.45, 132.22, 128.64, 127.96, 127.81, 127.72, 115.27, 114.70, 110.78, 105.33, 70.62, 67.45, 53.36, 47.89, 36.10, 30.89, 30.08, 26.21, 26.14.

HRMS-ESI calculated for C₂₈H₃₄NO₃ [M+H]⁺ 432.2533, found m/z 432.2506.

5-(Benzyloxy)-3-(4-methoxybenzoyl)-1-[(oxolan-3-yl)methyl]-1H-indole (2.22h)

A solution of 2.19d (87 mg, 0.28 mmol), 4-methoxybenzonitrile (130 mg, 0.98 mmol) and 2.21 (13 mg, 28.0 μmol) in H₂O (0.12 mL), glacial AcOH (0.18 mL) and 1,4-dioxane (0.6 mL) was reacted as described in the procedure for 2.22a. The crude residue was purified by flash silica
column chromatography (2:1 PE/EA) to yield 2.22h (84 mg, 0.19 mmol, 67%) as a light brown oil (Rf 0.12, 2:1 PE/EA).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.05 (d, $J = 2.5$ Hz, 1H, ArH indole), 7.83 (dt, $J = 8.7$, 2.8, 2.6 Hz, 2H, ArH MeOPh), 7.54 (s, 1H, ArH indole), 7.50 (d, $J = 6.9$ Hz, 2H, ArH Bn), 7.40 (t, $J = 7.2$ Hz, 2H, ArH Bn), 7.32 (dd, $J = 9.4$, 8.1 Hz, 2H, ArH Bn, ArH indole), 7.07 (dd, $J = 8.9$, 2.5 Hz, 1H, ArH indole), 7.00 (dt, $J = 8.7$, 2.8, 2.6 Hz, 2H, ArH MeOPh), 5.17 (s, 2H, CH$_2$Bn), 4.11 (d, $J = 7.8$ Hz, 2H, N-CH$_2$), 3.97 (t, $J = 8.3$, 5.4 Hz, 1H, O-CH$_2$ THF), 3.90 (s, 3H, O-CH$_3$), 3.77 (t, $J = 8.3$, 6.9 Hz, 1H, O-CH$_2$ THF), 3.71 (dd, $J = 9.1$, 6.4 Hz, 1H, O-CH$_2$ THF), 3.61 (dd, $J = 9.1$, 4.4 Hz, 1H, O-CH$_2$ THF), 2.92 – 2.79 (m, 1H, CH THF), 2.12 – 1.99 (m, 1H, CH$_2$Bn), 1.73 – 1.60 (m, 1H, CH$_2$ THF).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 189.91, 162.36, 155.77, 137.45, 136.23, 133.48, 132.00, 130.96, 128.65, 128.41, 127.97, 127.82, 115.71, 114.99, 113.73, 110.68, 105.34, 70.85, 70.69, 67.62, 55.58, 49.90, 39.74, 29.86.

HRMS-ESI calculated for C$_{28}$H$_{28}$NO$_4$ [M+H]$^+$ 442.2013, found m/z 442.1985.

5-(Benzyloxy)-3-(naphthalene-1-carbonyl)-1-[(oxolan-3-y1)methyl]-1H-indole (2.22i)

A solution of 2.19d (92 mg, 0.3 mmol), 1-naphthonitrile (159 mg, 1.0 mmol) and 2.21 (13 mg, 28.0 μmol) in H$_2$O (0.12 mL), glacial AcOH (0.18 mL) and 1,4-dioxane (0.6 mL) was reacted as described in the procedure for 2.22a. The crude residue was purified by flash silica column chromatography (2:1 PE/EA) to yield 2.22i (86 mg, 0.19 mmol, 62%) as a red-orange oil (Rf 0.2, 2:1 PE/EA).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.19 (ddt, $J = 8.2$, 1.6, 0.8 Hz, 1H, ArH naphthalene), 8.14 (d, $J = 2.5$ Hz, 1H, ArH indole), 7.98 (dt, $J = 8.3$, 1.1 Hz, 1H, ArH naphthalene), 7.92 (dt, $J = 8.1$, 0.9 Hz, 1H, ArH naphthalene), 7.65 (dd, $J = 7.0$, 1.3 Hz, 1H, ArH naphthalene), 7.56 – 7.45 (m, 5H, ArH Bn, ArH naphthalene), 7.41 (tt, $J = 6.6$, 0.9 Hz, 2H, ArH Bn), 7.37 – 7.28 (m, 3H, ArH Bn, ArH indole), 7.09 (dd, $J = 8.9$, 2.5 Hz, 1H,
ArH indole), 5.19 (s, 2H, CH₂ Bn), 4.01 (d, J = 7.8 Hz, 2H, N-CH₂), 3.90 (td, J = 8.3, 5.4 Hz, 1H, O-CH₂ THF), 3.75 – 3.62 (m, 2H, O-CH₂ THF), 3.52 (dd, J = 9.1, 4.4 Hz, 1H, O-CH₂ THF), 2.88 – 2.67 (m, 1H, CH THF), 2.08 – 1.92 (m, 1H, CH₂ THF), 1.64 – 1.54 (m, 1H, CH₂ THF).

¹³C NMR (101 MHz, CDCl₃) δ 192.16, 156.10, 139.07, 137.81, 137.40, 133.89, 132.22, 130.89, 130.19, 128.67, 128.34, 128.01, 127.92, 127.87, 126.90, 126.46, 126.06, 125.94, 124.70, 117.71, 115.12, 110.88, 105.53, 70.76, 70.72, 67.55, 49.89, 39.66, 29.81.

HRMS-ESI calculated for C₃₁H₂₈NO₃ [M+H]⁺ 462.2064, found m/z 462.2023.

3-(4-Methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-ol (2.23a)

A flask containing a stirred solution of 2.22a (497 mg, 1.1 mmol), EtOH (12 mL), MeOH (12 mL) and AcOH (1 mL) was evacuated and purged with N₂, followed by addition of Pd/C (10% by weight loading (dry basis), matrix carbon powder, wet support) (50 mg). The solution evacuated and then placed under an atmosphere of hydrogen (balloon) (evacuated and purged with H₂ three times) and stirred for 20 h at rt. The solution was then filtered through celite, the celite washed with 1:1 EtOH:MeOH and then MeOH, and the filtrate evaporated under reduced pressure. The crude residue was purified by precipitation in EA to yield the desired product 2.23a (54 mg, 0.14 mmol, 13%) as a pale fawn solid (Rᶠ 0.29, 95:5 DCM/MeOH).

¹H NMR (400 MHz, DMSO-d₆) δ 9.08 (s, 1H, OH), 7.93 (s, 1H, ArH indole), 7.77 (d, J = 8.3 Hz, 2H, ArH indole), 7.66 (s, 1H, ArH indole), 7.41 (d, J = 8.8 Hz, 1H, ArH indole), 7.06 (d, J = 8.3 Hz, 2H, ArH MeOPh), 6.76 (d, J = 8.7 Hz, 1H, ArH indole), 4.30 (t, J = 6.2 Hz, 2H, N1-CH₂), 3.85 (s, 3H, O-CH₃), 3.54 (t, J = 4.6 Hz, 4H, O-CH₂ morpholino), 2.67 (t, J = 6.3 Hz, 2H, N1-CH₂CH₂), 2.47 – 2.35 (br m, 4H, N-CH₂ morpholino).

¹³C NMR (101 MHz, DMSO-d₆) δ 188.12, 161.52, 153.35, 138.50, 133.22, 130.81, 130.44, 127.90, 113.54, 113.22, 112.71, 111.12, 106.06, 66.26, 57.30, 55.40, 53.17, 43.09.
HRMS-ESI calculated for C_{22}H_{25}N_{2}O_{4} [M+H]^+ 381.1809, found m/z 381.1779. Analytical RP-HPLC R_t = 12.89 min.

3-(4-Methoxybenzoyl)-1-[2-(mopholin-4-yl)ethyl]-1H-indol-6-ol (2.23b)

A mixture of 2.22b (75 mg, 0.16 mmol), EtOH (2.6 mL), EA (1.3 mL) and Pd/C (7.5 mg) was reacted as described in the procedure for 2.23a. The reaction mixture was filtered through celite, the celite washed with 2:1 EtOH:EA and then MeOH, and the filtrate evaporated under reduced pressure. The crude residue was purified by precipitation in EA to yield the desired product 2.23b (9.4 mg, 24.7 µmol, 16%), as a pale brown solid (R_f 0.21, 95:5 DCM/MeOH). Less than 95% purity necessitated semi-preparative RP-HPLC on a small amount of this material (6 mg) to yield a sample of 2.23b (4.8 mg) as a pale brown solid.

^1H NMR (400 MHz, DMSO-d_6) δ 9.38 (s, 1H, ArOH), 8.00 (d, J = 8.6 Hz, 1H, ArH indole), 7.84 (s, 1H, ArH indole), 7.81 – 7.75 (m, 2H, ArH MeOPh), 7.09 – 7.03 (m, 2H, ArH MeOPh), 6.88 (d, J = 2.1 Hz, 1H, ArH indole), 6.76 (dd, J = 8.5, 2.1 Hz, 1H, ArH indole), 4.25 (t, J = 6.3 Hz, 2H, N1-CH_2), 3.85 (s, 3H, O-CH_3), 3.55 (t, J = 4.6 Hz, 4H, O-CH_2 morpholino), 2.66 (t, J = 6.3 Hz, 2H, N1-CH_2CH_2), 2.44 (t, J = 4.6 Hz, 4H, N-CH_2 morpholino).

^13C NMR (126 MHz, DMSO-d_6) δ 188.10, 161.59, 154.39, 137.76, 137.37, 133.07, 130.45, 122.27, 119.71, 114.01, 113.54, 112.04, 95.81, 66.25, 57.07, 55.41, 53.18, 42.91.

HRMS-ESI calculated for C_{22}H_{25}N_{2}O_{4} [M+H]^+ 381.1809, found m/z 381.1804. Analytical RP-HPLC R_t = 13.78 min.
1-[2-(Morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indol-5-ol (2.23c)

A mixture of 2.22c (299 mg, 0.61 mmol), EtOH (11 mL), EA (5.5 mL) and Pd/C (30 mg) was reacted as described in the procedure for 2.23a. The reaction mixture was filtered through celite, the celite washed with 2:1 EtOH:EA and then MeOH, and the filtrate evaporated under reduced pressure. The crude residue was purified by precipitation in EA to yield the desired product 2.23c (95 mg, 0.24 mmol, 39%) as a white solid (Rf 0.35, 95:5 DCM/MeOH).

\(^{1}\)H NMR (400 MHz, MeOD-\(d_4\)) \(\delta\) 8.03 (dd, \(J = 8.2, 4.6\) Hz, 2H, ArH naphthalene), 7.99 – 7.94 (m, 1H, ArH naphthalene), 7.82 (d, \(J = 2.4\) Hz, 1H, ArH indole), 7.65 (dd, \(J = 7.0, 1.4\) Hz, 1H, ArH naphthalene), 7.62 – 7.57 (m, 1H, ArH naphthalene), 7.55 (s, 1H, ArH indole), 7.55 – 7.46 (m, 2H, ArH naphthalene), 7.39 (d, \(J = 8.8\) Hz, 1H, ArH indole), 6.88 (dd, \(J = 8.8, 2.5\) Hz, 1H, ArH indole), 4.21 (t, \(J = 6.1\) Hz, 2H, N1-CH\(_2\)), 3.53 – 3.40 (m, 4H, O-CH\(_2\) morpholino), 2.67 (t, \(J = 6.1\) Hz, 2H, N1-CH\(_2\)CH\(_2\)), 2.37 (t, \(J = 4.6\) Hz, 4H, N-CH\(_2\) morpholino).

\(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 194.66, 155.60, 141.78, 141.08, 135.85, 134.00, 132.88, 132.13, 130.36, 129.98, 128.93, 128.49, 128.07, 127.79, 126.68, 119.17, 115.54, 112.71, 109.78, 68.95, 59.58, 55.73, 46.54.

HRMS-ESI calculated for C\(_{25}\)H\(_{25}\)N\(_2\)O\(_3\) [M+H]\(^+\) 401.1860, found m/z 401.1850.

Analytical RP-HPLC \(R_t = 14.70\) min.

3-Cyclohexanecarbonyl-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-ol (2.23d)

A mixture of 2.22d (85 mg, 0.19 mmol), EtOH (3 mL), EA (1.5 mL) and Pd/C (8.5 mg) was reacted as described in the procedure for 2.23a. The reaction mixture was filtered through celite, the celite washed with 2:1 EtOH:EA and then MeOH, and the filtrate evaporated under reduced pressure. The crude residue was purified by flash silica gel column chromatography (1:1 PE/EA)
and precipitation in EA to yield the desired product 2.23d (11 mg, 30.9 µmol, 16%) as a pale pink solid (Rf 0.54, 95:5 DCM/MeOH).

\[ ^1\text{H NMR (400 MHz, DMSO-}d_6\text{) }\delta 9.01 (s, 1H, ArOH), 8.26 (s, 1H, ArH indole), 7.60 (d, J = 2.4 Hz, 1H, ArH indole), 7.35 (d, J = 8.8 Hz, 1H, ArH indole), 6.71 (dd, J = 8.8, 2.4 Hz, 1H, ArH indole), 4.27 (t, J = 6.4 Hz, 2H, N1-CH₂), 3.53 (t, J = 4.6 Hz, 4H, O-CH₂ morpholino), 3.12 – 2.99 (m, 1H, CH cyclohexane), 2.66 (t, J = 6.4 Hz, 2H, N1-CH₂CH₂), 2.43 (t, J = 4.7 Hz, 4H, N-CH₂ morpholino), 1.84 – 1.65 (m, 5H, CH₂ cyclohexane), 1.49 – 1.31 (m, 4H, CH₂ cyclohexane), 1.27 – 1.13 (m, 1H, CH₂ cyclohexane).\]

\[ ^13\text{C NMR (126 MHz, DMSO-}d_6\text{) }\delta 197.90, 153.24, 136.82, 130.92, 127.23, 113.26, 112.37, 110.97, 106.11, 66.23, 57.20, 53.14, 46.27, 43.26, 29.73, 25.67, 25.49.\]

HRMS-ESI calculated for C$_{21}$H$_{29}$N$_2$O$_3$ [M+H]$^+$ 357.2173, found m/z 357.2154.

Analytical RP-HPLC R$_t$ = 13.77 min.

3-(4-Methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-5-ol (2.23e)

A mixture of 2.22e (1.10 g, 2.4 mmol), EtOH (15 mL), CHCl$_3$ (40 mL), AcOH (1 mL) and Pd/C (110 mg) was reacted as described in the procedure for 2.23a. The reaction mixture was filtered through celite, the celite washed with 3:1 CHCl$_3$:EtOH and then MeOH, and the filtrate evaporated under reduced pressure. The crude residue was purified by precipitation in 1:1 EtOH:MeOH to yield the desired product 2.23e (588 mg, 1.6 mmol, 67%) as a creamy pearl solid (Rf 0.37, 95:5 DCM/MeOH).

\[ ^1\text{H NMR (400 MHz, DMSO-}d_6\text{) }\delta 9.11 (s, 1H, OH), 7.89 (s, 1H, ArH indole), 7.82 – 7.73 (m, 2H, ArH MeOPh), 7.68 (d, J = 2.4 Hz, 1H, ArH indole), 7.45 (d, J = 8.8 Hz, 1H, ArH indole), 7.12 – 7.03 (m, 2H, ArH MeOPh), 6.78 (dd, J = 8.8, 2.4 Hz, 1H, ArH indole), 4.11 (d, J = 7.2 Hz, 2H, N-CH₂), 3.85 (s, 3H, O-CH₃), 3.84 – 3.76 (m, 2H, O-CH₂ THP), 3.19 (td, J = 11.5, 2.3 Hz, 2H, O-CH₂ THP), 2.15 – 1.98 (m, 1H, CH THP), 1.41 – 1.19 (m, 4H O-CH₂CH₂ THP).\]

\[ ^13\text{C NMR (101 MHz, DMSO-}d_6\text{) }\delta 188.18, 161.54, 153.40, 138.08, 133.15, 131.11, \]
3-(Naphthalene-1-carbonyl)-1-[(oxan-4-yl)methyl]-1H-indol-5-ol (2.23f)

A mixture of 2.22f (54 mg, 0.11 mmol), EtOH (2.5 mL), EA (1.25 mL) and Pd/C (6 mg) was reacted as described in the procedure for 2.23a. The reaction mixture was filtered through celite, the celite washed with 2:1 EtO<sub>H</sub>:EA and then MeOH, and the filtrate evaporated under reduced pressure. The crude residue was purified by flash silica gel column chromatography (10:1 EA/hexane), recrystallisation in MeOH and semi-preparative RP-HPLC to yield the desired product 2.23f (14.7 mg, 38.1 µmol, 33%) as a brown solid (R<sub>f</sub> 0.65, 95:5 DCM/MeOH).

<sup>1</sup>H NMR (400 MHz, MeOD-<i>d</i><sub>4</sub>) δ 8.06 – 7.99 (m, 2H, ArH naphthalene), 7.98 – 7.92 (m, 1H, ArH naphthalene), 7.82 (d, <i>J</i> = 2.7 Hz, 1H, ArH indole), 7.65 – 7.60 (m, 1H, ArH naphthalene), 7.60 – 7.49 (m, 2H, ArH naphthalene), 7.48 – 7.42 (m, 2H, ArH indole & naphthalene), 7.40 – 7.36 (m, 1H, ArH indole), 6.88 (dd, <i>J</i> = 8.8, 2.5 Hz, 1H, ArH indole), 4.06 – 3.94 (m, 2H, N-CH<sub>2</sub>), 3.92 – 3.80 (m, 2H, O-CH<sub>2</sub> THP), 3.26 (dd, <i>J</i> = 11.7, 2.1 Hz, 2H, O-CH<sub>2</sub> THP), 2.12 – 1.98 (m, 1H, CH THP), 1.41 – 1.21 (m, 4H, O-CH<sub>2</sub>CH<sub>2</sub> THP).

HRMS-ESI calculated for C<sub>25</sub>H<sub>23</sub>NNaO<sub>3</sub> [M+Na]<sup>+</sup> 408.1570, found m/z 408.1535. Analytical RP-HPLC R<sub>t</sub> = 19.69 min.

3-(4-Methoxybenzoyl)-1-[(oxolan-3-yl)methyl]-1H-indol-5-ol (2.23g)

A mixture of 2.22h (72 mg, 0.16 mmol), EtOH (2.5 mL), EA (1.4 mL) and Pd/C (7 mg) was reacted as described in the procedure for 2.23a. The reaction mixture was filtered through celite, the celite washed with 2:1 EtOH:EA and then MeOH, and the filtrate evaporated under reduced pressure. The crude residue was
purified by flash silica gel column chromatography (1:1 hexane/EA) to give 2.23g (6.8 mg, 19.4 μmol, 12%) as a brown oil (Rf 0.27, 1:1 hexane/EA; shown to be pure by NMR spectra). Significant degradation upon long term storage (56% purity according to analytical RP-HPLC) necessitated further purification immediately prior to biological testing, therefore a small amount of this material (4 mg) was purified using semi-preparative RP-HPLC to yield a sample of 2.23g (1.58 mg) as a pale fawn solid.

^1^H NMR (400 MHz, CDCl\textsubscript{3}) δ 8.41 (d, J = 2.4 Hz, 1H, ArH indole), 7.83 (d, J = 8.5 Hz, 2H, ArH MeOPh), 7.52 (s, 1H, ArH indole), 7.28 (d, J = 6.1 Hz, 1H, ArH indole), 7.02 (d, J = 8.6 Hz, 2H, ArH MeOPh), 6.98 (dd, J = 8.8, 2.4 Hz, 1H, ArH indole), 4.10 (d, J = 7.8 Hz, 2H, N-CH\textsubscript{2}), 3.96 (td, J = 8.3, 5.4 Hz, 1H, O-CH\textsubscript{2}THF), 3.90 (s, 3H, O-CH\textsubscript{3}), 2.86 (td, J = 12.8, 6.2 Hz, 1H, CH THF), 2.05 (m, 1H, CH\textsubscript{2} THF), 1.66 (m, 1H, CH\textsubscript{2} THF).

