The Synthesis, Photophysical and Biological Evaluation of Glycosylated Naphthalimides for Medicinal and Material Applications

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Based on research carried out under the direction of Prof. Eoin M. Scanlan and Prof. Thorfinnur Gunnlaugsson

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Declaration

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_______________________________________
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Publications


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Abstract

This thesis entitled “Synthesis, Photophysical and Biological Evaluation of Glycosylated Naphthalimides for Medicinal and Materials Applications” is divided into 8 chapters, in which Chapter 1, the introduction, gives an overview of the importance of carbohydrates in biology and for prodrug development as well as their use to improve site-selectivity and water solubility. It also explains what glycosidase enzymes are and how they have been used for prodrugs development. A short review on the photophysical and biological applications of naphthalimides, and some previous examples of glycosylated naphthalimides that can be found in the literature are also included.

In Chapter 2, the synthesis of a family of glycosylated naphthalimides is described and their spectroscopic and biological properties examined. It was demonstrated that these compounds are good substrates for enzymatic (glycosidase) activity, which cleaves the carbohydrate moiety releasing the naphthalimide core. Interestingly, it was found that when the glycosylated compounds were incubated in vitro (in HeLa cells), no cell uptake occurs. However, the successful implementation of the enzymatic reaction (releasing the naphthalimide core) in vitro, allowed naphthalimide core to rapidly into the cells. Thus, Chapter 2 describes the development of a new class of compounds with interesting photophysical properties that via enzymatic release, could be used for the delivery of a probe inside the cells.

Chapter 3 exploits the results obtained in Chapter 2 and aims for the use of this enzymatic-dependent release to deliver selectively (inside or outside the cell) a naphthalimide containing an alkyne group that could undergo click chemistry with modified sugars containing an azide group. These modified sugars, previously fed into the cells, can be metabolically incorporated into the cells’ glycome. Interestingly, these compounds (when released by enzymatic exposure) were able to go into the cell nuclei, thus allowing for click chemistry with modified DNA bases as well.

In Chapter 4, the enzymatic-dependent delivery is used in order to deliver a known cytotoxic drug (Amonafide). Thus, Chapter 4 comprises the synthesis, photophysical and biological evaluation of two compounds that could act as prodrugs of Amonafide, as an
efficient delivery of Amonafide in vitro was demonstrated in three different cell lines as well as the cytotoxicity induced by the delivery of Amonafide.

Chapter 5 describes the use of glycosylated naphthalimides as protein-binding probes, and the affinity of two glycosylated naphthalimides, one being a Tröger’s base of the other one, was successfully assessed using the lectin Concanavalin A (Con A). The changes in the fluorescence emission intensity demonstrated that the binding between the glycosylated naphthalimides and the protein was occurring. The binding with similar proteins is also investigated.

Chapter 6 gives a brief description of the different self-assembled structures observed for these glycosylated naphthalimides, which range from microspheres to gels, and studies these different morphologies using confocal microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Chapter 6 gives an overview on the material side of these compounds, and how they could be relevant for different medicinal applications.

Chapter 7 gives overall conclusions on glycosylated naphthalimides based on the results obtained from the previous chapters.

Finally, Chapter 8 comprises the experimental procedures for the synthesis of the glycosylated naphthalimides described in the previous chapters, as well as the methods used for the different techniques employed.
Abbreviations