^13^C NMR (101 MHz, CDCl\textsubscript{3}) δ 191.02, 162.49, 154.10, 137.38, 133.26, 131.70, 131.01, 128.69, 115.23, 113.97, 113.95, 110.69, 107.61, 70.89, 67.65, 55.62, 50.05, 39.66, 29.90.

HRMS-ESI calculated for C\textsubscript{21}H\textsubscript{21}NNaO\textsubscript{4} \[\text{M+Na}\]^+ 374.1363, found m/z 374.1345.

Analytical RP-HPLC R\textsubscript{t} = 17.16 min.

tert-Butyl 2-[[3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-yl]oxy]acetate (2.24a)

A stirred solution of 2.23a (87 mg, 0.23 mmol) in anhydrous DMF (2.5 mL) was added dropwise to a mixture of NaH (60% by mass dispersion in mineral oil) (13 mg, 0.54 mmol) in anhydrous DMF (1 mL). The mixture was heated to 60°C for 1 h, then cooled to rt and a solution of tert-butylbromacetate (44 µL, 0.30 mmol) in anhydrous DMF (1 mL) was added. The reaction was stirred at 60°C for 3 h, then quenched with sat. aq. NH\textsubscript{4}CL (4 mL) and H\textsubscript{2}O (4 mL) and extracted with EA (4 × 4 mL). The combined organics were washed with H\textsubscript{2}O (3 × 7 mL), dried over MgSO\textsubscript{4} and evaporated under reduced pressure. The
crude product was purified by flash silica gel column chromatography (99:1 DCM/MeOH with 0.3% of 25% aq. ammonia solution to load silica) to yield 2.24a (51 mg, 0.10 mmol, 45%) as a pale yellow oil, (Rf 0.39, 95:5 DCM/MeOH).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.89 (d, $J = 2.6$ Hz, 1H, ArH indole), 7.87 – 7.80 (m, 2H, ArH MeOPh), 7.65 (s, 1H, ArH indole), 7.29 (d, $J = 8.9$ Hz, 1H, ArH indole), 7.07 (dd, $J = 8.9$, 2.6 Hz, 1H, ArH indole), 7.02 – 6.95 (m, 2H, ArH MeOPh), 4.62 (s, 2H, O-CH$_2$), 4.36 – 4.08 (br m, 2H, N1-CH$_2$), 3.89 (s, 3H, O-CH$_3$), 3.78 – 3.62 (br m, 4H, O-CH$_2$ morpholino), 2.91 – 2.65 (br m, 2H, N1-CH$_2$CH$_2$), 2.63 – 2.36 (br m, 4H, N-CH$_2$ morpholino), 1.51 (s, 9H, CH$_3$ t Bu).

$^{13}$C NMR (126 MHz, CDCl$_3$) δ 189.67, 168.39, 162.25, 154.71, 137.26, 133.53, 132.16, 130.89, 128.10, 115.42, 114.60, 113.60, 110.59, 105.12, 82.27, 66.91, 66.33, 57.73, 55.54, 53.73, 44.28, 28.19.

HRMS-ESI calculated for C$_{28}$H$_{35}$N$_2$O$_6$ [M+H]$^+$ 495.2490, found m/z 495.2457.

tert-Butyl 2-[(1-[2-(morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indol-5-yl]oxy)acetate (2.24b)

A solution of 2.23c (71 mg, 0.18 mmol), NaH (14 mg, 0.36 mmol) and tert-butylobromoacetate (34 µL, 0.23 mmol) in DMF (3 mL) was reacted as described in the procedure for 2.24a. The crude product was purified by flash silica gel column chromatography (99:1 DCM/MeOH with 0.3% of 25% aq. ammonia solution to load silica) to yield 2.24b (56 mg, 0.11 mmol, 61%) as a brown oil (Rf 0.6, 95:5 DCM/MeOH).

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.14 (d, $J = 8.3$ Hz, 1H, ArH naphthalene), 8.02 (d, $J = 2.5$ Hz, 1H, ArH indole), 7.96 (d, $J = 8.2$ Hz, 1H, ArH naphthalene), 7.90 (d, $J = 8.0$ Hz, 1H, ArH naphthalene), 7.63 (d, $J = 6.9$ Hz, 1H, ArH naphthalene), 7.56 – 7.42 (m, 3H, ArH naphthalene), 7.38 (s, 1H, ArH indole), 7.29 (d, $J = 8.9$ Hz, 1H, ArH indole), 7.10 (dd, $J = 9.0$, 2.6 Hz, 1H, ArH indole), 4.67 (s, 2H, O-CH$_2$), 4.25 – 4.00 (br m, 2H, N1-CH$_2$), 3.70 – 3.43 (br m, 4H, O-CH$_2$ morpholino), 2.80 – 2.57 (br m, 2H, N1-
CH₃CH₂), 2.54 – 2.24 (br m, 4H, N-CH₂ morpholino), 1.54 (s, 9H, CH₃ t Bu).

¹³C NMR (101 MHz, CDCl₃) δ 192.09, 168.44, 155.09, 139.13, 139.05, 133.85, 132.38, 130.85, 130.04, 128.33, 127.60, 126.88, 126.43, 125.99, 125.72, 124.61, 117.58, 114.92, 110.86, 105.20, 82.45, 66.79, 66.32, 57.49, 53.59, 44.28, 28.25.

HRMS-ESI calculated for C₃₁H₅₃N₂O₅ [M+H]+' 515.2540, found m/z 515.2497.

tert-Butyl 2-[[3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-5-yl]oxy]acetate (2.24c)

A solution of 2.23e (50 mg, 0.14 mmol), NaH (11 mg, 0.27 mmol) and tert-butylbromoacetate (26 µL, 0.18 mmol) in DMF (3 mL) was reacted as described in the procedure for 2.24a. The crude product was purified by flash silica gel column chromatography (1:1 hexane/EA) to yield 2.24c (50 mg, 0.10 mmol, 76%) as a colourless oil (Rf 0.41, 1:1 hexane/EA).

¹H NMR (400 MHz, CDCl₃) δ 7.87 (d, J = 2.5 Hz, 1H, ArH indole), 7.83 – 7.78 (m, 2H, ArH MeOPh), 7.48 (s, 1H, ArH indole), 7.27 (d, J = 7.8 Hz, 1H, ArH indole), 7.07 (dd, J = 8.9, 2.6 Hz, 1H, ArH indole), 7.02 – 6.95 (m, 2H, ArH MeOPh), 4.62 (s, 2H, O-CH₂), 4.03 – 3.91 (m, 4H, N-CH₂, O-CH₂ THP), 3.88 (s, 3H, O-CH₃), 3.30 (td, J = 11.8, 2.1 Hz, 2H, O-CH₂ THP), 2.16 – 2.04 (m, 1H, CH THP), 1.53 – 1.31 (m, 13H, O-CH₂CH₂ THP & CH₃ t-Bu).

¹³C NMR (101 MHz, CDCl₃) δ 189.71, 168.38, 162.28, 154.73, 136.82, 133.53, 132.39, 130.87, 128.20, 115.41, 114.82, 113.69, 110.93, 105.05, 82.30, 67.39, 66.32, 55.55, 53.31, 36.00, 30.83, 28.21.

HRMS-ESI calculated for C₂₈H₃₃NNaO₆ [M+Na]+' 502.2200, found m/z 502.2164.
2-[[3-(4-Methoxybenzoyl)-1-[(2-(morpholin-4-y1)ethyl]-1H-indol-5-yl]oxy]acetic acid (2.25a)

To a stirred solution of 2.24a (25 mg, 51.5 µmol) in anhydrous DCM (1.8 mL) at 0°C, was added TFA (0.9 mL). The reaction mixture was stirred at rt for 3 h, then evaporated under N₂ stream, followed by reduced pressure. The TFA salt of the crude product 2.25a (34 mg), was used in the next reaction without further purification. Some crude TFA salt 2.25a (approx. 3 mg) was purified for biological testing by semi-preparative RP-HPLC, yielding 2.25a (1.38 mg) as a white solid.


2-[(1-[2-(Morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indol-5-yl]oxy)acetic acid (2.25b)

A solution of 2.24b (18 mg, 35.9 µmol) and TFA (0.75 mL) in anhydrous DCM (1.5 mL) was reacted as described in the procedure for 2.25a. The TFA salt of the crude product 2.25b (24 mg) was used in the next reaction without further purification. Some crude TFA salt 2.25b (approx. 3 mg) was purified for biological testing by semi-preparative RP-HPLC, yielding 2.25b (1.1 mg) as a white solid.

2-{[3-(4-Methoxybenzoyl)-1-{(oxan-4-yl)methyl-1H-indol-5-yl]oxy}acetic acid (2.25c)

A solution of 2.24c (17 mg, 0.04 mmol) and TFA (0.75 mL) in anhydrous DCM (1.5 mL) was reacted as described in the procedure for 2.25a. The TFA salt of the crude product 2.25c (21 mg) was used in the next reaction without further purification. Some crude TFA salt 2.25c (approx. 3 mg) was purified for biological testing by semi-preparative RP-HPLC, yielding 2.25c (1.0 mg) as a white solid.

HRMS-ESI calculated for C_{24}H_{25}N_{2}NaO_{6} [M+Na]^+ 446.1574, found m/z 446.1561.

Analytical RP-HPLC R_t = 18.10.

Fmoc-Ala-Ala-1,2-diaminoethane trityl resin (2.26)

1,2-Diaminoethane trityl resin (300 mg, 1.8 mmol/g, 200-400 mesh) was swelled overnight in DMF. Fmoc-Ala-OH (504 mg, 1.6 mmol), HBTU (614 mg, 1.6 mmol) and DIPEA (0.56 mL, 3.2 mmol) were dissolved in DMF (3.2 mL), swirled for 1 min, then added to the drained, swelled resin in a SPPS vessel. This mixture was swirled and left standing for 1 h, after which the resin was drained and washed with DMF. The Fmoc-Ala-OH coupling procedure was repeated to double-couple, then the resin washed with DMF. The resin was capped by double treatment with acetic anhydride (500 µL) and DIPEA (500 µL) in DMF (1 mL) for 20 min, after which the resin was drained and washed with DMF and DCM and then dried under vacuum for 2 h. An Fmoc loading test was performed on the resin (6.9 mg), by adding a solution of piperidine in DMF (1 mL, 20% v/v). After 60 mins, 100 µL of this solution was diluted to 10 mL with DMF. The absorbance was measured at 301 nm and the substitution was calculated to be 0.68 mmol/g, using the formula:
Substitution (mmol/g) = \frac{100 \times \text{Absorbance}}{7.8 \times \text{resin mass (mg)}}

The resin was swelled in DMF for 1-2 h and the Fmoc was cleaved by treatment with 20% \text{v/v} piperidine/DMF (3 mL) for 20 min with occasional stirring, then the resin drained and the piperidine treatment repeated for another 20 min, then the resin was drained and washed thoroughly with DMF. A solution of Fmoc-Ala-OH (224 mg, 0.72 mmol), HBTU (273 mg, 0.72 mmol) and DIPEA (0.25 mL, 1.4 mmol) was dissolved in DMF (1.4 mL), swirled for 1 min, then added to the resin, swirled to mix and then left for 1 h. The resin was drained, washed with DMF, then DCM and left under vacuum for 2 h to dry to yield 2.26 (440 mg). Another Fmoc loading test was performed on the resin and the substitution was calculated to be 0.72 mmol/g, using the aforementioned formula.

tert-Butyl N-(8-aminooctyl)carbamate (2.28a)

To a vigorously stirred solution of 1,8-diamino-octane 2.27a (2 g, 13.9 mmol) in dioxane (60 mL) at 0°C was added dropwise over 2 h a solution of Boc\textsubscript{2}O (0.64 mL, 2.8 mmol) in dioxane (60 mL). The mixture was then stirred for 16 h, filtered, washed with dioxane and evaporated under reduced pressure. The residue was dissolved in DCM (90 mL), washed with warm H\textsubscript{2}O (15 x 90 mL) and sat. aq. NaCl (1 x 90 mL), dried over MgSO\textsubscript{4}, filtered and evaporated under reduced pressure, to yield the desired product 2.28a (470 mg, 1.9 mmol, 69%) as a colourless oil.

\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \delta 4.56 (br s, 1H, NH), 3.07 (q, \textit{J} = 6.8 Hz, 2H, NH-CH\textsubscript{2}), 2.65 (t, \textit{J} = 7.0 Hz, 2H, NH\textsubscript{2}-CH\textsubscript{2}), 1.66 – 1.52 (m, 2H, NH\textsubscript{2}), 1.47 – 1.37 (m, 13H, CH\textsubscript{2} & CH\textsubscript{3} t Bu), 1.34 – 1.23 (m, 8H, CH\textsubscript{2}). This matched literature data.

HRMS-ESI calculated for C\textsubscript{13}H\textsubscript{29}N\textsubscript{2}O\textsubscript{2} [M+H]\textsuperscript{+} 245.2224, found \textit{m/z} 245.2224.
**tert-Butyl N-[2-[2-(aminoethoxy)ethoxy]ethyl]carbamate (2.28b)**

To a vigorously stirred solution of 2-2’-(ethylenedioxy)bis(ethylamine) **2.27b** (2.0 mL, 13.5 mmol) in anhydrous DCM (30 mL) at 0°C was added dropwise over 2 h a solution of Boc₂O (0.43 mL, 1.89 mmol) in anhydrous DCM (60 mL). The mixture was stirred for 3 h, then washed with NaHCO₃ (20 mL), H₂O (2 x 20 mL) and sat. aq. NaCl (20 mL), dried over MgSO₄, filtered and evaporated under reduced pressure, to yield the desired product **2.28b** (322 mg, 1.3 mmol, 69%) as a colourless oil.

¹H NMR (400 MHz, CDCl₃) δ 5.18 (br s, 1H, NH), 3.68-3.56 (m, 4H, O-CH₂CH₂-O), 3.51 (dt, J = 9.0, 5.2 Hz, 4H, O-CH₂), 3.28 (q, J = 5.3 Hz, 2H, NH-CH₂), 2.95-2.77 (br m, 2H, NH₂-CH₂), 1.82 (br s, 2H, NH₂), 1.41 (s, 9H, CH₃ t Bu).
³C NMR (101 MHz, CDCl₃) δ 156.11, 79.25, 73.31, 70.30, 70.27, 41.73, 40.43, 28.51.
¹H and ³C NMR matched literature data.²²¹ HRMS-ESI calculated for C₁₁H₂₅N₂O₄ [M+H]⁺ 249.1809, found m/z 249.1812.

**tert-Butyl N-[8-[(3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-yl)oxy]acetamido]octyl]carbamate (2.29a)**

To a solution of the TFA salt of **2.25a** (27 mg, 41.2 μmol) in anhydrous DMF (2 mL) were added DIPEA (21.5 µL, 0.12 mmol) and HATU (16 mg, 41.2 μmol). After stirring for 5 min, a solution of **2.28a** (33 mg, 0.14 mmol) and DIPEA (21.5 µL, 0.12 mmol) in DMF (1.5 mL) was added. The reaction mixture was stirred for 14 h and then the solvent evaporated under reduced pressure. The crude product was purified by flash silica gel column chromatography (99:1 DCM/MeOH with 0.3% of 25% aq. ammonia solution to load silica) to yield **2.29a** (19 mg, 28.6 mmol, 69%) as a brown oil (Rₜ 0.28, 9:1 DCM/MeOH).
\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.98 (d, \(J = 2.5\) Hz, 1H, ArH indole), 7.88 – 7.81 (m, 2H, ArH MeOPh), 7.70 (br s, 1H, ArH indole), 7.33 (br s, 1H, ArH indole), 7.02 – 6.97 (m, 2H, ArH MeOPh), 6.70 (t, \(J = 5.8\) Hz, 1H, ArH indole), 4.56 (s, 2H, O-CH\(_2\)), 4.52 (br s, 1H, NH), 4.31 - 4.15 (br m, 2H, N1-CH\(_2\)), 3.90 (s, 3H, O-CH\(_3\)), 3.79 – 3.56 (br m, 4H, O-CH\(_2\) morpholino), 3.41 – 3.31 (m, 2H, CH\(_2\) octyl), 3.14 – 3.01 (m, 2H, CH\(_2\) octyl), 2.86 – 2.69 (br m, 2H, N1-CH\(_2\)CH\(_2\)), 2.63 – 2.31 (br m, 4H, N-CH\(_2\) morpholino), 1.60 – 1.51 (m, 2H, CH\(_2\) octyl), 1.50 – 1.39 (m, 11H, CH\(_2\) octyl & CH\(_3\) t-Bu), 1.37 – 1.23 (m, 8H, CH\(_2\) octyl).

HRMS-ESI calculated for C\(_{37}\)H\(_{52}\)N\(_4\)NaO\(_7\) [M+Na]\(^+\) 687.3728, found m/z 687.3717.

tert-Butyl N-(2-(2-([3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-yl]oxy)acetamido)ethoxy)ethoxy)ethyl carbamate (2.29b)

The TFA salt of 2.25a (23 mg, 34.4 \(\mu\)mol), 2.28b (26 mg, 0.11 mmol) DIPEA (36 \(\mu\)L, 0.21 mmol) and HATU (13 mg, 34.4 \(\mu\)mol) in DMF (3 mL) were reacted as described in the procedure for 2.29a. The crude product was purified by flash silica gel column chromatography (99:1 DCM/MeOH with 0.3% of 25% aq. ammonia solution to load silica) to yield 2.29b (22 mg, 32.9 \(\mu\)mol, 94%) as a brown oil (R\(_f\) 0.38, 9:1 DCM/MeOH).

\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.97 (d, \(J = 2.5\) Hz, 1H, ArH indole), 7.88 – 7.82 (m, 2H, ArH MeOPh), 7.69 (br s, 1H, ArH indole), 7.33 (br s, 1H, ArH indole), 7.18 – 7.10 (br m, 1H, ArH indole), 7.06 – 6.97 (m, 3H, ArH MeOPh & NH), 5.08 (br s, 1H, NH), 4.59 (s, 2H, indole-O-CH\(_2\)), 4.31 – 4.15 (br m, 2H, N1-CH\(_2\)), 3.90 (s, 3H, O-CH\(_3\)), 3.75 – 3.51 (m, 14H, ([CH\(_2\)]\(_2\)O\(_2\))(CH\(_2\))\(_2\) & O-CH\(_2\) morpholino), 3.32 – 3.25 (br m, 2H, ([CH\(_2\)]\(_2\)O\(_2\))(CH\(_2\))\(_2\)), 2.82 – 2.71 (br m, 2H, N1-CH\(_2\)CH\(_2\)), 2.61 – 2.38 (br m, 4H, N-CH\(_2\) morpholino), 1.42 (s, 9H, CH\(_3\) t-Bu).

\(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 162.37, 156.57, 137.17, 136.76, 133.55, 131.98, 131.04, 131.00, 128.45, 114.67, 114.38, 113.80, 113.73, 110.81, 105.54, 103.99, 74.21, 67.44,
tert-Butyl \(N\)-\(8\)-(1-\(2\)-(morpholin-4-yl)ethyl)-3-(napthalene-1-carbonyl)-1\(H\)-indol-5-yl)oxy)acetamido]octyl carbamate (2.29c)

The TFA salt of 2.25b (12 mg, 18.1 \(\mu\)mol), 2.28a (16 mg, 67.1 \(\mu\)mol), DIPEA (19 \(\mu\)L, 0.11 mmol) and HATU (6.8 mg, 18.1 \(\mu\)mol) in DMF (1.8 mL) were reacted as described in the procedure for 2.29a. The crude product was purified by flash silica gel column chromatography (97:3 DCM/MeOH with 0.3% of 25% aq. ammonia solution to load silica) to yield 2.29c (8.3 mg, 12.1 \(\mu\)mol, 67%) as a brown oil (\(R_f\) 0.48, 9:1 DCM/MeOH).

\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.14 (d, \(J = 8.4\) Hz, 1H, ArH naphthalene), 8.08 (br s, 1H, ArH indole), 7.97 (dt, \(J = 8.3, 1.0\) Hz, 1H, ArH naphthalene), 7.94 – 7.87 (m, 1H, ArH naphthalene), 7.63 (dd, \(J = 7.0, 1.2\) Hz, 1H, ArH naphthalene), 7.57 – 7.50 (m, 2H, ArH naphthalene), 7.47 (ddd, \(J = 8.3, 6.8, 1.5\) Hz, 1H, ArH naphthalene), 7.44 (s, 1H, ArH indole), 7.32 (d, \(J = 13.2\) Hz, 1H, ArH indole), 7.03 (d, \(J = 8.8\) Hz, 1H, ArH indole), 6.79 – 6.67 (m, 1H, NH), 4.58 (s, 2H, indole-O-CH\(_2\)), 4.51 (s, 1H, NH), 4.21-4.05 (br m, 2H, N1-CH\(_2\)), 3.71 – 3.42 (br m, 4H, O-CH\(_2\) morpholino), 3.41 – 3.32 (m, 2H, CH\(_2\) octyl), 3.08 (q, \(J = 6.8\) Hz, 2H, CH\(_2\) octyl), 2.78 – 2.60 (br m, 2H, N1-CH\(_2\)CH\(_2\)), 2.56 – 2.22 (br m, 4H, N-CH\(_2\) morpholino), 1.78 – 1.52 (m, 4H, CH\(_2\) octyl), 1.43 (s, 9H, CH\(_3\) t Bu), 1.38 – 1.16 (m, 8H, CH\(_2\) octyl).

\(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 192.05, 168.33, 156.12, 154.48, 139.48, 133.89, 130.80, 130.29, 128.43, 127.95, 127.01, 126.53, 125.89, 124.63, 113.09, 110.90, 107.55, 79.12, 68.48, 66.92, 57.63, 53.66, 44.53, 40.74, 39.19, 30.17, 29.71, 29.32, 29.30, 28.58, 26.96, 26.86 (four quaternary carbons were not observed).

HRMS-ESI calculated for \(C_{40}H_{53}N_{6}O_{6}\) [M+H]\(^+\) 685.3960, found \(m/z\) 685.3955.
tert-Butyl N-[2-{(2-{2-((1-[(1-(morpholin-4-yl)ethyl)-3-(naphthalene-1-carbonyl)-1H-indol-5-yl]oxy)acetamido)ethoxy)ethoxy)ethyl]carbamate (2.29d)

The TFA salt of 2.25b (27 mg, 39.1 μmol), 2.28b (33 mg, 0.13 mmol), DIPEA (41 μL, 0.23 mmol) and HATU (15 mg, 39.1 μmol) in DMF (3.5 mL) were reacted as described in the procedure for 2.29a. The crude product was purified by flash silica gel column chromatography (98:2 DCM/MeOH with 0.3% of 25% aq. ammonia solution to load silica) to yield 2.29d (13 mg, 18.9 μmol, 50%) as a brown oil (Rf 0.36, 9:1 DCM/MeOH).

1H NMR (400 MHz, CDCl3) δ 8.19 – 8.12 (m, 1H, ArH naphthalene), 8.09 (d, J = 2.6 Hz, 1H, ArH indole), 7.96 (d, J = 8.0 Hz, 1H, ArH naphthalene), 7.91 (dd, J = 7.8, 1.5 Hz, 1H, ArH naphthalene), 7.64 (dd, J = 7.0, 1.3 Hz, 1H, ArH naphthalene), 7.57 – 7.44 (m, 3H, ArH naphthalene), 7.42 (s, 1H, ArH indole), 7.33 (d, J = 9.1 Hz, 1H, ArH indole), 7.17 (br s, 1H, NH), 7.03 (dd, J = 8.9, 2.6 Hz, 1H, ArH indole), 5.08 (br s, 1H, NH), 4.62 (s, 2H, indole-O-CH2), 4.29 – 4.03 (br m, 2H, N1-CH2), 3.68 – 3.50 (m, 14H, ([CH2]2O)2(CH2)2 & O-CH2 morpholino), 3.36 – 3.24 (m, 2H, ([CH2]2O)2(CH2)2), 2.75 – 2.61 (br m, 2H, N1-CH2CH2), 2.52 – 2.25 (br m, 4H, N-CH2 morpholino), 1.41 (s, 9H, CH3 tBu).

13C NMR (126 MHz, CDCl3) δ 192.04, 168.56, 156.11, 154.50, 139.38, 138.88, 133.86, 132.70, 130.78, 130.24, 128.39, 127.86, 126.97, 126.49, 125.87, 125.83, 124.61, 117.72, 113.69, 110.96, 107.14, 79.33, 70.44, 70.36, 70.34, 69.93, 68.47, 66.84, 57.47, 53.52, 44.44, 40.48, 38.94, 28.53.

HRMS-ESI calculated for C38H49N4O8 [M+H]+ 689.3545, found m/z 689.3546.
N-(8-Aminooctyl)-2-[[3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-yl]oxy]acetamide (2.30a)

2.29a (7.3 mg, 10.9 µmol) was dissolved in DCM (1.6 mL) and TFA (1.6 mL) was added. After 1 h stirring, the reaction mixture was evaporated under N₂ stream, followed by reduced pressure. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of 2.30a (5.2 mg, 5.7 µmol, 52%) as a white solid.

HRMS-ESI calculated for C₃₂H₄₅N₄O₅ [M+H]⁺ 565.3384, found m/z 565.3338.
Analytical RP-HPLC Rₜ = 13.43 min.

N-[[2-[2-(2-Aminoethoxy)ethoxy]ethyl]2-[[3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-yl]oxy]acetamide (2.30b)

A solution of 2.29b (8.4 mg, 12.6 µmol) and TFA (1.6 mL) in DCM (1.6 mL) was reacted as described in the procedure for 2.30a. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of 2.30b (8.8 mg, 9.7 µmol, 77%) as a white solid.

HRMS-ESI calculated for C₃₀H₄₁N₄O₇ [M+H]⁺ 569.2970, found m/z 569.2926.
Analytical RP-HPLC Rₜ = 11.92 min.
**N-(8-Aminooctyl)-2-((1-[2-(morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indol-5-yl)oxy)acetamide (2.30c)**

A solution of **2.29c** (7 mg, 10.2 μmol) and TFA (1 mL) in DCM (1 mL) was reacted as described in the procedure for **2.30a**. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of **2.30c** (5.3 mg, 5.7 μmol, 56%) as a white solid.

HRMS-ESI calculated for C_{35}H_{45}N_{4}O_{4} [M+H]^+ 585.3435, found m/z 585.3384.
Analytical RP-HPLC R_t = 14.32 min.

**N-{2-[2-(2-Aminoethoxy)ethoxy]ethyl}-2-((1-[2-(morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indol-5-yl)oxy)acetamide (2.30d)**

A solution of **2.29d** (12 mg, 17.4 μmol) and TFA (1 mL) in DCM (1 mL) was reacted as described in the procedure for **2.30a**. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of **2.30d** (4.3 mg, 4.6 μmol, 27%) as a white solid.

HRMS-ESI calculated for C_{33}H_{41}N_{4}O_{6} [M+H]^+ 589.3021, found m/z 589.2984.
Analytical RP-HPLC R_t = 12.95 min.
(2S)-N-[(1S)-1-[(2-aminoethyl)carbamoyl]ethyl]-2-(2-[[3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-yl]oxy]acetamido)propanamide (2.30e)

2.26 (58 mg, 41.4 μmol) was swelled in DMF, Fmoc-deprotected using 20% v/v piperidine/DMF and then washed thoroughly with DMF. A solution of the TFA salt of 2.25a (23 mg, 34.3 μmol), HATU (16 mg, 41.4 μmol) and DIPEA (29 μL, 0.17 mmol) in DMF (83 μL) was swirled for 1 min and then added to the drained resin and left for 2 h. The resin was drained, washed with DMF and DCM and dried under vacuum. The resin was transferred to a round bottom flask and cleaved using a solution of TFA in DCM (5 mL, 5% v/v), stirring for 1 h. The mixture was filtered, washed with DCM and the filtrate dried under reduced pressure. The crude residue was purified using semi-preparative RP-HPLC, to yield the TFA salt of 2.30e (9.9 mg, 10.2 μmol, 30%) as a white solid.

HRMS-ESI calculated for C₃₂H₄₃N₆O₇ [M+H]⁺ 623.3188, found m/z 623.3133.
Analytical RP-HPLC Rₜ = 11.83 min.

(2S)-N-[(1S)-1-[(2-aminoethyl)carbamoyl]ethyl]-2-[2-[[1-[(2-(morpholin-4-yl)ethyl)-3-(naphthalene-1-carbonyl)-1H-indol-5-yl]oxy]acetamido]propanamide (2.30f)

2.26 (38 mg, 27.3 μmol), piperidine in DMF (20% v/v), 2.25b (16 mg, 22.7 μmol), HATU (10 mg, 27.3 μmol), DIPEA (19 μL, 0.11 mmol), TFA in DCM (5 mL 5% v/v) were used as in the procedure for 2.30e. The crude was purified using semi-preparative RP-HPLC, to yield the TFA salt of 2.30f (5.14 mg, 6.2 μmol, 27%) as a white solid.
HRMS-ESI calculated for C_{35}H_{43}N_{6}O_{6} [M+H]^+ 643.3239, found m/z 643.3194. Analytical RP-HPLC R_t = 13.21 min.

(2S)-N-[(1S)-1-[(2-aminoethyl)carbamoyl]ethyl]-2-(2-[(3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-5-yl]oxy)acetamido)propanamide (2.30g)

2.26 (44 mg, 31.7 μmol), piperidine in DMF (20% v/v), the TFA salt of 2.25e (17 mg, 31.7 μmol), HATU (12 mg, 31.7 μmol), DIPEA (22 μL, 0.13 mmol), TFA in DCM (5 mL 5% v/v) were used as described in the procedure for 2.30e. The crude was purified using semi-preparative RP-HPLC, to yield the TFA salt of 2.30g (7.45 mg, 8.9 μmol, 28%) as a white solid.

HRMS-ESI calculated for C_{32}H_{42}N_{5}O_{7} [M+H]^+ 608.3079, found m/z 608.3031. Analytical RP-HPLC R_t = 15.63 min.

N-2-[(2-(2-Acetamidoethoxy)ethoxy]ethyl]-2-[(3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-yl]oxy)acetamide (2.31)

To a solution of the TFA salt of 2.30b (2.6 mg, 2.9 μmol) and DIPEA (1.71 μL, 9.8 μmol, added as a 1:10 solution in DCM) was added acetic anhydride (0.34 μL 3.6 μmol, added as a 1:10 solution in DCM) and the mixture was stirred at rt for 1 h. The reaction solvent was evaporated under N_2 stream. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 2.31 (1.69 mg, 2.8 μmol, 97%) as a white solid.
HRMS-ESI calculated for C_{32}H_{43}N_{4}O_{8} [M+H]^+ 611.3075, found m/z 611.3068.
Analytical RP-HPLC R_t = 13.49 min.

6-(2-{4-[(E)-2-[2,2-Difluoro-4-(thiophen-2-yl)-1\lambda4,3-diaza-2\lambda4-boratricyclo[7.3.0.0^{3,7}]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy}acetamido)-N-[8-(2-[[3-(4-methoxybenzoyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indol-5-yl]oxy]acetamido)octyl]hexanamide (2.32a)

To a solution of the TFA salt of 2.30a (2.82 mg, 3.11 µmol) in anhydrous DMF (500 µL), was added a solution of DIPEA (1.91 µL, 11.0 µmol) in anhydrous DMF (30.59 µL), followed by a solution of BODIPY 630/650-OSu 2.15 (1.25 mg, 1.89 µmol) in anhydrous DMF (300 µL). The mixture was swirled, left standing for 12 h, then evaporated under reduced pressure. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 2.32a (1.56 mg, 1.41 µmol, 74%) as a bright blue solid.

HRMS-ESI calculated for C_{61}H_{70}BF_{2}N_{7}NaO_{8}S [M+Na]^+ 1132.4970, found m/z 1132.5064.
Analytical RP-HPLC R_t = 22.33 min.
6-(2-[(E)-2-[2,2-Difluoro-4-(thiophen-2-yl)-1\,4,3-diaza-2\,4-
boratricyclo[7.3.0.0\,3,7]dodeca-1(12),4,6,8,10-pentaen-12-
yl]ethenyl]phenoxy)acetamido)-N-(2-[2-[2-([3-(4-methoxybenzoyl)-1-[2-
(morpholin-4-yl)ethyl]-1H-indol-5-
yl]oxy)acetamido]ethoxy]ethoxy]ethyl)hexanamide (2.32b)

The TFA salt of 2.30b (3.59 mg, 3.94 µmol), DIPEA (2.24 µL, 12.9 µmol), BODIPY
630/650-X-Osu 2.15 (1.25 mg, 1.89 µmol) and DMF (836 µL) were reacted as
described in the procedure for 2.32a. The crude product was purified by semi-
preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to
remove TFA, yielding 2.32b (1.26 mg, 1.13 µmol, 60%) as a bright blue solid.

HRMS-ESI calculated for C_{59}H_{66}BF_{2}N_{7}NaO_{10}S [M+Na]^+ 1136.4555, found m/z
1136.4618.
Analytical RP-HPLC R_t = 21.36 min.
6-(2-[4-[(E)-2-[2,2-Difluoro-4-(thiophen-2-yl)-1\_4,3-diaza-2\_4-boratricyclo[7.3.0.0^{3,7}]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy]acetamido)-N-[8-[2-[[1-[[2-(morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indol-5-yl]oxy]acetamido]octyl]hexanamide (2.32c)

The TFA salt of 2.30c (3.22 mg, 3.47 µmol), DIPEA (2.04 µL, 11.7 µmol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 µmol) and DMF (832 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 2.32c (1.88 mg, 1.66 µmol, 88%) as a bright blue solid.

HRMS-ESI calculated for C_{64}H_{70}BF_{2}N_{7}NaO_{7}S [M+Na]^+ 1152.5021, found m/z 1152.5090.
Analytical RP-HPLC R_t = 23.05 min.
6-(2-{(E)-2-[2,2-Difluoro-4-(thiophen-2-yl)-1\(\lambda\)4,3-diaza-2\(\lambda\)4-boratricyclo[7.3.0.0\(3^3\)]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl}phenoxy)acetamido)-N-[2-((2-2-2-[1-((2-(morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indol-5-yl)oxy)acetamido]ethoxy]ethoxy)ethyl]hexanamide (2.32d)

![Chemical Structure]

The TFA salt of 2.30d (2.41 mg, 2.59 \(\mu\)mol), DIPEA (1.71 \(\mu\)L, 9.8 \(\mu\)mol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 \(\mu\)mol) and DMF (827 \(\mu\)L) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 2.32d (1.75 mg, 1.54 \(\mu\)mol, 82\%) as a bright blue solid.

HRMS-ESI calculated for C\(_{62}\)H\(_{66}\)BF\(_2\)N\(_7\)NaO\(_9\)S [M+Na]\(^+\) 1156.4606, found \(m/z\) 1156.4661.

Analytical RP-HPLC \(R_t = 22.02\) min.
6-(2-[4-[(E)-2-[2,2-Difluoro-4-(thiophen-2-yl)-1\ı4,3-diaza-2\ı4-
boratricyclo[7.3.0.0\ı3,7]dodeca-1(12),4,6,8,10-pentaen-12-
yl]ethenyl]phenoxy)acetamido)-N-(2-[2-[2-([3-(4-methoxybenzoyl)-1-[2-
(morpholin-4-yl)ethyl]-1H-indol-5-
yl]oxy)acetamido)propanamido]propanamido)ethyl)hexanamide (2.32e)

The TFA salt of 2.30e (3.67 mg, 3.80 µmol), DIPEA (2.37 µL, 13.6 µmol), BODIPY
630/650-X-OSu 2.15 (1.67 mg, 2.52 µmol) and DMF (840 µL) were reacted as
described in the procedure for 2.32a. The crude product was purified by semi-
preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to
remove TFA, yielding 2.32e (1.56 mg, 1.34 µmol, 53%) as a bright blue solid.

HRMS-ESI calculated for C_{61}H_{68}BF_{2}N_{9}NaO_{10}S [M+Na]^+ 1190.4773, found m/z
1190.4840.
Analytical RP-HPLC R_t = 21.18 min.
6-(2-{4-[{(E)-2-[2,2-Difluoro-4-(thiophen-2-yl)-1:4,3-diaza-2:4-boratricyclo[7.3.0.0\textsuperscript{3,7}]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy}acetamido)-N-[2-(2-[2-[2-[(1-[2-(morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indol-5-yl]oxy)acetamido]propanamido]propanamido)ethyl]hexanamide (2.32f)

The TFA salt of 2.30f (3.32 mg, 3.37 µmol), DIPEA (2.19 μL, 12.6 µmol), BODIPY 630/650-X-OSu 2.15 (1.67 mg, 2.52 µmol) and DMF (837 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 2.32f (2.08 mg, 1.75 µmol, 70%) as a bright blue solid.

HRMS-ESI calculated for C\textsubscript{64}H\textsubscript{69}BF\textsubscript{2}N\textsubscript{9}O\textsubscript{9}S [M+H]\textsuperscript{+} 1188.5005, found m/z 1188.5012. Analytical RP-HPLC R\textsubscript{t} = 21.76 min.
6-(2-[4-\((E)\)-2,2-Difluoro-4-(thiophen-2-yl)-1\,4,3-diaza-2\,4-boratricyclo[7.3.0.0^{3,7}]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy)acetamido)-N-(2-[2-[(2-[(3-((4-methoxybenzoyl)1-[((oxan-4-yl)methyl]-1H-indol-5-yl)oxy)acetamido)propanamido]propanamido]ethyl)hexanamide (2.32g)

The TFA salt of 2.30g (3.89 mg, 4.65 µmol), DIPEA (2.46 µL, 14.1 µmol), BODIPY 630/650-X-OSu 2.15 (1.67 mg, 2.52 µmol) and DMF (842 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 2.32g (1.06 mg, 0.92 µmol, 36%) as a bright blue solid.

HRMS-ESI calculated for C_{61}H_{67}BF_{2}N_{8}NaO_{10}S [M+Na]^+ 1175.4664, found m/z 1175.4655.

Analytical RP-HPLC R_t = 23.14 min.

7-(Benzyloxy)-1-[(oxan-4-yl) methyl]-1H-indole (3.2)

7-Benzylxyindole 3.1 (335 mg, 1.5 mmol), NaH (268 mg, 6.7 mmol) and 2.17b (582 mg, 3.0 mmol) in DMF (14 mL) were reacted as described in the procedure for 2.19a. The crude product was purified using flash silica column chromatography (4:1 hexane/EA) to yield the desired 3.2 (418 mg, 1.3 mmol, 87%), as a pale violet wax (R_f 0.63, 2:1 PE/EA).
$^1$H NMR (400 MHz, CDCl$_3$) δ 7.51 – 7.44 (m, 2H, ArH Bn), 7.44 – 7.33 (m, 3H, ArH Bn), 7.24 (dt, $J$ = 8.0, 0.7 Hz, 1H, ArH indole), 7.00 (t, $J$ = 7.8 Hz, 1H, ArH indole), 6.91 (d, $J$ = 2.9 Hz, 1H, ArH indole), 6.73 (dd, $J$ = 7.8, 0.8 Hz, 1H, ArH indole), 6.40 (d, $J$ = 1.2 Hz, 1H, ArH indole), 5.14 (s, 2H, CH$_2$Bn), 4.08 (d, $J$ = 7.2 Hz, 2H, N-CH$_2$), 3.87 – 3.76 (m, 2H, O-CH$_2$THP), 3.13 (td, $J$ = 11.6, 2.5 Hz, 2H, O-CH$_2$THP), 2.11 – 1.92 (m, 1H, CH THP), 1.26 – 1.03 (m, 4H, O-CH$_2$CH$_2$THP).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 146.85, 136.98, 131.35, 130.02, 128.71, 128.56, 128.41, 125.57, 119.87, 114.12, 103.02, 100.83, 70.60, 67.70, 55.41, 37.68, 30.43.

HRMS-ESI calculated for C$_{21}$H$_{23}$NNaO$_2$ [M+Na]$^+$ 344.1621, found m/z 344.1591.

7-(Benzyloxy)-3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indole (3.3)

A solution of 3.2 (104 mg, 0.32 mmol), 4-methoxybenzonitrile (129 mg, 0.97 mmol) and 2.21 (13 mg, 32.0 μmol) in H$_2$O (0.12 mL), glacial AcOH (0.18 mL) and 1,4-dioxane (0.6 mL) was reacted as described in the procedure for 2.22a. The crude residue was purified by flash silica column chromatography (2:1 hexane/EA) to yield 3.3 (85 mg, 0.19 mmol, 58%) as a pale pinkish-white solid ($R_f$ 0.44, 1:1 hexane/EA).