- **AcOH**: acetic acid
- **ADEPT**: Antibody-Directed Enzyme Prodrug Therapy
- **AIE**: aggregation induced emission
- **Ar**: aromatic
- **ASPG-R**: asialoglycoprotein receptor
- **bs**: broad singlet
- **BSA**: Bovine Serum Albumin
- **BTC**: bis(trichloromethyl)carbonate
- **CD**: Circular Dichroism
- **CDI**: N,N’-carbonyldiimidazole
- **cm**: centimetre
- **Con A**: Concanavalin A
- **CT**: charge transfer
- **CuAAC**: Copper-Catalysed Azide-Alkyne Cycloaddition
- **CYP1A**: cytochrome P450
- **d**: doublet
- **dd**: Double doublet
- **DAPI**: (4′,6-diamidino-2-phenylindole
- **DBU**: 1,8-Diazabicyclo[5.4.0]undec-7-ene
- **DCE**: Dichloroethane
- **DMAP**: 4-Dimethylaminopyridine
- **DMF**: dimethylformamide
- **DNA**: deoxyribonucleic acid
- **DPBS**: Dulbecco's phosphate-buffered saline
- **Dox**: Doxorubicin
- **DRAQ5**: 1,5-bis{[2-(di-methylamino)ethyl]amino}-4,8-dihydroxyanthracene-9, 10-dione. (nuclear stain)
- **DsRed**: Discosoma sp. Red (red fluorescence marker)
- **EtOAc**: ethyl acetate
ESI  electron spray Ionization
equiv  equivalents
FRET  Fluorescence resonance energy transfer
GGT  gamma-glutamyl transpeptidase
h  hour
HCT-116  human colon cancer cell line
HepG2  human liver cancer cell line
HeLa  cervical cancer cells
HIB  Helium-ion Beam microscopy
HIV  human immunodeficiency virus
HMBC  heteronuclear multiple bond correlation
HOBt  Hydroxybenzotriazole
HPLC  high performance liquid chromatography
HRMS  high resolution mass-spectroscopy
HSQC  Heteronuclear single quantum coherence spectroscopy
Hz  Hertz
hνA  energy of absorption process
hνF  energy of fluorescence process
hνP  energy of phosphorescence process
IC_{50}  half maximal inhibitory concentration
ICT  Internal Charge Transfer
IR  infrared
ISC  intersystem crossing
J  coupling constant
LRMS  low resolution mass-spectroscopy
m  multiplet
MALDI  matrix-assisted laser desorption/ionization
MCF-7  breast cancer cell line
MHz  mega-hertz
min  minutes
mL  millilitre
mM  milimolar
M.p.  melting point
Mtb  Mycobacterium tuberculosis
MS  mass spectrometry
m/z  metal to charge ratio
NaOAc  sodium acetate
NAT2  N-acetyl transferase 2
NBS  N-Bromosuccinimide
NET3  triethylamine
nm  nanometre
NMR  nuclear magnetic resonance
NTR  nitroreductase enzyme
NR  Non-radiative relaxation
ns  nanoseconds
O-Ac  O-acetyl
OD  optical density
p  para
PBS  phosphate-buffered saline
Pd/C  Palladium on carbon catalyst
PDT  Photodynamic therapy
PEG  polyethylene glycol
PET  photoinduced electron transfer
PFA  paraformaldehyde
pH  -log[H3O]^+
PNA  Peanut agglutinin
p-HBADs  p-hydroxybenzoic acid derivatives
ppm  parts per million
PSGL-1  P-Selectin glycoprotein ligand-1
PTMS  post-translational modifications
PTT  Photothermal therapy
q  Quartet
x
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>$R$</td>
<td>Enantiomer configuration “right”</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
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<tr>
<td>S</td>
<td>electron spin</td>
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<td>$S$</td>
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<td>$S_0$</td>
<td>singlet ground state</td>
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<tr>
<td>$S_1$</td>
<td>first singlet excited state</td>
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<tr>
<td>SAR</td>
<td>Structural activity relationship</td>
</tr>
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<td>Ser</td>
<td>Serine</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SKBR-3</td>
<td>human breast cancer cell</td>
</tr>
<tr>
<td>sLe$^x$</td>
<td>Sialyl-Lewis$^x$</td>
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<tr>
<td>$Sx2$</td>
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<tr>
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<td>Strain Promoted Azide Alkyne Cycloadditions</td>
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<td>salmon testes DNA</td>
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<td>t</td>
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<td>T2</td>
<td>triplet state</td>
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<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoracetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>THPTA</td>
<td>tris(3-hydroxypropyl-triazoylmethyl)amine</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TNP</td>
<td>tri-nitrophenol</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total Correlation Spectroscopy</td>
</tr>
<tr>
<td>TPM</td>
<td>Two photon microscopy</td>
</tr>
<tr>
<td>UV-vis</td>
<td>ultraviolet-visible</td>
</tr>
<tr>
<td>VR</td>
<td>Vibrational relaxation</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical shift</td>
</tr>
<tr>
<td>ε</td>
<td>molar extinction coefficient</td>
</tr>
<tr>
<td>Φ</td>
<td>quantum yield</td>
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<td>Φ_F</td>
<td>fluorescence quantum yield</td>
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<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>v_{max}</td>
<td>maximum frequency</td>
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Chapter 1

Introduction
1 Introduction
This thesis will discuss the applications of glycosylated naphthalimide derivatives for different medicinal purposes, from cellular or protein fluorescence probes to prodrug development. Therefore, a wide range of techniques such as organic synthesis, UV-vis and fluorescence spectroscopy, in vitro evaluation, and confocal fluorescence microscopy will be described in this thesis. This introduction aims to give an overview on all these topics; however, a more concise introduction on specific topics can be found in each chapter.