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.01 (dd, $J$ = 8.1, 0.9 Hz, 1H, ArH indole), 7.85 – 7.78 (m, 2H, ArH MeOPh), 7.51 – 7.46 (m, 2H, ArH Bn), 7.45 – 7.37 (m, 3H, ArH Bn), 7.37 (s, 1H, ArH indole), 7.23 (t, $J$ = 8.0 Hz, 1H, ArH indole), 7.02 – 6.95 (m, 2H, ArH MeOPh), 6.87 (dd, $J$ = 7.9, 0.9 Hz, 1H, ArH indole), 5.15 (s, 2H, CH$_2$ Bn), 4.10 (d, $J$ = 7.2 Hz, 2H, N-CH$_2$), 3.89 (s, 3H, O-CH$_3$), 3.86 – 3.77 (m, 2H, O-CH$_2$ THP), 3.12 (td, $J$ = 11.6, 2.4 Hz, 2H, O-CH$_2$ THP), 2.14 – 1.93 (m, 1H, CH THP), 1.22 – 0.99 (m, 4H, O-CH$_2$CH$_2$ THP).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 189.81, 162.33, 146.60, 137.85, 136.49, 133.54, 131.04, 130.25, 128.82, 128.74, 128.66, 126.27, 123.20, 115.56, 115.32, 113.64, 105.14, 70.84, 67.54, 56.29, 55.55, 37.10, 30.22.

HRMS-ESI calculated for C$_{29}$H$_{30}$NO$_4$ [M+H]$^+$ 456.2169, found m/z 456.2129.
3-(4-Methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-7-ol (3.4)

A mixture of 3.3 (723 mg, 1.6 mmol), EtOH (12 mL), CHCl₃ (32 mL) and Pd/C (72 mg) was reacted as described in the procedure for 2.23a. The crude residue was purified by flash silica gel column chromatography (2:1 hexane/EA) to yield the desired product 3.4 (322 mg, 0.88 mmol, 55%) as a brown oil, (Rf 0.47, 1:1 PE/EA).

¹H NMR (400 MHz, CDCl₃) δ 7.96 – 7.89 (m, 1H, ArH indole), 7.87 – 7.79 (m, 2H, ArH MeOPh), 7.44 (s, 1H, ArH indole), 7.07 (t, J = 7.9 Hz, 1H, ArH indole), 7.02 – 6.95 (m, 2H, ArH MeOPh), 6.69 (dt, J = 7.6, 1.1 Hz, 1H, ArH indole), 4.31 (d, J = 7.2 Hz, 2H, N-CH₂), 4.02 – 3.91 (m, 2H, O-CH₂ THP), 3.89 (s, 3H, O-CH₃), 3.31 (td, J = 11.7, 2.3 Hz, 2H, O-CH₂ THP), 2.31 – 2.07 (m, 1H, CH THP), 1.58 – 1.27 (m, 4H, O-CH₂CH₂ THP).

¹³C NMR (101 MHz, CDCl₃) δ 190.53, 162.47, 143.72, 143.72, 138.20, 133.38, 131.19, 130.33, 126.12, 123.47, 115.56, 114.63, 113.71, 109.48, 67.65, 55.72, 55.59, 37.41, 30.45.

HRMS-ESI calculated for C₂₂H₂₃NNaO₄ [M+Na]⁺ 388.1519, found m/z 388.1553.

Analytical RP-HPLC Rᵣ = 18.19 min.

tert-Butyl 2-[(3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-7-yloxyl]oxy]acetate (3.5)

A solution of 3.4 (11 mg, 30.1 µmol), NaH (2.4 mg, 60.2 µmol) and tert-butylbromoacetate (5.8 µL, 39.1 µmol) in DMF (1.8 mL) was reacted as described in the procedure for 2.24a. The crude product was purified by flash silica gel column chromatography (1:1 hexane/EA) to yield 3.5 (4.7 mg, 9.8 µmol, 33%) as a yellow oil (Rf 0.6, 1:2 hexane/EA).

¹H NMR (400 MHz, CDCl₃) δ 8.02 (dd, J = 8.1, 0.8 Hz, 1H, ArH indole), 7.87 – 7.79 (m, 2H, ArH MeOPh), 7.43 (s, 1H, ArH indole), 7.17 (t, J = 8.0 Hz, 1H, ArH indole), 7.03 – 6.95 (m, 2H, ArH MeOPh), 6.64 (dd, J = 8.0, 0.8 Hz, 1H, ArH indole), 4.64 (s,
2H, O-CH$_2$), 4.39 (d, $J = 7.2$ Hz, 2H, N-CH$_2$), 3.94 (dd, $J = 11.5$, 4.0 Hz, 2H, O-CH$_2$
THP), 3.90 (s, 3H, O-CH$_3$), 3.30 (td, $J = 11.8$, 2.1 Hz, 2H, O-CH$_2$
THP), 2.28 – 2.11
(m, 1H, CH THP), 1.57 – 1.31 (m, 13H, O-CH$_2$CH$_2$ THP & CH$_3$t Bu).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 189.86, 167.36, 162.39, 145.50, 138.02, 133.53, 131.11,
130.38, 126.44, 123.03, 116.18, 115.46, 113.69, 105.22, 82.60, 67.68, 65.99, 56.19,
55.60, 37.29, 30.53, 28.25.

HRMS -ESI calculated for C$_{28}$H$_{34}$NO$_6$ [M+H]$^+$ 480.2381, found m/z 480.2346.

2-[(3-(4-Methoxybenzoyl)-1-[(oxan-4-yI)methyl]-1H-indol-7-yl]oxy}acetic acid (3.6)

A solution of 3.5 (3.1 mg, 6.46 µmol) and TFA (0.4 mL) in DCM (0.8 mL) was reacted as described in the procedure for 2.25a. The crude was purified by semi-preparative RP-HPLC
and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 3.6 (1.45 mg, 3.4 µmol, 53%), a white solid.

HRMS -ESI calculated for C$_{24}$H$_{24}$NO$_6$ [M-H]$^-$ 422.1609, found m/z 422.1598.

Analytical RP-HPLC $R_t$ = 17.17 min.

3-(4-Methoxybenzoyl)-1-[(oxan-4-yl)methyl]-5-propoxy-1H-indole (3.7a)

To a solution of 2.23e (50 mg, 0.14 mmol) in anhydrous
DMF (3 mL) was added NaH (60% by mass dispersion
in mineral oil) (20 mg, 0.50 mmol), followed by
dropwise addition of 1-bromopropane (18.6 µL, 0.21
mmol) in anhydrous DMF (2 mL). After stirring for 20
h, the reaction was quenched with sat. aq. NH$_4$Cl (2 mL) and H$_2$O (3 mL) and extracted
with EA (4 × 5 mL). The combined organics were washed with H$_2$O (4 × 20 mL), dried
over MgSO$_4$, filtered and evaporated under reduced pressure. The crude product was
purified using flash silica column chromatography (99:1 DCM/MeOH) to yield the
desired 3.7a (46 mg, 0.11 mmol, 83%), as a white-opaque solid (Rf 0.57, 99:1 DCM/MeOH).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.93 (d, J = 2.5 Hz, 1H, ArH indole), 7.86 – 7.79 (m, 2H, ArH MeOPh), 7.48 (s, 1H, ArH indole), 7.25 (dd, J = 8.9, 0.5 Hz, 1H, ArH indole), 7.03 – 6.95 (m, 3H, ArH indole & ArH MeOPh), 4.07 – 3.92 (m, 6H, O-CH$_2$ propyl, N-CH$_2$ & O-CH$_2$ THP), 3.89 (s, 3H, O-CH$_3$), 3.31 (td, J = 11.8, 2.2 Hz, 2H, O-CH$_2$ THP), 2.19 – 2.03 (m, 1H, CH THP), 1.93 – 1.76 (m, 2H, O-CH$_2$-CH$_2$ propyl), 1.55 – 1.30 (m, 4H, O-CH$_2$CH$_3$ THP), 1.05 (t, J = 7.4 Hz, 3H, CH$_3$ propyl).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 189.83, 162.29, 156.02, 136.66, 133.58, 131.88, 130.93, 128.43, 115.32, 114.72, 113.67, 110.71, 104.90, 70.25, 67.41, 55.55, 53.29, 36.03, 30.84, 22.80, 10.71.

HRMS-ESI calculated for C$_{25}$H$_{29}$NNaO$_4$ [M+Na]$^+$ 430.1989, found m/z 430.1954.

Analytical RP-HPLC R$_f$ = 22.17 min.

3-(4-Methoxybenzoyl)-1-[(oxan-4-y1)methyl]-7-propoxy-1H-indole (3.7b)

A solution of 3.4 (9.5 mg, 25.9 μmol), NaH (3.1 mg, 77.7 μmol), and 1-bromopropane (3.5 μL, 38.9 μmol) in DMF (0.95 mL) was reacted as described in the procedure for 3.7a. The crude product was purified using flash silica column chromatography (2:1 hexane/EA) to yield the desired 3.7b (6.1 mg, 15.0 μmol, 57%), as a colourless oil (Rf 0.56, 1:1 hexane/EA).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.96 (dd, J = 8.1, 0.9 Hz, 1H, ArH indole), 7.86 – 7.77 (m, 2H, ArH MeOPh), 7.40 (s, 1H, ArH indole), 7.18 (t, J = 7.9 Hz, 1H, ArH indole), 7.03 – 6.95 (m, 2H, ArH MeOPh), 6.74 (dd, J = 7.9, 0.9 Hz, 1H, ArH indole), 4.29 (d, J = 7.3 Hz, 2H, N-CH$_2$), 4.10 (t, J = 6.5 Hz, 2H, O-CH$_2$ propyl), 4.01 – 3.92 (m, 2H, O-CH$_2$ THP), 3.89 (s, 3H, O-CH$_3$), 3.30 (td, J = 11.7, 2.3 Hz, 2H, O-CH$_2$ THP), 2.26 – 2.11 (m, 1H, CH THP), 1.99 – 1.82 (m, 2H, O-CH$_2$-CH$_2$ propyl), 1.52 – 1.28 (m, 4H, O-CH$_2$CH$_3$ THP), 1.12 (t, J = 7.4 Hz, 3H, CH$_3$ propyl).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 189.88, 162.34, 146.87, 137.66, 133.65, 131.09, 130.10, 126.36, 123.25, 115.50, 115.13, 113.67, 105.03, 69.83, 67.63, 56.21, 55.59, 37.29,
30.58, 22.97, 11.04.
HRMS-ESI calculated for C_{25}H_{29}NNaO_{4} [M+Na]^+ 430.1989, found m/z 430.1994.
Analytical RP-HPLC R_t = 22.55 min.

tert-Butyl N-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)carbamate (3.9)

To a stirred solution of 2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethanol 3.8 (100 µL, 0.55 mmol) in dioxane (1 mL) was added Boc₂O (152 µL, 0.66 mmol). The mixture was stirred for 18 h at rt and then evaporated under reduced pressure. The crude was taken up in DCM (6 mL), washed with H₂O (2 x 6 mL) and sat. aq. NaCl (1 x 5 mL), dried over MgSO₄, filtered and evaporated under reduced pressure. The crude product was purified using flash silica column chromatography (99:1 DCM/MeOH) to yield the desired 3.9 (24 mg, 81.8 µmol, 15%), as a pale yellow oil (R_f 0.52, 9:1 DCM/MeOH).

1H NMR (400 MHz, CDCl₃) δ 3.75 – 3.67 (m, 4H, O-CH₂), 3.66 – 3.57 (m, 8H, O-CH₂CH₂-O), 3.52 (t, J = 5.2 Hz, 2H, O-CH₂), 3.29 (t, J = 5.2 Hz, 2H, NH-CH₂), 1.43 (s, 9H, CH₃ t Bu).
13C NMR (101 MHz, CDCl₃) δ 156.31, 79.19, 72.77, 70.72, 70.67, 70.55, 70.36, 70.20, 61.76, 40.67, 28.56.
HRMS-ESI calculated for C_{13}H_{27}NNaO₆ [M+Na]^+ 316.1731, found m/z 316.1738.

tert-Butyl N-(2-{2-[2-(2-bromoethoxy)ethoxy]ethoxy}ethyl)carbamate (3.10)

A solution of bromine (7.3 µL, 0.14 mmol) in anhydrous DCM (0.2 mL) at 0°C was added to a solution of triphenylphosphine (37 mg, 0.14 mmol) and Et₃N (20 µL, 0.14 mmol) in anhydrous DCM (0.2 mL) at 0°C. Following stirring at 0°C for 30 min, a solution of 3.9 (42 mg, 0.14 mmol) in anhydrous DCM (0.2 mL) was added dropwise. After stirring at 0°C for 2 h, the mixture was evaporated under reduced pressure. The crude product was purified using flash silica column chromatography (5:1 hexane/EA) to yield the desired 3.10 (13 mg, 36.5 µmol, 26%) as a colourless oil.

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1H NMR (400 MHz, CDCl₃) δ 5.03 (br s, 1H, NH), 3.81 (t, J = 6.3 Hz, 2H, O-CH₂), 3.73 – 3.58 (m, 8H, O-CH₂CH₂-O), 3.54 (t, J = 5.1 Hz, 2H, O-CH₂), 3.47 (t J = 6.3, 1.2 Hz, 2H, CH₂Br), 3.31 (q, J = 5.6 Hz, 2H, NH-CH₃), 1.44 (s, 9H, CH₃ t Bu).

HRMS-ESI calculated for C₁₃H₂₆BrNNaO₅ [M+Na]⁺ 378.0887, found m/z 378.0894.

tert-Butyl N-(2-[2-[2-[3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-5-yl]oxy]ethoxy]ethoxy)ethyl)carbamate (3.11)

To a solution of 2.23e (13 mg, 35.1 μmol) in anhydrous DMF (0.7 mL) was added NaH (60% by mass dispersion in mineral oil) (5.5 mg, 0.14 mmol). After stirring for 30 min, a solution of 3.10 (12.5 mg, 35.1 μmol) in anhydrous DMF (0.6 mL) was added. The reaction mixture was stirred for 18 h, then quenched with H₂O (1.5 mL), extracted with EA (4 × 2 mL), washed with H₂O (2 × 7 mL), dried over MgSO₄, filtered and evaporated under reduced pressure. The crude product was purified using flash silica column chromatography (99:1 DCM/MeOH) and semi-preparative RP-HPLC to yield the desired 3.11 (6.88 mg, 10.7 μmol, 31%), as a white solid.

1H NMR (400 MHz, DMSO-d₆) δ 7.97 (s, 1H, ArH indole), 7.81 – 7.75 (m, 3H, ArH indole and MeOPh), 7.58 (d, J = 9.0 Hz, 1H, ArH indole), 7.12 – 7.05 (m, 2H, ArH MeOPh), 6.95 (dd, J = 8.9, 2.5 Hz, 1H, ArH indole), 6.74 (br s, 1H, NH), 4.21 – 4.07 (m, 4H, N1-CH₂ & indole-O-CH₂), 3.86 (s, 3H, O-CH₃), 3.85 – 3.75 (m, 4H, O-CH₂ & O-CH₂ THP), 3.65 – 3.45 (m, 8H, O-CH₂CH₂-O), 3.37 (t, J = 6.1 Hz, 2H, O-CH₂), 3.19 (td, J = 11.7, 2.0 Hz, 2H, O-CH₂ THP), 3.05 (q, J = 6.0 Hz, 2H, NH-CH₂), 2.13 – 2.02 (m, 1H, CH THP), 1.41 – 1.21 (m, 13H, O-CH₂CH₂ THP, CH₃ t Bu).

HRMS-ESI calculated for C₃₅H₄₆N₂NaO₉ [M+Na]⁺ 663.3252, found m/z 663.3247. Analytical RP-HPLC Rₛ = 21.00 min.
To a solution of 3.11 (1.81 mg, 2.8 µmol) in DCM (0.45 mL) was added TFA (0.45 mL). The reaction mixture was swirled, left to stand for 1 h and then evaporated under reduced pressure. The residue was then dissolved in a solution of DCM (0.4 mL) and a solution of DIPEA (1.48 µL, 8.5 µmol, added as a 1:50 solution in DCM) added, followed by a solution of acetic anhydride (0.29 µL, 3.1 µmol, added as a 1:50 solution in DCM). The reaction mixture was swirled and then left to stand for 1.5 h, after which the reaction solvent was evaporated under air. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 3.12 (1.17 mg, 2.0 µmol, 71%), as a white solid.

HRMS-ESI calculated for C_{32}H_{42}N_{2}NaO_{8} [M+Na]^+ 605.2833, found m/z 605.2828.

Analytical RP-HPLC R_t = 18.12 min.

**Methyl 5-[(3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-7-yl]oxy]pentanoate (3.13)**

A solution of 3.4 (261 mg, 0.71 mmol), NaH (86 mg, 2.1 mmol) and methyl 5-bromovalerate (153 µL, 1.1 mmol) in DMF (20 mL) was reacted as described in the procedure for 3.7a. The crude product was purified using flash silica column chromatography (4:1 hexane/EA) to yield the desired 3.13 (103 mg, 0.28 mmol, 30%), as a white solid (R_t 0.32, 1:1 hexane/EA).

^1H NMR (400 MHz, CDCl_3) δ 7.97 (dd, J = 8.1, 0.8 Hz, 1H, ArH indole), 7.84 – 7.80 (m, 2H, ArH MeOPh), 7.40 (s, 1H, ArH indole), 7.18 (t, J = 8.0 Hz, 1H, ArH indole).
7.01 – 6.96 (m, 2H, ArH MeOPh), 6.73 (dd, J = 7.9, 0.9 Hz, 1H, ArH indole), 4.28 (d, J = 7.3 Hz, 2H, N-CH2), 4.15 (t, J = 5.8 Hz, 2H, O-CH2 butyl), 3.98 – 3.92 (m, 2H, O-CH2 THP), 3.89 (s, 3H, Ph-O-CH3), 3.69 (s, 3H, COOCH3), 3.29 (td, J = 11.6, 2.4 Hz, 2H, O-CH2 THP), 2.43 (t, J = 6.9 Hz, 2H, CH2COO), 2.22 – 2.08 (m, 1H, CH THP), 1.96 – 1.83 (m, 4H, CH2CH2), 1.48 – 1.30 (m, 4H, O-CH2CH2 THP).

13C NMR (101 MHz, CDCl3) δ 189.89, 173.66, 162.36, 146.64, 137.77, 133.52, 131.10, 130.14, 126.27, 123.24, 115.45, 115.30, 113.66, 105.06, 67.63, 67.57, 56.20, 55.58, 51.79, 37.28, 33.71, 30.56, 29.06, 21.94.

HRMS-ESI calculated for C28H34NO6 [M+H]+ 480.2381, found m/z 480.2366.

Analytical RP-HPLC Rₜ = 20.92 min.

5-[[3-(4-Methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-7-yl]oxy]pentanoic acid (3.14)

To a stirred solution of 3.13 (91 mg, 0.19 mmol) in THF (1.6 mL) at 0°C was added dropwise 0.2 M aq. lithium hydroxide monohydrate solution (2.4 mL). The mixture was stirred at 0°C for 18 h, then quenched with biphase of 0.2 M aq. HCl/EA (1:1 v/v) until pH 4. The aqueous layer was extracted with EA (4 × 4 mL), dried over MgSO₄, filtered and evaporated under reduced pressure to yield the hydrochloride salt of 3.14 (84 mg, 0.17 mmol, 88%) as a brown solid.

1H NMR (400 MHz, CDCl3) δ 7.97 (dd, J = 8.1, 0.8 Hz, 1H, ArH indole), 7.85 – 7.79 (m, 2H, ArH MeOPh), 7.40 (s, 1H, Ar indole), 7.18 (t, J = 8.0 Hz, 1H, ArH indole), 7.02 – 6.95 (m, 2H, ArH MeOPh), 6.74 (dd, J = 8.2, 0.7 Hz, 1H, ArH indole), 4.29 (d, J = 7.3 Hz, 2H, N-CH2), 4.16 (t, J = 5.6 Hz, 2H, O-CH2 butyl), 3.99 – 3.92 (m, 2H, O-CH2 THP), 3.89 (s, 3H, O-CH2), 3.30 (td, J = 11.6, 2.5 Hz, 2H, O-CH2 THP), 2.48 (t, J = 6.9 Hz, 2H, CH2COOH), 2.21 – 2.11 (m, 1H, CH THP), 2.00 – 1.84 (m, 4H, CH2CH2), 1.48 – 1.31 (m, 4H, O-CH2CH2 THP).

HRMS-ESI calculated for C27H33NNaO6 [M+Na]+ 488.2044, found m/z 488.2007.

Analytical RP-HPLC Rₜ = 18.68 min.
**tert-Butyl N-(2-[2-[5-[[3-(4-methoxybenzoyl)-1-[((oxan-4-yl)methyl]-1H-indol-7-yl]oxy]pentanamido]ethoxy]ethoxy]ethyl)carbamate (3.15)**

The hydrochloride salt of 3.14 (38 mg, 74.9 μmol), DIPEA (85 μL, 0.49 mmol), HATU (31 mg, 80.8 μmol) and 2.28b (60 mg, 0.24 mmol) in DMF (5.8 mL) were reacted as described in the procedure for 2.29a. The crude product was purified by flash silica gel column chromatography (99:1 DCM/MeOH) to yield 3.15 (46 mg, 66.1 μmol, 88%) as a brown solid (R<sub>f</sub> 0.56, 9:1 DCM/MeOH).

1H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.97 (dd, J = 8.1, 0.8 Hz, 1H, ArH indole), 7.86 – 7.78 (m, 2H, ArH MeOPh), 7.39 (s, 1H, ArH indole), 7.18 (t, J = 8.0 Hz, 1H, ArH indole), 7.03 – 6.95 (m, 2H, ArH MeOPh), 6.73 (dd, J = 7.9, 1.0 Hz, 1H, ArH indole), 6.10 (br s, 1H, NH), 5.02 (br s, 1H, NH), 4.28 (d, J = 7.3 Hz, 2H, N1-CH<sub>2</sub>), 4.20 – 4.11 (m, 2H, O-CH<sub>2</sub> butyl), 3.95 (dd, J = 11.6, 3.9 Hz, 2H, O-CH<sub>2</sub> THP), 3.89 (s, 3H, O-CH<sub>3</sub>), 3.65 – 3.52 (m, 8H, CH<sub>2</sub>-O-(CH<sub>2</sub>)<sub>2</sub>-O-CH<sub>2</sub>), 3.47 (d, J = 4.8 Hz, 2H, CH<sub>2</sub>CONH), 3.35 – 3.23 (m, 4H, O-CH<sub>2</sub> THP & CH<sub>2</sub>NHCOO), 2.30 (t, J = 6.9 Hz, 2H, CH<sub>2</sub>NHCO), 2.22 – 2.09 (m, 1H, CH THP), 1.90 (q, J = 4.2, 3.5 Hz, 4H, O-CH<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>), 1.48 – 1.30 (m, 13H, O-CH<sub>2</sub>CH<sub>2</sub> THP, CH<sub>3</sub> t Bu).

13C NMR (101 MHz, CDCl<sub>3</sub>) δ 189.84, 172.62, 162.28, 156.07, 146.63, 137.72, 133.48, 131.00, 130.03, 126.24, 123.16, 115.34, 115.12, 113.60, 105.02, 79.55, 70.38, 70.32, 70.25, 70.20, 67.69, 67.51, 56.11, 55.51, 40.48, 39.36, 37.20, 35.86, 30.48, 29.09, 28.48, 22.45.

HRMS-ESI calculated for C<sub>38</sub>H<sub>53</sub>N<sub>3</sub>O<sub>9</sub> [M+Na]<sup>+</sup> 718.3674, found m/z 718.3704.
A solution of 3.15 (15 mg, 21.9 µmol) and TFA (1.1 mL) in DCM (1.1 mL) was reacted as described in the procedure for 2.30a. The crude product was purified by semi-preparative RP-HPLC to yield the TFA salt of 3.16 (17 mg, 20.7 µmol, 95%) as a white solid.

HRMS-ESI calculated for C_{33}H_{46}N_{3}O_{7} [M+H]^+ 596.3330, found m/z 596.3286.
Analytical RP-HPLC R_t = 16.28 min.

The TFA salt of 3.16 (3.4 mg, 4.3 µmol), DIPEA (2.3 µL, 13.0 µmol), acetic anhydride (0.45 µL, 4.8 µmol) and DCM (0.27 mL) were reacted as described in the procedure for 2.31. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 3.17 (2.67 mg, 4.2 µmol, 96%) as a white solid.

HRMS-ESI calculated for C_{35}H_{47}N_{3}NaO_{8} [M+Na]^+ 660.3255, found m/z 660.3193.
Analytical RP-HPLC R_t = 17.97 min.
6-(2-[4-[(E)-2-[2,2-Difluoro-4-(thiophen-2-yl)-1λ4,3-diaza-2λ4-boratricyclo[7.3.0.03,7]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy]acetamido)-N-(2-[2-(2-[2-(3-[(4-methoxybenzoyl)-1-[((oxan-4-yl)methyl]-1H-indol-7-yl]oxy]pentanamido)ethoxy]ethoxy]ethyl)hexanamide (3.18)

The TFA salt of 3.16 (5.88 mg, 7.1 µmol), DIPEA (3.26 µL, 18.7 µmol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 µmol) and DMF (852 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 3.18 (2.01 mg, 1.76 µmol, 93%) as a bright blue solid.

HRMS-ESI calculated for C_{62}H_{71}BF_{2}N_{8}NaO_{10}S [M+Na]^+ 1163.4916, found m/z 1163.4991.
Analytical RP-HPLC R_t = 23.50 min.

tert-Butyl N-(6-hydroxyhexyl)carbamate (3.20)

To a solution of 6-amino-1-hexanol 3.19 (96 mg, 0.82 mmol) in dioxane (1.4 mL), was added Boc\textsubscript{2}O (226 µL, 0.99 mmol). The mixture was stirred for 18 h at rt, then evaporated under reduced pressure. The crude was taken up in EA (8 mL), washed with H\textsubscript{2}O (2 x 8 mL) and sat. aq. NaCl (1 x 6 mL), dried over MgSO\textsubscript{4}, filtered and evaporated under reduced pressure. The crude product was purified by flash silica gel column chromatography (2:1 EA/hexane) to yield 3.20 (141 mg, 0.65 mmol, 79%) as a colourless oil, (R_t 0.58, 3:1 EA/hexane).
1H NMR (400 MHz, CDCl3) δ 4.50 (br s, 1H, NH), 3.64 (t, J = 6.5 Hz, 2H, CH₂OH), 3.11 (t, J = 7.0 Hz, 2H, NHCH₃), 1.62 – 1.27 (m, 18H, CH₂, OH, CH₃ t Bu).

13C NMR (101 MHz, CDCl₃) δ 156.25, 79.37, 62.79, 40.66, 32.69, 30.19, 28.55, 26.50, 25.40.

HRMS-ESI calculated for C₁₁H₂₄NO₃ [M+H]⁺ 218.1751, found m/z 218.1730.

**tert-Butyl N-(6-bromohexyl)carbamate (3.21)**

A solution of 3.20 (139 mg, 0.64 mmol), bromine (33 µL, 0.64 mmol), triphenylphosphine (168 mg, 0.64 mmol) and triethylamine (89 µL, 0.64 mmol) in DCM (1.8 mL) was reacted as described in the procedure for 3.10. The crude product was purified using flash silica column chromatography (4:1 hexane/EA) to yield the desired 3.21 (103 mg, 0.37 mmol, 57%), as a yellow oil (Rf 0.37, 4:1 hexane/EA).

1H NMR (400 MHz, CDCl₃) δ 4.50 (br s, 1H, NH), 3.40 (t, J = 6.8 Hz, 2H, CH₂Br), 3.11 (q, J = 6.7 Hz, 2H, NHCH₃), 1.86 (p, J = 7.0 Hz, 2H, CH₂CH₂Br), 1.54 – 1.40 (m, 13H, CH₂, CH₃ t Bu), 1.39 – 1.27 (m, 2H, CH₂).

13C NMR (101 MHz, CDCl₃) δ 156.12, 79.28, 40.66, 33.91, 32.79, 30.08, 28.56, 27.96, 26.08. The 1H and 13C NMR matched literature reports.³²² HRMS-ESI calculated for C₁₁H₂₂BrNNaO₂ [M+Na]⁺ 302.0726, found m/z 302.0708.

**tert-Butyl N-(6-[[3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-7-yl]oxy]hexyl)carbamate (3.22)**

A solution of 3.4 (22 mg, 61.4 µmol), NaH (7.4 mg, 0.18 mmol) and 3.21 (26 mg, 92.1 µmol) in DMF (2.7 mL) was reacted as described in the procedure for 3.7a. The crude product was purified using flash silica column chromatography (1:1 hexane/EA) to yield the desired 3.22 (27 mg, 47.8 µmol, 79%), as a brown solid (Rf 0.31, 1:1 hexane/EA).
1H NMR (400 MHz, CDCl$_3$) $\delta$ 7.96 (dd, $J = 8.1$, 0.8 Hz, 1H, ArH indole), 7.86 – 7.78 (m, 2H, ArH MeOPh), 7.40 (s, 1H, ArH indole), 7.18 (t, $J = 8.0$ Hz, 1H, ArH indole), 7.02 – 6.95 (m, 2H, ArH MeOPh), 6.73 (dd, $J = 7.9$, 0.9 Hz, 1H, ArH indole), 4.56 (br s, 1H, NH), 4.28 (d, $J = 7.3$ Hz, 2H, N1-CH$_2$), 4.12 (t, $J = 6.5$ Hz, 2H, O-CH$_2$ hexyl), 4.02 – 3.91 (m, 2H, O-CH$_2$ THP), 3.89 (s, 3H, O-CH$_3$), 3.29 (td, $J = 11.7$, 2.5 Hz, 2H, O-CH$_2$ THP), 3.13 (t, $J = 7.0$ Hz, 2H, NHCH$_3$), 2.23 – 2.09 (m, 1H, CH THP), 1.94 – 1.81 (m, 2H, O-CH$_2$CH$_3$ hexyl), 1.61 – 1.23 (m, 19H, (CH$_2$)$_3$, O-CH$_2$CH$_2$ THP & t Bu CH$_3$).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 189.86, 162.32, 156.15, 146.76, 137.66, 133.55, 131.07, 130.06, 126.31, 123.22, 115.46, 115.14, 113.64, 105.05, 79.32, 68.03, 67.57, 56.12, 55.56, 40.71, 37.27, 30.54, 30.28, 29.56, 28.55, 26.72, 26.19.

HRMS-ESI calculated for C$_{33}$H$_{44}$N$_2$NaO$_6$ [M+Na]$^+$ 587.3092, found m/z 587.3057.

6-[[3-(4-Methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-7-yl]oxy]hexan-1-amine (3.23)

A solution of 3.22 (14 mg, 25.7 μmol) and TFA (1 mL) in DCM (1 mL) was reacted as described in the procedure for 2.30a. The crude product was purified by semi-preparative RP-HPLC to yield the TFA salt of 3.23 (16 mg, 23.7 μmol, 92%) as a white solid.

HRMS-ESI calculated for C$_{28}$H$_{37}$N$_2$O$_4$ [M+H]$^+$ 465.2748, found m/z 465.2710. Analytical RP-HPLC $R_t$ = 16.79 min.
\[N-(6-[(3-(4-Methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-7-yl]oxy)hexyl)acetamide \ (3.24)\]

The TFA salt of \textit{3.23} (3.2 mg, 4.6 µmol), DIPEA (2.5 µL, 14.4 µmol), and acetic anhydride (0.5 µL, 5.3 µmol) in DCM (297 µL) were treated as in the procedure for \textit{2.31}. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding \textit{3.24} (2.3 mg, 4.6 µmol, 98%) as a white solid.

HRMS-ESI calculated for C\textsubscript{30}H\textsubscript{38}N\textsubscript{2}NaO\textsubscript{5} [M+Na]\textsuperscript{+} 529.2673, found \textit{m/z} 529.2661.

Analytical RP-HPLC \textit{R}\textsubscript{t} = 20.0 min.

\[6-(2-[(E)-2-[(2,2-Difluoro-4-(thiophen-2-yl)-1\lambda4,3-diaza-2\lambda4-boratricyclo[7.3.0.0\textsubscript{3,7}]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy)acetamido)-N-(6-[(3-(4-methoxybenzoyl)-1-[(oxan-4-yl)methyl]-1H-indol-7-yl]oxy)hexyl)hexanamide \ (3.25)\]

The TFA salt of \textit{3.23} (6.05 mg, 8.73 µmol), DIPEA (3.90 µL, 22.4 µmol), BODIPY 630/650-X-OSu \textit{2.15} (1.25 mg, 1.89 µmol) and DMF (862 µL) were reacted as described in the procedure for \textit{2.32a}. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding \textit{3.25} (1.46 mg, 1.45 µmol, 77%) as a bright blue solid.
HRMS-ESI calculated for C\textsubscript{57}H\textsubscript{62}BF\textsubscript{2}N\textsubscript{5}NaO\textsubscript{7}S [M+Na]\textsuperscript{+} 1032.4333, found m/z 1032.4411.

Analytical RP-HPLC R\textsubscript{t} = 24.73 min.

2-Amino-5-bromopyridine-3-carbaldehyde (4.13)

To a solution of 2-amino-3-pyridinecarbaldehyde 4.12 (3.66 g, 30.0 mmol) in glacial acetic acid (70 mL) was added a solution of bromine (1.54 mL, 30.0 mmol) in acetic acid (20 mL) dropwise. The reaction mixture was stirred for 24 h and the precipitate formed was filtered and washed with diethyl ether. The precipitate was then added to water and treated with solid NaOH until pH 7-8, then extracted with DCM, dried over MgSO\textsubscript{4} and evaporated under reduced pressure. The crude was purified by recrystallisation in ACN, to yield 4.13 (3.68 g, 18.3 mmol, 61%), as a canary yellow solid.

\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}) \text{\delta} 9.83 (s, 1H, CHO), 8.31 (d, J = 2.6 Hz, 1H, ArH), 8.24 (d, J = 2.6 Hz, 1H, ArH), 7.69 (br s, 2H, NH\textsubscript{2}).

\textsuperscript{13}C NMR (101 MHz, DMSO-\textit{d}\textsubscript{6}) \text{\delta} 192.91, 156.79, 155.01, 145.72, 114.17, 104.29.

HRMS-ESI calculated for C\textsubscript{6}H\textsubscript{6}BrN\textsubscript{2}O [M+H]\textsuperscript{+} 200.9658, found m/z 200.9661.

Ethyl 6-bromo-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylate (4.14)

To a stirred solution of 4.13 (4.49 g, 22.33 mmol) in EtOH (55 mL) were added diethyl malonate (5.1 mL, 33.6 mmol) and piperidine (0.63 mL, 6.4 mmol). The mixture was stirred under reflux for 18 h and the resulting solid filtered, washed with EtOH and dried, to yield 4.14 (6.22 g, 20.9 mmol, 94%), as a yellow solid, which was used without further purification.

\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}) \text{\delta} 12.63 (br s, 1H, NH), 8.70 (d, J = 2.4 Hz, 1H, ArH), 8.55 (d, J = 2.4 Hz, 1H, ArH), 8.44 (s, 1H, ArH), 4.28 (q, J = 7.1 Hz, 2H, CH\textsubscript{2}), 1.30 (t, J = 7.1 Hz, 3H, CH\textsubscript{3}). This matches the literature reference.\textsuperscript{145}

\textsuperscript{13}C NMR (101 MHz, DMSO-\textit{d}\textsubscript{6}) \text{\delta} 163.78, 159.11, 152.78, 149.10, 141.27, 139.55,
125.69, 114.50, 112.43, 61.07, 14.09.
HRMS-ESI calculated for C₁₁H₉BrN₂NaO₃ [M+Na]^+ 318.9689, found m/z 318.9665.

6-Bromo-N-(4-methylcyclohexyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (4.15)

In a sealed tube, 4.14 (424 mg, 1.4 mmol) and 4-methylcyclohexylamine (equal mixture of cis and trans isomers) (0.94 mL, 7.1 mmol) were heated to 150°C for 24 h. Upon cooling, diethyl ether was added to the reaction mixture and the solid residue was collected by filtration. The crude product was purified by precipitation in EA, to give 4.15 (376 mg, 1.0 mmol, 72%) as a white solid.

HRMS-ESI calculated for C₁₆H₁₈BrN₃NaO₂ [M+Na]^+ 386.0475, found m/z 386.0450.

6-Bromo-N-(4-methylcyclohexyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (4.8)

To a stirred solution of 4.15 (22 mg, 59.9 µmol) in DMF (1 mL) was added caesium carbonate (55 mg, 0.17 mmol). After stirring for 1 h, 4-(2-chloroethyl)morpholin hydrochloride (22 mg, 0.12 mmol) was added. The mixture was stirred at 50°C for 12 h and upon cooling was evaporated under reduced pressure. Saturated aq. NaHCO₃ was added until pH 10-11 and then extracted with DCM (3 x 20 mL). The combined organics were washed with H₂O (1 x 60 mL), dried over MgSO₄ and evaporated under reduced pressure. The crude product was purified by recrystallisation in ACN to yield 4.8 (10.5 mg, 22.0 µmol, 37%), as a yellow solid.

¹H NMR (400 MHz, CDCl₃) δ 9.80 (m, 1H, NH isomer A), 9.41 (m, 1H, NH isomer B) 8.80 (s, 1H, ArH), 8.69 (d, J = 2.3 Hz, 1H, ArH), 8.19 (t, J = 2.1 Hz, 1H, ArH), 5.04 – 4.58 (m, 2H, N1-CH₂), 4.32 – 3.91 (m, 1H, NHCH isomer A and NHCH isomer B), 3.92 – 3.55 (m, 4H, N-CH₂ morpholino), 3.40 – 2.22 (m, 6H, N1-CH₂CH₂ & O-CH₂
morpholino), 2.12 – 0.88 (m, 12H, CH & CH₂ cyclohexyl, CH₃). Isomers A and B represent cis/trans (in no defined order) isomers. This matches the literature reference.¹⁴⁵

¹³C NMR (101 MHz, CDCl₃) δ 162.50, 162.36, 161.26, 152.67, 152.61, 148.21, 141.02, 140.10, 124.54, 116.46, 55.51, 53.40, 49.06, 45.76, 33.99, 33.07, 32.11, 31.24, 30.25, 29.84, 29.74, 22.36, 21.80. Isomers A and B resolved as separate peaks in some instances, but are not assigned.

HRMS-ESI calculated for C₂₂H₃₀BrN₄O₃ [M+H]⁺ 477.1496, found m/z 477.1462. Analytical RP-HPLC Rₜ = 15.57 min (57%) and 15.84 min (43%).

Methyl 5-{6-bromo-3-[(4-methylcyclohexyl)carbamoyl]-2-oxo-1,2-dihydro-1,8-naphthyridin-1-yl}pentanoate (4.9)

To a stirred solution of 4.15 (319 mg, 0.88 mmol) in DMF (4 mL), was added caesium carbonate (799 mg, 2.5 mmol). After stirring for 1 h, methyl 5-bromovalerate (0.25 mL, 1.8 mmol) was added. The mixture was stirred at 50°C for 12 h and upon cooling was evaporated under reduced pressure. The solid was taken up in ACN and filtered. The filtrate was evaporated and then purified by recrystallisation in ACN to yield 4.9 (94 mg, 0.20 mmol, 22%), as a white solid.

¹H NMR (400 MHz, CDCl₃) δ 9.93 (d, J = 7.8 Hz, 1H, NH isomer A), 9.55 (d, J = 8.1 Hz, 1H, NH isomer B), 8.79 (s, 1H, ArH), 8.70 (d, J = 2.4 Hz, 1H, ArH), 8.17 (dd, J = 3.5, 2.4 Hz, 1H, ArH), 4.65 – 4.46 (m, 2H, N-CH₂), 4.25 (m, 1H, NHCH isomer A), 3.9 (m, 1H, NHCH isomer B), 3.67 (s, 3H, O-CH₃), 2.47 – 2.34 (m, 2H, CH₂COOMe), 2.11 – 0.87 (m, 16H, N-CH₂(CH₂)₂, CH & CH₂ cyclohexyl & CH₃). Isomers A and B represent cis/trans (in no defined order) isomers.