1.1 Glycoconjugates
The term glycoconjugate is used to describe any molecule covalently linked to a carbohydrate. Naturally occurring glycoconjugates are classified into two major classes of biomolecules; glycolipids and glycoproteins, both of which are important components of cell walls and extracellular structures in plants, animals and bacteria.¹ Glycoconjugates participate in recognition processes between cells or in the recognition of cellular structures by other molecules.¹ These recognition events are necessary for fertilisation, cell growth and a variety of other critical cellular processes.

Due to the extend variety of possible combinations, glycoconjugates structures are often very complex, and yet can control very specific biological processes. Understanding the role of these glycoconjugates is relevant for several disciplines such as glycobiology, molecular biology, proteomics and medicine, as changes in the glycoconjugates structures can modify their function and therefore lead to diseases.² ³ For instance, Lewis X is specifically expressed on neural stem cells, serving as marker for neural stem cells differentiation.⁴ Defects in the carbohydrate attachment to proteins are associated to certain diseases, that are mostly characterised by neurological and developmental disorders.⁵ For instance, disorders in the N-glycosylation can be related to pathological diseases such as botulism, gas gangrene or pseudomembranous colitis.⁴ ⁵

Glycolipids are generally found on the exterior of eukaryotic cellular membranes, contributing to membrane stability and helping with cell-cell interactions. Gangliosides, which exert these functions, have been used as markers for cancerous cells as they are overexpressed and can shed from the tumour cells altering the function of normal cells near to the tumour, interfering with the critical anti-tumour response.⁶ Gangliosides are glycosphingolipids formed from a negatively charged sialic-acid containing carbohydrate linked to a ceramide that is attached to the outer layer of the lipid cellular membrane.
Gangliosides are known to change dramatically during development and have been used as markers of bone marrow stem cells by their extraction and subsequent analysis through high-performance TLC.\(^7\)

Glycoproteins can be found in the cell membrane but also in the cytoplasm and nucleus. The structure of these biomolecules can show high levels of diversity, but the most important glycoproteins are formed by N- and O-glycosylation. Errors in the biosynthesis of these glycopeptides are related to a range of diseases such as diabetes and mental disability.\(^8,9\)

Furthermore, modifications of natural glycoconjugates can be employed to tag or label cellular processes. A common method consists of modification of glycoconjugates displayed on the cell surface, which gives a wide range of information about internal cellular processes or about how the cells interact with their environment.

A tumour marker can be defined as any substance produced by the body in response to a tumour or by the tumour itself.\(^10\) In order to be used successfully, markers need to be sufficiently distinct to their natural counterpart. For instance, the antigen Tn (GalNAc\(\alpha_1\)-Ser/Thr) has been found to be sialylated and in fact, most colon cancer tissues present an increased content of \(\alpha\,2,6\)-sialyl-\(\beta\)-galactosides (Figure 1.1.1).\(^11\) Therefore, the antigen Tn has proved to be an ideal marker for colon cancer.

TNF-\(\alpha\) (Tumor Necrosis Factor) activates cells at sites of injury to recruit immune cells in acute and chronic inflammatory processes. TNF-\(\alpha\) stimulates endothelial expression of N-glycans, such as \(\beta\,1,6\)-GlcNAc (Figure 1.1.1) branching, as these epitopes play an important role in monocyte rolling and adhesion to the endothelium. Toyoda and co-workers demonstrated that in pathological conditions, \(\beta\,1,6\)-GlcNAc branching initiates endothelial cell contraction and gap formation, leading to subsequent biological events such as tumorigenesis.\(^12\)

![Figure 1.1.1](image-url) Different \(\text{\textit{O}-glycoside derivates}}\) used as tumour markers.
These are just a few examples that resemble the importance of natural glycoconjugates for cancer detection as well as for their use targeting other diseases.

1.1.1 Synthetic Glycoconjugates
The necessity to fully understand the mechanism of glycan recognition and their biological activity has driven the development of synthetic glycoconjugates. They have emerged as versatile therapeutic agents, being utilised for a wide range of medicinal applications, from vaccine and drug delivery systems to probes for biological functions and mechanisms.\textsuperscript{13}

The influence of carbohydrates in many biological processes and their ability to bind lectins and transport molecules inside of cells has rendered the field of synthetic fluorophore-containing glycoconjugates of great interest for the development of imaging tools and probes for different enzymes and diseases.\textsuperscript{14-16} The synthetic approach for these glycoconjugates typically involves the use of Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) chemistry as these reactions are cheap, rapid and result in high yields.\textsuperscript{17-20} Ideal glycoconjugate imaging tools should be physiologically stable, non-toxic and possess a strong absorption and emission in the near-infrared.