¹³C NMR (101 MHz, CDCl₃) δ 173.92, 173.87, 162.34, 162.29, 161.55, 161.53, 152.77, 152.72, 148.34, 148.28, 140.76, 140.58, 139.83, 139.79, 124.47, 124.38, 116.25, 116.24, 114.27, 114.21, 51.71, 51.70, 49.03, 45.83, 41.80, 41.72, 34.01, 33.83, 33.82, 33.07, 32.12, 31.21, 30.30, 29.72, 27.48, 27.47, 22.47, 22.45, 22.36, 21.67. Isomers A and B resolved as separate peaks in some instances, but are not assigned.
Methyl 4-({6-bromo-3-[4-methylcyclohexyl]carbamoyl}-2-oxo-1,2-dihydro-1,8-naphthyridin-1-yl}methyl)benzoate (4.10)

A solution of 4.15 (75 mg, 0.23 mmol), caesium carbonate (206 mg, 0.63 mmol) and methyl 4-bromomethylbenzoate (104 mg, 0.45 mmol) in DMF (1.1 mL) was reacted as described in the procedure for 4.9. The crude residue was purified by flash silica gel column chromatography (99:1 DCM/MeOH) and a portion (6 mg) of the solid obtained (20 mg) was further purified by semi-preparative RP-HPLC to yield 4.10 (3.54 mg, 6.93 µmol), as a white solid.

$^1$H NMR (400 MHz, CDCl$_3$) δ 9.92 – 9.75 (m, 1H, NH isomer A), 9.49 – 9.38 (m, 1H, NH isomer B), 8.84 (s, 1H, ArH), 8.73 – 8.65 (m, 1H, ArH), 8.22 – 8.16 (m, 1H, ArH), 8.00 – 7.89 (m, 2H, ArH Ph), 7.48 – 7.38 (m, 2H, ArH Ph), 6.95 (d, J = 8.6 Hz, 2H, N1-CH$_2$), 4.37 – 4.16 (m, 1H, NHCH), 2.11 – 0.89 (m, 15H, CH & CH$_2$ cyclohexyl, CH$_3$).

Isomers A and B represent cis/trans (in no defined order) isomers.

HRMS-ESI calculated for C$_{25}$H$_{26}$BrN$_3$NaO$_4$ [M+Na]$^+$ 534.0999, found m/z 534.1008. Analytical RP-HPLC $R_t$ = 24.16 min (50%) and 24.44 min (50%).

Methyl 5-[6-(4-methoxyphenyl)-3-[4-methylcyclohexyl]carbamoyl]-2-oxo-1,2-dihydro-1,8-naphthyridin-1-yl]pentanoate (4.11)

4.9 (16 mg, 32.8 µmol), 4-methoxyphenylboronic acid (6.5 mg, 42.7 µmol) and NaN$_2$CO$_3$ (9.1 mg, 85.4 µmol) were dissolved in a 1:4 v:v mixture of H$_2$O and DMF (1.5 mL). Pd(OAc)$_2$ (0.08 mg, 0.33 µmol) was added and the reaction mixture heated to 110°C and stirred for 3 h, then cooled to rt and diluted with H$_2$O (1.5 mL). The aqueous phase was extracted with EA (4 x 1.5 mL) and the
combined organics washed with H₂O (3 x 6 mL) and sat. aq. NaCl (1 x 6 mL), dried over MgSO₄ and evaporated under reduced pressure. The crude product was purified by flash silica gel column chromatography (2:1 hexane/EtOAc) to yield the desired 4.11 (9.2 mg, 18.1 µmol, 55%), as a yellow solid (Rf 0.5, 1:1 hexane/EtOAc).

¹H NMR (400 MHz, CDCl₃) δ 10.04 (d, J = 7.8 Hz, 1H, NH isomer A), 9.67 (d, J = 8.0 Hz, 1H, NH isomer B), 8.91 (s, 1H, ArH), 8.89 (d, J = 2.4 Hz, 1H, ArH), 8.19 – 8.12 (m, 1H, ArH), 7.63 – 7.50 (m, 2H, ArH MeOPh), 7.11 – 6.99 (m, 2H, ArH MeOPh), 4.73 – 4.54 (m, 2H, N-CH₂), 4.26 (m, 1H, NHCH isomer A), 3.93 (m, 1H, NHCH isomer B), 3.88 (m, 1H, NHC₃H isomer B), 3.67 (m, 1H, COOC₃H isomer A), 2.52 – 2.40 (m, 2H, CH₂COOMe), 2.36 (m, 1H, CHCH₃ isomer A), 2.09 (m, 1H, CHCH₃ isomer B), 1.90 – 0.87 (m, 15H, N-CH₂(CH₂)₂, CH₂ cyclohexyl, CH₃). Isomers A and B represent cis/trans (in no defined order) isomers.

¹³C NMR (101 MHz, CDCl₃) δ 174.04, 173.99, 162.56, 162.52, 162.10, 160.07, 160.06, 150.63, 150.58, 148.57, 148.51, 142.07, 141.89, 135.46, 135.43, 132.28, 132.22, 128.96, 128.92, 128.27, 123.56, 123.47, 114.93, 114.83, 55.58, 51.73, 51.70, 48.94, 45.80, 41.66, 41.57, 34.21, 34.05, 33.93, 33.91, 33.61, 33.11, 32.14, 31.20, 30.34, 29.75, 28.21, 27.61, 22.56, 22.53, 22.39, 21.65, 21.36. Isomers A and B resolved as separate peaks in some instances, but are not assigned.

HRMS-ESI calculated for C₂₉H₃₅N₃NaO₅ [M+Na]⁺ 528.2469, found m/z 528.2475.

Analytical RP-HPLC R₁ = 24.33 min (50%) and 25.08 min (50%).

5-{6-Bromo-3-[(4-methylcyclohexyl)carbamoyl]-2-oxo-1,2-dihydro-1,8-naphthyridin-1-yl}pentanoic acid (4.16)

A solution of 4.9 (60 mg, 0.13 mmol) and 10% NaOH (10 mL) was heated to 110°C for 5 h. The mixture was cooled and conc. HCl added until pH 2-3. The precipitate was filtered and treated with diethyl ether to yield 4.16 (25 mg, 54.5 µmol, 43%) as a white solid (Rf 0.34, 1:1 hexane/EtOAc).
$^1$H NMR (400 MHz, CDCl$_3$) δ 9.95 (d, $J = 7.7$ Hz, 1H, NH isomer A), 9.57 (d, $J = 7.9$ Hz, 1H, NH isomer B), 8.81 (s, 1H, ArH), 8.70 (d, $J = 2.1$ Hz, 1H, ArH), 8.18 (d, $J = 2.1$ Hz, 1H, ArH), 4.64 – 4.46 (m, 2H, N-CH$_2$), 4.25 (m, 1H, NHCH isomer A), 3.89 (m, 1H, NHCH isomer B), 2.56 – 2.35 (m, 2H, CH$_2$COOH), 2.15 – 0.87 (m, 16H, N-CH$_2$(CH$_2$)$_2$, CH & CH$_2$ cyclohexyl & CH$_3$). Isomers A and B represent cis/trans (in no defined order) isomers.

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 178.34, 178.28, 162.35, 162.30, 161.70, 161.69, 152.84, 152.80, 148.31, 148.28, 140.98, 140.81, 139.92, 139.88, 124.30, 124.21, 116.26, 116.25, 114.34, 114.28, 49.11, 45.91, 41.75, 41.67, 33.99, 33.65, 33.64, 33.04, 32.10, 31.20, 30.28, 29.84, 29.71, 27.40, 22.36, 22.21, 21.19, 21.18. Isomers A and B resolved as separate peaks in some instances, but are not assigned.

HRMS-ESI calculated for C$_{21}$H$_{26}$BrN$_3$NaO$_4$ [M+Na]$^+$ 486.0999, found m/z 486.1007.

tert-Butyl N-[8-(5-{6-bromo-3-[(4-methylcyclohexyl)carbamoyl]-2-oxo-1,2-dihydro-1,8-naphthyridin-1-yl}pentanamido)octyl]carbamate (4.17a)

4.16 (13 mg, 27.8 µmol), 2.28a (22 mg, 88.8 µmol), DIPEA (29 µL, 0.17 mmol) and HATU (11 mg, 27.8 µmol) in DMF (3.5 mL) were used as described in the procedure for 2.29a. The crude residue was purified by flash silica gel column chromatography (1:1 hexane/EtAl) to yield 4.17a (14 mg, 19.6 µmol, 71%) as a grey solid (R$_f$ 0.484 1:2 hexane/EtAl).

$^1$H NMR (400 MHz, MeOD-$d_4$) δ 10.33 (d, $J = 7.9$ Hz, 1H, NH isomer A), 9.89 (d, $J = 7.9$ Hz, 1H, NH isomer B), 8.83 – 8.75 (m, 2H, ArH), 8.52 (dd, $J =$ 4.2, 2.4 Hz, 1H, ArH), 4.65 – 4.51 (m, 2H, N1-CH$_2$), 4.21 (m, 1H, NHCH isomer A), 3.81 (m, 1H, NHCH isomer B), 3.14 (t, $J =$ 7.0 Hz, 2H, CH$_2$), 3.04 – 2.86 (m, 2H, CH$_2$), 2.32 – 2.20 (m, 2H, CH$_2$), 2.10 – 0.90 (m, 37H, CH, CH$_2$, CH$_3$). Isomers A and B represent cis/trans (in no defined order) isomers.

$^{13}$C NMR (101 MHz, MeOD-$d_4$) δ 175.66, 163.62, 163.41, 154.11, 149.57, 142.26, 141.71, 124.66, 117.54, 117.51, 115.28, 115.24, 79.74, 50.33, 49.64, 49.43, 49.21,
Isomers A and B resolved as separate peaks in some instances, but are not assigned.

HRMS-ESI calculated for C_{34}H_{52}BrN_{5}NaO_{5} [M+Na]^+ 712.3044, found m/z 712.3065.

tert-Butyl N-(2-{2-[5-{6-bromo-3-[4-methylcyclohexyl]carbamoyl}-2-oxo-1,2-dihydro-1,8-naphthyridin-1-yl]pentanamido}ethoxy)ethoxy)ethyl)carbamate (4.17b)

**4.16** (8.9 mg, 19.2 µmol), DIPEA (20.1 µL, 0.12 mmol), HATU (7.3 mg, 19.2 µmol) and **2.28b** (15 mg, 62.0 µmol) in DMF (1.8 mL) were used as described in the procedure for **2.29a**. The crude product was purified by flash silica gel column chromatography (1:7 hexane/EtOAc) to yield **4.17b** (10 mg, 14.7 µmol, 77%) as a grey solid (R_f 0.19 1:2 hexane/EtOAc).

^{1}H NMR (400 MHz, CDCl₃) δ 9.95 (d, J = 7.7 Hz, 1H, NH isomer A), 9.57 (d, J = 8.0 Hz, 1H, NH isomer B), 8.76 (s, 1H, ArH), 8.69 (d, J = 2.1 Hz, 1H, ArH), 8.20 – 8.12 (m, 1H, ArH), 6.16 (s, 1H, NH), 4.98 (s, 1H, NH), 4.62 – 4.43 (m, 2H, N1-CH₂), 4.22 (m, 1H, NHCH isomer A), 3.87 (m, 1H, NHCH isomer B), 3.74 – 3.38 (m, 10H, CH₂), 3.36 – 3.20 (br m, 2H, CH₂), 2.36 – 2.21 (br m, 2H, CH₂), 2.10 – 0.85 (m, 25H, CH, CH₂, CH₃).

Isomers A and B represent cis/trans (in no defined order) isomers.

^{13}C NMR (101 MHz, CDCl₃) δ 162.31, 162.27, 161.65, 152.89, 152.86, 148.27, 140.83, 140.66, 139.86, 139.82, 124.33, 124.24, 116.24, 114.31, 114.25, 70.47, 70.36, 70.25, 70.10, 49.07, 45.88, 41.87, 39.60, 36.14, 33.99, 33.07, 32.11, 30.29, 29.71, 28.55, 27.53, 23.33, 22.36, 21.69. Isomers A and B resolved as separate peaks in some instances, but are not assigned.

HRMS-ESI calculated for C_{32}H_{49}BrN_{5}O_{7} [M+H]^+ 694.2810, found m/z 694.2870.
1-{4-[\(8\text{-Aminoctyl}\)carbamoyl]butyl}-6-bromo-\(N\)-(4-methylcyclohexyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (4.18a)

A solution of 4.17a (11 mg, 16.1 μmol) and TFA (0.9 mL) in DCM (0.9 mL) was reacted as described in the procedure for 2.30a. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of 4.18a (11.9 mg, 14.6 μmol, 91%) as a white solid.

HRMS-ESI calculated for \(C_{29}H_{45}BrN_5O_3\) [M+H]^\+ 590.2700, found \(m/z\) 590.2703.

Analytical RP-HPLC \(R_t = 18.12\) min (40%) and 18.27 min (60%).

1-{4-\([2-\{2\text{-Aminoethoxy}\}ethoxy\}ethyl\)carbamoyl]butyl}-6-bromo-\(N\)-(4-methylcyclohexyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (4.18b)

A solution of 4.17b (8.0 mg, 11.5 μmol) and TFA (1.2 mL) in DCM (1.2 mL) was reacted as described in the procedure for 2.30a. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of 4.18b (8.1 mg, 9.8 μmol, 86%) as a white solid.

HRMS-ESI calculated for \(C_{27}H_{41}BrN_5O_5\) [M+H]^\+ 594.2286, found \(m/z\) 594.2294.

Analytical RP-HPLC \(R_t = 16.84\) min (51%) and 17.01 min (49%).
6-Bromo-1-{4-[(8-acetamidoctyl)carbamoyl]butyl}-N-(4-methylcyclohexyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (4.19a)

The TFA salt of 4.18a (2.4 mg, 2.9 µmol), DIPEA (1.9 µL, 10.9 µmol), and acetic anhydride (0.28 µL, 3.0 µmol) in DCM (516 µL) were treated as in the procedure for 2.31. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 4.19a (1.76 mg, 2.8 µmol, 95%) as a white solid.

HRMS-ESI calculated for C₃₁H₄₆BrN₅NaO₄ [M+Na]+ 654.2625, found m/z 654.2612.
Analytical RP-HPLC Rₜ = 21.04 min (40%) and 21.20 min (60%).

6-Bromo-1-{4-[(2-[2-(2-acetamidoethoxy)ethoxy]ethyl)carbamoyl]butyl}-N-(4-methylcyclohexyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (4.19b)

The TFA salt of 4.18b (1.53 mg, 1.9 µmol), DIPEA (1.2 µL, 6.9 µmol), and acetic anhydride (0.18 µL, 1.9 µmol) in DCM (437 µL) were treated as in the procedure for 2.31. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 4.19b (1.14 mg, 1.8 µmol, 96%) as a white solid.

HRMS-ESI calculated for C₂₉H₄₂BrN₅NaO₆ [M+Na]+ 658.2211, found m/z 658.2191.
Analytical RP-HPLC Rₜ = 18.90 min (53%) and 19.11 min (47%).
6-Bromo-1-{4-[(8-[6-(2-[(E)-2-2,2-difluoro-4-(thiophen-2-yl)-1λ4,3-diaza-2λ4-boratricyclo[7.3.0.0^3^7]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy)acetamido]hexanamido}octyl]carbamoyl}butyl]-N-(4-methycyclohexyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (4.20a)

The TFA salt of 4.18a (4.9 mg, 6.0 µmol), DIPEA (2.6 µL, 14.9 µmol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 µmol) and DMF (842 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 4.20a (1.98 mg, 1.7 µmol, 92%) as a bright blue solid.

HRMS-ESI calculated for C_{58}H_{70}BBrF_{2}N_{8}NaO_{6}S [M+Na]^+ 1157.4285, found m/z 1157.4175.
Analytical RP-HPLC R_t = 25.44 min (cis trans isomers resolved as one peak in standard analytical method).
6-bromo-1-(4-([2-([2-([6-([4-([E]-[2,2-difluoro-4-(thiophen-2-yl)-1\text{\textsuperscript{1}}4,3-diaza-2\text{\textsuperscript{2}}4-boratricyclo[7.3.0.0\text{\textsuperscript{3,7}}]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy)acetamido]hexanamido]ethoxy)ethoxy)ethyl]carbamoyl)butyl)\text{-N-(4-methylcyclohexyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (4.20b)

The TFA salt of 4.18b (5.32 mg, 6.5 μmol), DIPEA (2.8 μL, 15.9 μmol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 μmol) and DMF (844 μL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 4.20b (1.9 mg, 1.7 μmol, 88%) as a bright blue solid.

HRMS-ESI calculated for C\text{\textsubscript{56}}H\text{\textsubscript{66}}BBr\text{\textsubscript{2}}N\text{\textsubscript{8}}NaO\text{\textsubscript{8}}S [M+Na]\text{\textsuperscript{+}} 1161.3870, found \textit{m/z} 1161.3780.

Analytical RP-HPLC \textit{R}t = 24.39 min (55%) and 24.50 min (45%).
Mixture of Ethyl 6-bromo-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylate (5.10a) and 6-bromo-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylic acid (5.10b)

To a stirred solution of 4.14 (6.08 g, 20.5 mmol) in anhydrous DMF (150 mL) was added caesium carbonate (18.67 g, 57.3 mmol). The reaction mixture was stirred for 1 h at rt, then 4-(2-chloroethyl)morpholinhydrochloride (7.62 g, 40.1 mmol) was added and the mixture was heated to 50°C and stirred for 12 h. The DMF was evaporated under reduced pressure and then saturated aq. NaHCO₃ (80 mL) was added to the residue which was then extracted with DCM (3 x 80 mL). The combined organics were washed with H₂O (2 x 80 mL), dried over MgSO₄, filtered and evaporated under reduced pressure. The crude was purified by precipitation in ACN, yielding a 1:3 mixture (5.17 g) of the ethyl formate 5.10a and carboxylic acid 5.10b, as a yellow solid.

Characterisation of the ethyl formate 5.10a (yielded at 41% in smaller scale reaction):

$^1$H NMR (400 MHz, CDCl₃) δ 8.66 (d, $J = 2.4$ Hz, 1H, ArH), 8.24 (s, 1H, ArH), 8.07 (d, $J = 2.4$ Hz, 1H, ArH), 4.66 (t, 2H, N1-CH₂), 4.42 (q, $J = 7.1$ Hz, 2H, O-CH₂), 3.66 (t, $J = 4.6$ Hz, 4H, O-CH₂ morpholino), 2.70 (t, $J = 7.2$ Hz, 2H, N1-CH₂CH₂), 2.60 (t, $J = 4.8$ Hz, 4H, N-CH₂ morpholino), 1.40 (t, $J = 7.1$ Hz, 3H, CH₃).

$^{13}$C NMR (101 MHz, CDCl₃) δ 164.17, 158.99, 152.77, 149.16, 140.38, 139.45, 125.56, 115.37, 113.58, 67.02, 62.08, 55.80, 53.94, 38.89, 14.38.

MS-ESI calculated for C₁₇H₂₁BrN₃O₄ [M+H]$^+$ 410.0715, found 410.1.
Mixture of Ethyl 6-(4-methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylate (5.11a) and 6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylic acid (5.11b)

A 1:3 mixture (4.07 g) of the ethyl formate 5.10a and carboxylic acid 5.10b, 4-methoxyphenylboronic acid (2.03 g, 13.3 mmol), Na₂CO₃ (2.83 g, 26.7 mmol) and Pd(OAc)$_2$ (23 mg, 0.10 mmol) in H₂O (25 mL) and DMF (100 mL) were reacted as described in the procedure for 4.11. Upon completion of the reaction, aq. HCl was added until the reaction mixture was pH 1-2, then diluted with H₂O (100 mL) and extracted with DCM (3 x 100 mL). The combined organics were washed with H₂O (2 x 150 mL) and sat. aq. NaCl (1 x 150 mL), dried over MgSO₄, filtered and rotary evaporated. The crude residue was washed with EtOH and filtered and the solid dried under reduced pressure yielding a 1:15 mixture (1.99 g) of the ethyl formate 5.11a and carboxylic acid 5.11b, as a yellow solid.

6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylic (5.11b)

A stirred solution of the 1:15 mixture of 5.11a and 5.11b (1.9 g) in THF (30 mL) was cooled to 0°C and 0.2 M lithium hydroxide monohydrate solution (49 mL) added dropwise. The reaction mixture was stirred at 0°C for 1 h and then quenched with a biphase of 0.2 M aq. HCl/EA (1:1 v:v, 200 mL) was added. The aqueous layer was extracted with DCM (10 x 100 mL), dried over MgSO₄, filtered and evaporated under reduced pressure to yield 5.11b (1.66 g) as a yellow solid.
$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.97 (d, $J = 2.4$ Hz, 1H, ArH), 8.93 (s, 1H, ArH), 8.22 (d, $J = 2.4$ Hz, 1H, ArH), 7.58 – 7.51 (m, 2H, ArH MeOPh), 7.08 – 7.01 (m, 2H, ArH MeOPh), 4.86 (t, $J = 6.8$ Hz, 2H, N1-CH$_2$), 3.87 (s, 3H, O-CH$_3$), 3.77 – 3.64 (m, 4H, O-CH$_2$ morpholino), 2.93 – 2.83 (m, 2H, N1-CH$_2$CH$_2$), 2.82 – 2.56 (m, 4H, N-CH$_2$ morpholino).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 164.63, 164.56, 160.41, 152.08, 148.49, 144.86, 135.94, 133.49, 128.33, 128.14, 119.28, 115.08, 115.07, 66.93, 56.00, 55.59, 53.96, 39.37.

HRMS-ESI calculated for C$_{22}$H$_{24}$N$_3$O$_5$ [M+H]$^+$ 410.1710, found m/z 410.1701.

**trans-N-(4-Hydroxycyclohexyl)-6-(4-methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5.1a)**

To a stirred solution of **5.11b** (800 mg, 2.0 mmol) in anhydrous DMF (40 mL) was added DIPEA (1 mL, 5.9 mmol) and HATU (743 mg, 2.0 mmol). The reaction mixture was stirred for 5 min, then *trans*-4-aminocyclohexanol (675 mg, 5.9 mmol) and DIPEA (1 mL, 5.9 mmol) was added and the mixture stirred for 42 h. The DMF was evaporated under reduced pressure and the residue taken up in H$_2$O (50 mL) and extracted with DCM (3 x 50 mL). The combined organics were washed with H$_2$O (2 x 60 mL), dried over MgSO$_4$, filtered and evaporated under reduced pressure. The crude was purified by precipitation in ACN, yielding **5.1a** (815 mg, 1.6 mmol, 82%) as a yellow solid.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.72 (d, $J = 7.9$ Hz, 1H, NH), 8.91 (s, 1H, ArH), 8.89 (d, $J = 2.4$ Hz, 1H, ArH), 8.17 (d, $J = 2.4$ Hz, 1H, ArH), 7.60 – 7.51 (m, 2H, ArH MeOPh), 7.10 – 7.00 (m, 2H, ArH MeOPh), 4.83 – 4.71 (m, 2H, N-CH$_2$), 4.07 – 3.93 (m, 1H, CH), 3.87 (s, 3H, O-CH$_3$), 3.75 – 3.64 (m, 5H, CH & O-CH$_2$ morpholino), 2.78 – 2.69 (m, 2H, N-CH$_2$), 2.68 – 2.57 (m, 4H, N-CH$_2$ morpholino), 2.18 – 2.09 (m, 2H, CH$_2$), 2.09 – 1.99 (m, 2H, CH$_2$), 1.54 – 1.36 (m, 4H, CH$_2$).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 162.57, 162.30, 160.11, 150.64, 148.65, 142.24, 135.44, 132.36, 128.85, 128.26, 123.24, 114.95, 114.78, 69.93, 67.15, 56.04, 55.58, 54.05, 47.94, 39.13, 34.04, 30.67.
HRMS-ESI calculated for C_{28}H_{35}N_{4}O_{5} [M+H]^+ 507.2602, found m/z 507.2571.
Analytical RP-HPLC R_t = 13.85 min.

cis-N-(4-Hydroxycyclohexyl)-6-(4-methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5.1b)

A stirred solution of 5.11b (800 mg, 2.0 mmol), DIPEA (2 mL, 11.8 mmol), HATU (743 mg, 2.0 mmol), and cis-4-aminocyclohexanol hydrochloride (889 mg, 5.9 mmol) in DMF (40 mL) was reacted as described in the procedure for 5.1a. Upon completion, the reaction mixture was diluted with NaHCO_3 (50 mL) and the aqueous phase extracted with DCM (4 x 50 mL). The combined organics were washed with H_2O (2 x 60 mL), dried over MgSO_4, filtered and evaporated under reduced pressure. The crude was purified by precipitation in ACN, yielding 5.1b (784 mg, 1.5 mmol, 79%) as a yellow solid.

^1^H NMR (400 MHz, CDCl_3) δ 9.95 (d, J = 7.8 Hz, 1H, NH), 8.91 (s, 1H, ArH), 8.88 (d, J = 2.4 Hz, 1H, ArH), 8.16 (d, J = 2.4 Hz, 1H, ArH), 7.55 (d, J = 8.5 Hz, 2H, ArH MeOPh), 7.04 (d, J = 8.5 Hz, 2H, ArH MeOPh), 4.78 (t, J = 7.1 Hz, 2H, N-CH_2), 4.18 – 4.08 (m, 1H, CH), 3.96 – 3.88 (m, 1H, CH), 3.87 (s, 3H, O-CH_3), 3.69 (t, J = 4.6 Hz, 4H, O-CH_2 morpholino), 2.74 (t, J = 7.1 Hz, 2H, N-CH_2), 2.63 (t, J = 4.5 Hz, 4H, N-CH_2 morpholino), 1.94 – 1.68 (m, 8H, CH_2).

^13^C NMR (101 MHz, CDCl_3) δ 162.56, 162.15, 160.08, 150.57, 148.68, 142.09, 135.40, 132.27, 128.89, 128.24, 123.35, 114.93, 114.77, 67.23, 67.16, 56.04, 55.57, 54.04, 46.58, 39.09, 31.50, 27.64.

HRMS-ESI calculated for C_{28}H_{35}N_{4}O_{5} [M+H]^+ 507.2602, found m/z 507.2552.
Analytical RP-HPLC R_t = 14.13 min.

7-[(tert-Butoxy)carbonyl]amino]heptanoic acid (5.19)

A stirred solution of 7-aminoheptanoic acid 5.18 (100 mg, 0.69 mmol) and NaOH (28 mg, 0.69 mmol) in
dioxane and H₂O (2:1, 3 mL) was cooled to 0°C and a solution of Boc₂O (165 mg, 0.76 mmol) in dioxane (1.5 mL) was slowly added. The mixture was stirred for 15 h at rt, then evaporated under reduced pressure. The resulting residue was taken up in H₂O (3 mL) and extracted with EA (2 x 2 mL). The aqueous phase was acidified to pH 4-5 with aqueous 1 M HCl and extracted with EA (3 x 3 mL). The combined organics were dried over MgSO₄, filtered and evaporated under reduced pressure to yield 5.19 (102 mg, 0.42 mmol, 60%) as a colourless oil.

^1^H NMR (400 MHz, CDCl₃) δ 10.69 (s, 1H, COOH), 4.55 (s, 1H, NH), 3.16 – 2.98 (m, 2H, CH₂NH), 2.33 (t, J = 7.5 Hz, 2H, CH₂COOH), 1.62 (quint, J = 7.4 Hz, 2H, CH₂), 1.52 – 1.25 (m, 15H, CH₂ and CH₃).

^1^C NMR (101 MHz, CDCl₃) δ 179.35, 156.17, 79.29, 40.62, 34.09, 29.97, 28.83, 28.55, 26.54, 24.74.

HRMS-ESI calculated for C₁₂H₂₃NaO₄ [M+Na]^+ 268.1519, found m/z 268.1500.

trans-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 2-[(tert-butoxy)carbonyl]amino]acetate (5.20a)

A stirred solution of Boc-glycine (10 mg, 59 µmol) and TFFH (16 mg, 59 µmol) in anhydrous DCM (1.6 mL) was cooled to 0°C and Et₃N (41 µL, 0.30 mmol) was added. The mixture was warmed to rt and stirred for 30 min and then 5.1a (30 mg, 59 µmol) and DMAP (0.7 mg, 5.9 µmol) were added. The mixture was stirred for 48 h at rt and then evaporated under reduced pressure. The residue was taken up in EA (3 mL) and washed with H₂O (3 x 3 mL) and sat. aq. NaCl (1 x 3 mL), dried over MgSO₄, filtered and evaporated under reduced pressure. The crude residue was purified by flash silica column chromatography (100% EA) to yield 5.20a (8.5 mg, 12.8 µmol, 22%) as a yellow solid.
\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 9.71 (d, \(J = 7.9\) Hz, 1H, NH), 8.97 – 8.86 (m, 2H, ArH), 8.17 (d, \(J = 2.4\) Hz, 1H, ArH), 7.61 – 7.52 (m, 2H, ArH MeOf), 7.11 – 7.00 (m, 2H, ArH MeOf), 5.05 – 4.98 (m, 1H, NH), 4.51 – 4.33 (m, 1H, CH), 4.07 – 3.91 (m, 2H, CH), 3.87 (s, 3H, O-CH\(_3\)), 3.74 – 3.64 (m, 5H, CH, N-CH\(_2\) morpholino), 2.76 (t, \(J = 7.2\) Hz, 2H, N1-CH=CH), 2.66 (t, \(J = 4.5\) Hz, 4H, O-CH\(_2\) morpholino), 2.19 – 1.99 (m, 4H, CH), 1.70 – 1.06 (m, 13H, CH, tBu CH\(_3\)).

\(^13\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 171.12, 162.56, 162.31, 160.13, 150.68, 148.61, 142.31, 135.48, 132.41, 128.84, 128.27, 123.22, 114.96, 114.80, 84.41, 69.94, 66.98, 55.91, 55.58, 53.90, 47.95, 47.47, 38.91, 34.03, 30.66, 28.47 (one quaternary carbon was not observed).

HRMS-ESI calculated for C\(_{35}\)H\(_{46}\)N\(_5\)O\(_8\) [M+H]\(^+\) 664.3341, found 664.3313.

cis-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 2-[(tert-butoxy)carbonyl]amino]acetate (5.20b)

A solution of 5.1b (50 mg, 99 \(\mu\)mol), Boc-glycine (17 mg, 99 \(\mu\)mol), TFFH (26 mg, 99 \(\mu\)mol), Et\(_3\)N (69 \(\mu\)L, 0.49 mmol) and DMAP (1.2 mg, 9.9 \(\mu\)mol) in DCM (3 mL) was reacted as described in the procedure for 5.20a.

The crude residue was purified by flash silica column chromatography (100% EA) to yield 5.20b (4.8 mg, 7.23 mmol, 9\%) as a yellow solid.

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 9.89 (d, \(J = 7.7\) Hz, 1H, NH), 8.95 – 8.86 (m, 2H, ArH), 8.17 (d, \(J = 2.4\) Hz, 1H, ArH), 7.62 – 7.47 (m, 2H, ArH MeOf), 7.11 – 6.99 (m, 2H, ArH MeOf), 5.07 – 4.99 (m, 2H, NH, CH), 4.80 (t, \(J = 7.1\) Hz, 2H, N1-CH\(_2\)), 4.20 – 4.07 (m, 1H, CH), 3.93 (d, \(J = 5.7\) Hz, 2H, CH\(_2\)), 3.88 (s, 3H, O-CH\(_3\)), 3.76 – 3.63 (m, 4H, N-CH\(_2\) morpholino), 2.76 (t, \(J = 7.1\) Hz, 2H, N1-CH=CH\(_2\)), 2.70 – 2.58 (m, 4H, O-CH\(_2\) morpholino), 1.98 – 1.71 (m, 8H, CH\(_2\)), 1.47 (s, 9H, tBu CH\(_3\)).

\(^13\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 169.94, 162.61, 162.24, 160.13, 150.68, 148.69, 142.25, 135.45, 132.38, 128.87, 128.27, 123.24, 114.97, 114.79, 80.11, 70.85, 67.18, 56.06,
55.59, 54.07, 46.82, 42.82, 39.17, 28.53, 28.49, 27.74 (one quaternary carbon was not observed).

HRMS-ESI calculated for C_{35}H_{46}N_{5}O_{8} [M+H]^+ 664.3341, found 664.3343.

**trans-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 7-[(tert-butoxycarbonyl)amino]heptanoate (5.20c)**

A solution of 5.1a (28 mg, 55 µmol), 5.19 (27 mg, 0.11 mmol), TFFH (27 mg, 0.11 mmol), Et\textsubscript{3}N (77 µL, 0.55 mmol) and DMAP (0.7 mg, 5.5 µmol) in DCM (1 mL) was reacted as described in the procedure for 5.20a. The crude residue was purified by flash silica column chromatography (100% EA) to yield 5.20c (32 mg, 44 µmol, 80%) as a yellow brown oil.

\(^1\)H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 9.77 (d, \(J = 7.9\) Hz, 1H, NH), 8.93 – 8.86 (m, 2H, ArH), 8.17 (d, \(J = 2.4\) Hz, 1H, ArH), 7.61 – 7.50 (m, 2H, ArH MeOPh), 7.08 – 7.00 (m, 2H, ArH MeOPh), 4.84 – 4.71 (m, 3H, NH and N1-CH\textsubscript{2}), 4.58 – 4.46 (m, 1H, CH), 4.12 – 3.97 (m, 1H, CH), 3.88 (s, 3H, O-CH\textsubscript{3}), 3.69 (t, \(J = 4.6\) Hz, 4H, N-CH\textsubscript{2} morpholino), 3.11 (q, \(J = 6.7\) Hz, 2H, CH\textsubscript{2}), 2.74 (t, \(J = 7.2\) Hz, 2H, N1-CH\textsubscript{2}CH\textsubscript{2}), 2.69 – 2.58 (m, 4H, O-CH\textsubscript{2} morpholino), 2.29 (t, \(J = 7.5\) Hz, 2H, CH\textsubscript{2}), 2.20 – 1.96 (m, 4H, CH\textsubscript{2}), 1.66 – 1.47 (m, 8H, CH\textsubscript{2}), 1.44 (s, 9H, tBu CH\textsubscript{3}), 1.37 – 1.30 (m, 4H, CH\textsubscript{2}).

\(^{13}\)C NMR (101 MHz, CDCl\textsubscript{3}) \(\delta\) 173.38, 162.58, 162.37, 160.14, 156.11, 150.69, 148.69, 142.26, 135.46, 132.39, 128.87, 128.28, 123.22, 114.97, 114.78, 71.78, 67.16, 56.05, 55.59, 54.07, 47.63, 40.66, 39.15, 34.67, 30.27, 30.07, 30.04, 28.92, 28.59, 26.60, 25.07 (one quaternary carbon was not observed).

HRMS-ESI calculated for C_{40}H_{55}N_{5}O_{8} [M+H]^+ 734.4123, found \(m/z\) 734.4072.
cis-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 7-[(tert-butoxycarbonyl)amino]heptanoate (5.20d)

A solution of 5.1b (50 mg, 99 µmol), 5.19 (48 mg, 0.20 mmol), TFFH (49 mg, 0.20 mmol), Et$_3$N (137 µL, 0.99 mmol) and DMAP (1.2 mg, 9.9 µmol) in DCM (4 mL) was reacted as described in the procedure for 5.20a. The crude residue was purified by flash silica column chromatography (1:2 hexane/EA) to yield 5.20d (48 mg, 66 µmol, 66%) as a yellow brown oil.

$^1$H NMR (400 MHz, CDCl$_3$) δ 9.85 (d, J = 7.8 Hz, 1H, NH), 8.97 – 8.88 (m, 2H, ArH), 8.17 (d, J = 2.4 Hz, 1H, ArH), 7.60 – 7.52 (m, 2H, ArH MeO), 7.09 – 7.01 (m, 2H, ArH MeO), 4.98 (s, 1H, NH), 4.80 (t, J = 7.1 Hz, 2H, N$_1$-CH$_2$), 4.68 – 4.49 (m, 1H, CH), 4.17 – 4.05 (m, 1H, CH), 3.88 (s, 3H, O-CH$_3$), 3.69 (t, J = 4.6 Hz, 4H, N-CH$_2$ morpholino), 3.11 (q, J = 6.7 Hz, 2H, CH$_2$), 2.76 (t, J = 7.1 Hz, 2H, N1-CH$_2$CH$_2$), 2.70 – 2.52 (m, 4H, O-CH$_2$ morpholino), 2.33 (t, J = 7.5 Hz, 2H, CH$_2$), 1.93 – 1.60 (m, 10H, CH$_2$), 1.51 – 1.32 (m, 15H, CH$_2$ and tBu CH$_3$).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 173.28, 162.66, 162.18, 160.14, 156.12, 150.67, 148.66, 142.30, 135.45, 132.41, 128.85, 128.27, 123.29, 114.97, 114.81, 114.81, 69.19, 67.13, 56.06, 55.59, 54.06, 47.05, 40.68, 39.16, 34.74, 30.11, 29.84, 28.98, 28.66, 28.58, 27.84, 26.63, 25.14.

HRMS-ESI calculated for C$_{40}$H$_{56}$N$_5$O$_8$ [M+H]$^+$ 734.4123, found m/z 734.4144.
trans-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 2-aminoacetate (5.21a)

A solution of 5.20a (4.1 mg, 6.2 μmol) and TFA (0.2 mL) in DCM (0.8 mL) was reacted as described in the procedure for 2.30a. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of 5.21a (3.1 mg, 3.42 μmol, 55%) as a yellow solid.

Analytical RP-HPLC Rt = 12.83 min.

cis-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 2-aminoacetate (5.21b)

A solution of 5.20b (7.0 mg, 10.4 μmol) and TFA (0.2 mL) in DCM (0.8 mL) was reacted as described in the procedure for 2.30a. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of 5.21b (4.3 mg, 4.75 μmol, 45%) as a yellow solid.

Analytical RP-HPLC Rt = 12.83 min.

trans-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 7-aminoheptanoate (5.21c)

A solution of 5.20c (15 mg, 20.8 μmol) and TFA (0.25 mL) in DCM (1 mL) was reacted as described in the procedure for 2.30a. The crude was purified by
semi-preparative RP-HPLC to yield the TFA salt of 5.21c (11.1 mg, 11.4 μmol, 55%) as a yellow solid.

HRMS-ESI calculated for C_{38}H_{48}N_{5}O_{6} [M+H]^+ 634.3599, found m/z 634.3583. Analytical RP-HPLC R_t = 14.42 min.

cis-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 7-aminoheptanoate (5.21d)

A solution of 5.20d (22 mg, 29.6 μmol) and TFA (0.25 mL) in DCM (1 mL) was reacted as described in the procedure for 2.30a. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of 5.21d (13 mg, 13.3 μmol, 45%) as a yellow solid.

HRMS-ESI calculated for C_{38}H_{48}N_{5}O_{6} [M+H]^+ 634.3599, found m/z 634.3595. Analytical RP-HPLC R_t = 14.41 min.

trans-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 2-acetamidoacetate (5.22a)

The TFA salt of 5.21a (1.4 mg, 1.5 μmol), DIPEA (0.81 μL, 4.6 μmol), and acetic anhydride (0.16 μL, 1.7 μmol) in DCM (496 μL) were treated as in the procedure for 2.31. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 5.22a (0.9 mg, 1.49 μmol, 96%) as a yellow solid.
MS-ESI calculated for C\textsubscript{32}H\textsubscript{40}N\textsubscript{5}O\textsubscript{7} [M+H]\textsuperscript{+} 606.2922, found 606.2.

Analytical RP-HPLC R\textsubscript{t} = 14.70 min.

cis-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 2-acetamidoacetate (5.22b)

The TFA salt of 5.21b (1.5 mg, 1.7 µmol), DIPEA (0.87 µL, 5.0 µmol), and acetic anhydride (0.17 µL, 1.8 µmol) in DCM (503 µL) were treated as in the procedure for 2.31. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 5.22b (0.97 mg, 1.6 µmol, 96%) as a yellow solid.

HRMS-ESI calculated for C\textsubscript{32}H\textsubscript{40}N\textsubscript{5}O\textsubscript{7} [M+H]\textsuperscript{+} 606.2922, found m/z 606.2933.

Analytical RP-HPLC R\textsubscript{t} = 14.73 min.

trans-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 7-acetamidoheptanoate (5.22c)

The TFA salt of 5.21c (3.5 mg, 3.6 µmol), DIPEA (1.87 µL, 10.8 µmol), and acetic anhydride (0.7 µL, 4.0 µmol) in DCM (622 µL) were treated as in the procedure for 2.31. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 5.22c (2.36 mg, 3.5 µmol, 97%) as a yellow solid.
cis-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 7-acetamidoheptanoate (5.22d)

The TFA salt of 5.21d (3.3 mg, 3.4 µmol), DIPEA (1.77 µL, 10.2 µmol), and acetic anhydride (0.35 µL, 3.7 µmol) in DCM (610 µL) were treated as in the procedure for 2.31. The product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 5.22d (2.23 mg, 3.3 µmol, 98%) as a yellow solid.

HRMS-ESI calculated for C_{37}H_{50}N_{5}O_{7} [M+H]^+ 676.3705, found m/z 676.3737.
Analytical RP-HPLC R_t = 16.76 min.

trans-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 2-[6-2-{4-[(E)-2-[2,2-difluoro-4-(thiophen-2-yl)-1,4,5,6,7,12,13,14-octahydro-1,10,11,11a-tetrahydro-1H-cyclopenta[a]indenin]-3-yl]hexanamido}acetamide (5.9a)

HRMS-ESI calculated for C_{37}H_{50}N_{5}O_{7} [M+H]^+ 676.3705, found m/z 676.3737.
Analytical RP-HPLC R_t = 16.81 min.
The TFA salt of 5.21a (1.7 mg, 1.9 µmol), DIPEA (1.3 µL, 7.6 µmol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 µmol) and DMF (721 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 5.9a (1.51 mg, 1.36 µmol, 72%) as a bright blue solid.

HRMS-ESI calculated for C_{59}H_{64}BF_{2}N_{8}O_{9}S [M+H]^+ 1109.4582, found m/z 1109.4580. Analytical RP-HPLC R_t = 20.39 min.

cis-4-[6-(4-Methoxyphenyl)-1-[2-(morpholin-4-y1)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 2-[6-(2-{4-[(E)-2-[2,2-difluoro-4-(thiophen-2-yl)-1λ4,3-diaza-2λ4-boratricyclo[7.3.0.03,7]dodeca-1(12),4,6,8,10-pentaen-12-yl]ethenyl]phenoxy}acetamido)hexanamido]acetate (5.9b)

The TFA salt of 5.21b (2.8 mg, 3.1 µmol), DIPEA (1.7 µL, 10.0 µmol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 µmol) and DMF (728 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 5.9b (2.03 mg, 1.83 µmol, 97%) as a bright blue solid.

HRMS-ESI calculated for C_{59}H_{64}BF_{2}N_{8}O_{9}S [M+H]^+ 1109.4582, found m/z 1109.4556. Analytical RP-HPLC R_t = 20.47 min.
trans-4-[6-(4-methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 7-[6-[(2-[2,2-difluoro-4-(thiophen-2-yl)-1,4,3-diaza-2λ4-boratricyclo[7.3.0.0³,⁷]dodeca-1(12),4,6,8,10-pentaen-12-y]ethenyl]phenoxy]acetamido]heptanoate (5.9c)

The TFA salt of 5.21c (4.2 mg, 4.3 µmol), DIPEA (2.2 µL, 12.5 µmol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 µmol) and DMF (735 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 5.9c (2.16 mg, 1.83 µmol, 97%) as a bright blue solid.

HRMS-ESI calculated for C₆₄H₇₄BF₂N₈O₉S [M+H]^+ 1179.5366, found m/z 1179.5275.
Analytical RP-HPLC Rₜ = 21.43 min.

cis-4-[6-(4-methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl 7-[6-(2-[4-[(E)-2-[2,2-difluoro-4-(thiophen-2-yl)-1,4,3-diaza-2λ4-boratricyclo[7.3.0.0³,⁷]dodeca-1(12),4,6,8,10-pentaen-12-y]ethenyl]phenoxy]acetamido]hexanamido]heptanoate (5.9d)
The TFA salt of 5.21d (3.7 mg, 3.8 µmol), DIPEA (2.0 µL, 11.4 µmol), BODIPY 630/650-X-OSu 2.15 (1.25 mg, 1.89 µmol) and DMF (732 µL) were reacted as described in the procedure for 2.32a. The crude product was purified by semi-preparative RP-HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA, yielding 5.9d (1.74 mg, 1.48 µmol, 78%) as a bright blue solid.


tert-Butyl N-[(1s,4s)-4-[6-(4-methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl]carbamate (5.23)

5.11b (50 mg, 0.12 mmol), DIPEA (128 µL, 0.73 mmol), HATU (46 mg, 0.12 mmol) and 1-N-Boc-cis-1,4-cyclohexyldiamine (79 mg, 0.37 mmol) in DMF (2.5 mL) were used as described in the procedure for 2.29a. The reaction mixture was evaporated under reduced pressure and the residue taken up in EA (5 mL), washed with H₂O (3 x 5 mL), dried over MgSO₄, filtered and evaporated under reduced pressure. The crude product was purified by flash silica gel column chromatography (4:1 EA/hexane) to yield 5.23 (53 mg, 87.2 µmol, 71%) as a yellow solid (Rᶠ 0.34, 100% EA).

¹H NMR (400 MHz, CDCl₃) δ 10.00 (d, J = 7.8 Hz, 1H, NH), 9.01 – 8.81 (m, 2H, ArH), 8.25 – 8.11 (m, 1H, ArH), 7.62 – 7.44 (m, 2H, ArH MeOPh), 7.09 – 6.95 (m, 2H, ArH MeOPh), 4.77 (t, J = 7.2 Hz, 2H, N1-CH₂), 4.72 – 4.59 (m, 1H, NH), 4.28 – 4.14 (m, 1H, CH), 3.86 (s, 3H, O-CH₃), 3.68 (t, J = 4.5 Hz, 5H, CH, N-CH₂ morpholino), 2.74 (t, J = 7.2 Hz, 2H, N1-CH₂CH₂), 2.64 (t, J = 4.4 Hz, 4H, O-CH₂ morpholino), 1.82 (d, J = 14.6 Hz, 6H, CH₂), 1.68 – 1.54 (m, 2H, CH₂), 1.45 (d, J = 1.4 Hz, 9H, tBu CH₃).

¹³C NMR (101 MHz, CDCl₃) δ 162.61, 162.11, 160.09, 155.28, 150.60, 148.63, 142.13, 135.39, 132.33, 128.81, 128.23, 123.26, 114.93, 114.76, 79.33, 67.12, 55.60, 55.55, 54.02, 47.41, 45.60, 39.09, 28.91, 28.64, 28.57.

HRMS calculated for C₃₃H₄₄N₅O₆ [M+H]+ 606.3286, found m/z 606.3246.
**6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-N-[(1s,4s)-4-aminocyclohexyl]-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5.24)**

A solution of 5.23 (43 mg, 71.3 μmol) and TFA (0.3 mL) in DCM (3 mL) was reacted as described in the procedure for 2.30a, to yield the TFA salt of 5.24 (60 mg) as a yellow solid, which was used in the next step without further purification.

HRMS calculated for C_{28}H_{36}N_{5}O_{4} [M+H]^+ 506.2762, found m/z 506.2767.

**tert-Butyl N-([(1s,4s)-4-[6-(4-methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-1,2-dihydro-1,8-naphthyridine-3-amido]cyclohexyl]carbamoyl)methyl)carbamate (5.25)**

The TFA salt of 5.24 (55 mg, 65.3 μmol), DIPEA (68 μL, 0.39 mmol), HATU (25 mg, 65.3 μmol) and Boc-glycine (34 mg, 0.20 mmol) in DMF (2 mL) were used as described in the procedure for 2.29a. The reaction mixture was evaporated under reduced pressure and the residue taken up in EA (5 mL), washed with H2O (3 x 5 mL), sat. aq. NaCl (3 mL) and sat. NaHCO₃ (1 mL), dried over MgSO₄, filtered and evaporated under reduced pressure. The crude product was purified by flash silica gel column chromatography (95:5 EA/MeOH) to yield 5.25 (13.4 mg, 20.3 μmol, 31%) as a yellow solid (Rf 0.10, 100% EA).

^1H NMR (400 MHz, CDCl₃) δ 10.01 (d, J = 7.5 Hz, 1H, NH), 8.94 – 8.87 (m, 2H, ArH), 8.17 (d, J = 2.4 Hz, 1H, ArH), 7.59 – 7.51 (m, 2H, ArH MeOPh), 7.08 – 7.01 (m, 2H, ArH MeOPh), 6.24 (d, J = 7.7 Hz, 1H, NH), 5.31 – 5.16 (m, 1H, NH), 4.79 (t, J = 6.8 Hz, 2H, N1-CH₂), 4.29 – 4.16 (m, 1H, CH), 4.03 – 3.90 (m, 1H, CH), 3.88 (s, 3H, O-CH₃), 3.78 (d, J = 5.8 Hz, 2H, COCH₂NH), 3.69 (t, J = 4.6 Hz, 4H, N-CH₂)
morpholino), 2.82 – 2.72 (m, 2H, N1-CH2CH2), 2.71 – 2.57 (m, 4H, O-CH2 morpholino), 1.90 – 1.75 (m, 6H, CH2), 1.68 – 1.56 (m, 2H, CH2), 1.47 (s, 9H, tBu CH3).

13C NMR (101 MHz, CDCl3) δ 168.81, 162.67, 162.22, 160.14, 156.30, 150.68, 148.65, 142.23, 135.46, 132.42, 128.82, 128.27, 123.24, 114.97, 114.80, 80.47, 67.14, 56.05, 55.58, 53.98, 46.50, 45.56, 44.85, 39.07, 29.83, 28.65, 28.49.

HRMS calculated for C35H47N6O7 [M+H]+ 663.3501, found m/z 663.3508.

6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-N-[(1s,4s)-4-(2-aminoacetamido)cyclohexyl]-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5.26)

A solution of 5.25 (12 mg, 18.1 μmol) and TFA (0.4 mL) in DCM (1.6 mL) was reacted as described in the procedure for 2.30a. The crude was purified by semi-preparative RP-HPLC to yield the TFA salt of 5.26 (9.27 mg, 10.3 μmol, 57%) as a yellow solid.

HRMS calculated for C30H39N6O5 [M+H]+ 563.2904, found m/z 563.2943.

Analytical RP-HPLC Rt = 12.47 min.
6-(4-Methoxyphenyl)-1-[2-(morpholin-4-yl)ethyl]-2-oxo-N-[(1s,4s)-4-{2-[6-(2-[(E)-2-[2,2-difluoro-4-(thiophen-2-yl)-1λ4,3-diaza-2λ4-
boratricyclo[7.3.0.03,7]dodeca-1(12),4,6,8,10-pentaen-12-
yl]ethenyl]phenoxy)acetamido]hexanamido]acetamido]cyclohexyl]-1,2-dihydro-
1,8-naphthyridine-3-carboxamide (5.27)

The TFA salt of 5.26 (3.39 mg, 3.8 μmol), DIPEA (1.85 μL, 10.6 μmol), BODIPY
630/650-X-OSu 2.15 (1 mg, 1.51 μmol) and DMF (731 μL) were reacted as described
in the procedure for 2.32a. The crude product was purified by semi-preparative RP-
HPLC and passed through an Amberlyst A21 ion exchange resin to remove TFA,
yielding 5.27 (2.29 mg, 2.07 μmol, quantitative yield) as a bright blue solid.

HRMS calculated for C_{59}H_{65}BF_{2}N_{9}O_{8}S [M+H]^{+} 1108.4742, found m/z 1108.4777.
Analytical RP-HPLC R_{t} = 20.12 min.

***** The greater than 100% calculated yield for 5.27 (137%) was likely due to the commercially
supplied container of BODIPY 630/650-X-OSu 2.15 containing more than the stated 1 mg. Previous
synthesis of dye compounds have used a larger container of BODIPY 630/650-X-OSu 2.15 (e.g. 5 mg)
split between three or four reactions, and therefore any excess in BODIPY 630/650-X-OSu 2.15 would
be spread across compounds.
7.2 Pharmacology

Pharmacological evaluation of compounds was carried out in the laboratory of Professor Michelle Glass at the University of Auckland. All pharmacological experiments were carried out by myself, except:

- Preparation of membranes for radioligand binding assays was carried out by various members of the Glass lab.
- The 10 μM BRET screen of 2.23a, 2.23c, 2.23d, 2.23e, 2.23f and 2.25c in the presence and absence of CP55,940 1.10 at hCB2 receptor (n = 1) was carried out by Christa Macdonald.
- Radioligand binding assays of 3.7a, 3.7b, 3.4 and 3.12 at hCB2 and hCB1 receptor (n = 4 or 5) were carried out by Christa Macdonald.
- Concentration response radioligand binding assays for 4.8, 4.9 and 4.11 at hCB2 receptor (n = 4) were carried out by Jamie Manning.

7.2.1 Radioligand binding assays

Competition binding assays were carried out on membranes prepared from HEK293 cells and protein concentrations were quantified using the DC Protein Assay Kit (Bio-Rad, Hercules, CA) by other members of the Glass lab as previously described.224 These cells were stably transfected with either hCB1 or hCB2 receptors as previously described,81,231 which was also carried out by researchers in the Glass lab. The following pharmacology experimental describes the work I carried out myself, after training by Christa Macdonald.

Membranes were resuspended in binding buffer (50 mM HEPES, 1 mM MgCl2, 1 mM CaCl2, 0.2% [w/v] fatty acid free bovine serum albumin [FAF BSA; MP Biomedicals, Auckland, New Zealand], pH 7.4) to give a final assay concentration of 10 μg/well (Chapters 2-4) or 7.5 μg/well (Chapter 5) for hCB2 or 7.5 μg/well (Chapters 2-4) or 5 μg/well (Chapter 5) for hCB1. Test compounds (10 mM in DMSO) were diluted with binding buffer containing EtOH to match CP55,940 1.10 (5-(1,1-dimethylheptyl)-2-[5-
hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenol) (Cayman Chemical, Michigan, USA) vehicle and serial dilutions were prepared using binding buffer containing the requisite amount of EtOH and DMSO to maintain vehicle levels throughout the dilution series. For CP55,940 1.10 control points, a stock aliquot in EtOH was diluted with binding buffer containing DMSO to match compound vehicle. All compounds were prepared at 4 × the required assay concentration and 50 μL added to a v-bottom 96-well plate and made up to a total volume of 200 μL with [3H]CP55,940 1.17 (PerkinElmer) and membrane. Similarly, [3H]CP55,940 1.17 was prepared at 10 nM or 4 nM, with 50 μL added to each well to give a final concentration of 2.5 nM or 1 nM. For vehicle control points, binding buffer containing matched concentrations of EtOH and DMSO was used in place of ligand or CP55,940 1.10. The v-bottom plates containing hCB2 or hCB1 membranes, [3H]CP55,940 1.17 and ligand (or CP55,940 1.10 or vehicle) were incubated at 30°C for 1 h.

GF/C 96-well harvest plates (PerkinElmer) were soaked in 50 μL/well of 0.1% polyethylenimine for 1 h, then washed with 200 μL/well of ice-cold wash buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 0.1% FAF BSA). The contents of the v-bottom plates were filtered through the harvest plates, followed by a 200 μL/well wash of the v-bottom plates with ice-cold wash buffer. The harvest plates were promptly washed three times with 200 μL/well of ice-cold wash buffer and dried overnight at 24°C. Scintillation fluid (50 μL/well) (IRGASAFE PLUS, PerkinElmer) was added to the harvest plates and incubated for 30 mins in darkness, after which the harvest plates were read for 2 min/well in a MicroBeta TriLux (PerkinElmer). Binding experiments were performed a minimum of three times in triplicate.

Data was analysed with GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA) and competition binding curves fit by nonlinear regression using one site competition binding. Dissociation constants (Ki) of compounds were determined using [3H]CP55,940 1.17 Kd = 2 nM (hCB1) or 3 nM (hCB2). Ki values are expressed as mean ± SEM. In cases where less than 50% displacement of [3H]CP55,940 1.17 was observed with 10 μM compound, affinity of the compound was deemed too low to be able to generate an accurate competition binding curve. Therefore, a one sample t-test (P <0.05) was used to determine if there was significant difference between
displacement in the absence (vehicle normalised to 0%) and presence of compound (with CP55,940 1.10 normalised to 100%), in which case the ligand was determined to have a $K_i > 10 \, \mu M$, otherwise it was determined to show no significant binding.

7.2.2 cAMP assays

Function of compounds at hCB$_2$ and hCB$_1$ receptor was determined using a BRET assay measuring forskolin-stimulated cellular cAMP in the appropriate HEK293 cells transfected with a plasmid that encodes for the cAMP sensor YFP-Epac-RLuc (CAMYEL) as previously described.$^{231}$ One or two days prior to transfection, HEK 3HA-hCB$_1$ pEF4A, HEK 3HA-hCB$_2$ S4 low, HEK S4 low WT, HEK-Flp pcDNA5/FRT HA-3TCS-hCB$_2$ 63Q or HEK-Flp WT cells, generated as previously described$^{81,231}$ were seeded in 10 cm sterile tissue culture dishes. Cells were transfected with 5 μg of pcDNA3L-His-CAMYEL plasmid (ATCC, Manassas, VA, USA) using 30 μg of linear polyethylenimine (PEI) (molecular weight 25 kDa; Polysciences, Warrington, PA, USA) in 150 mM NaCl. After 24 h, transfected cells were plated in poly-β-lysine (PDL) (0.05 mg mL$^{-1}$ in phosphate buffered saline (PBS); Sigma-Aldrich, St Louis, MO, USA) treated 96-Well Solid White Flat Bottom Polystyrene TC-Treated Microplates (Corning) at a density of 60-80,000 cells/well in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After 16 h, cells were serum-starved in Hank’s balanced salt solution (HBSS) containing 1 mg mL$^{-1}$ FAF BSA, pH 7.4 for 30 mins. Cells were then treated with 5 or 7.5 μM coelenterazine-h (Nanolight Technology) for 5 mins, followed by addition of ligand or matched vehicle in HBSS plus 1mg mL$^{-1}$ FAF BSA and 5 μM forskolin (Tocris, Bristol, UK). A LUMIstar plate reader (BMG) was used to immediately measure emission signals at 37 °C following ligand addition, which were simultaneously detected at 460/25 nM (RLuc) and 535/25 nM (YFP). The inverse BRET ratio of emission at 460/535 nm is presented in the raw data, and hence an increase in ratio correlates to an increase in cAMP production. Assays were carried out a minimum of three times (except where stated) in duplicate.

Data analysis was performed using GraphPad Prism 7, with dose response curves fit by nonlinear regression. AUC analysis was achieved using values normalised to the
vehicle (0%) or forskolin (100%) values for individual experiments and $E_{\text{max}}$ was determined as a percentage of the normalised forskolin values. A $t$-test ($P < 0.05$) was used to determine if there was a significant difference in response for compounds at 10 $\mu$M in HEK-hCB$_2$ or -hCB$_1$ cells in the presence or absence of CP55,940 1.10 and in WT HEK cells to determine receptor mediated signalling.
7.3 Modelling

All receptor sequences used were downloaded from UniProt. All CB2 receptor homology models were generated using MODELLER 9.19. Model A was generated using the structure of the inverse agonist-bound human β2-adrenoceptor (PDB ID: 2RH1) as a template, based on the modified sequence alignment between hCB2 receptor (P34972), human β2-adrenoceptor (P07550), bovine rhodopsin (P02699), human D3 receptor (P35462-3) and human β1-adrenoceptor (P08588) from the T-Coffee server. Models B, C and D were generated using either the structure of the antagonist-bound hCB1 receptor (PDB ID: 5U09), the inverse-agonist bound hCB1 receptor (PDB ID: 5TGZ) or the agonist bound hCB1 receptor (PDB ID: 5XRA) as templates, respectively, based on a modified sequence alignment between hCB2 receptor (P39472) and hCB1 receptor (P21554) from the T-coffee server.

Three dimensional models of ligands were generated using Avogadro 1.2 and minimised using the universal force field (UFF). Ligand docking was performed using GOLD v5.5 (CCDC Software) centred on S285 extending for a distance of up to 10 or 15 Å and visualised in PyMOL (The PyMOL Molecular Graphics System, Version 1.8.6.0 Schrödinger, LLC.).
Appendix

NMR spectra for 2.22h (Chapter Two, section 2.3.1.2, pg 55)

2.22h: HSQC
2.22h: HMBC
2.22h: NOESY
References


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