Modification of commercially available proteins with fluorophores to monitor lectins, enzymes and cellular processes is possible with the technology developed these days. Yu et al. modified Bovine Serum Albumin (BSA), as it possesses excellent biocompatibility, with a rhodamine derivative (fluorophore) and $\alpha$-mannose units to selectively detect Concanavalin A (Con A), a lectin that selectively binds to $\alpha$-mannose units, pili of \textit{E. Coli}, and lysosomes in MCF-7 as mannose-coated nanoparticles accumulate in the lysosomes. These are just some examples of the versatility imparted by the carbohydrate unit, where only by a modification with a monosaccharide like mannose allows for the development of a probe for multiple purposes, highlighting the broad-ranging applicability of this approach.\textsuperscript{21}

1.1.2 Recent Development of Synthetic Glycoconjugates Within the Scanlan Group.
An important research line of the Scanlan group have focussed on the development of glycoconjugates for the treatment of mycobacterium tuberculosis (Mtb). Mtb bacterial cell wall is formed by a complex mixture of glycosylated biomolecules that are immunomodulators during the infection process. Amongst these are found $p$-hydroxybenzoic acid derivatives or $p$-HBADs. Derivatives of this class of compounds have
been synthesised within the Scanlan group and their immunomodulatory effect tested in vitro. The results demonstrated that they can inhibit the production of inflammatory cytokines such as interferon-γ by T-cells.\textsuperscript{22}

The development of a family of glycosylated porphyrins (glycoporphyrins) and their applications for photodynamic therapy (PDT) have also been explored in the Scanlan group.\textsuperscript{23} Glycoporphyrins containing mono-, di- and trisaccharides (compounds 1a-d, Figure 1.1.2) were synthesised to investigate the impact of the carbohydrate moiety on their biological behaviour, as the introduction of larger and more complex carbohydrate structures opens the possibility of specific carbohydrate-lectin interactions. The group was able to demonstrate that the larger and partially protected saccharides improve biological compatibility as they increase water solubility, which is often an issue for therapeutic porphyrins. However, it was found that a low amount of singlet oxygen was produced and therefore the compounds exhibited low cytotoxicity towards human oesophageal squamous carcinoma cell line.\textsuperscript{23}

![Figure 1.1.2. Selection of the glycoporphyrins developed by the Scanlan group.\textsuperscript{23}](image)

In collaboration with the Gunnlaugsson group, glycosylated cyclen lanthanides complexes were developed as probes for glycosidase enzyme activity.\textsuperscript{24} It was envisioned that the excellent photophysical properties of lanthanides cyclen complexes would allow for simple fluorescence analysis. A range of glycosylated Tb(III) compounds (Scheme 1.1.1) were tested for glycosidase activity in real time. This type of probe was able to detect glycosidic hydrolysis at high concentrations, however complex aggregation
behaviour was observed at low concentrations thus limiting the scope of these type of probes and therefore this line of research was abandoned.²⁴

![Scheme 1.1.1] Scheme 1.1.1. Lanthanide cyclen complexes used as probes for glycosidase activity developed in a collaborative project between the Gunnlaugsson and Scanlan group. The system was based on an increase in the emission intensity upon enzymatic cleavage of the carbohydrate.²⁴

### 1.2 Importance of Carbohydrates in Biology

Carbohydrates are widely distributed throughout nature and play a pivotal role in essential cellular processes, from energy storage to fertilisation and neuronal development.²⁵,²⁶ Their capacity to modulate a plethora of different biological processes relies on their structural, stereochemical and conformational diversity, allowing them to proportionate information that regulates the functions of proteins.²⁷ It is estimated that just 4 different monosaccharides can virtually create 35,560 unique tetrasaccharides due to the multiple combinations that can be formed, as each monosaccharide contains five hydroxyl groups.²⁸

Proteins are post-translationally modified via glycosylation and aberrant glycosylation patterns are characteristic of some diseases, such as cancer or chronic inflammatory disease, and therefore can be used as biomarkers.¹⁵,²⁹,³⁰

Some carbohydrates have been used directly as drugs; for instance, D-mannose is used to treat urinary tract infections as it competitively inhibits the binding of *Escherichia Coli* to the endothelial wall.³¹ However, their high polarity diminishes the membrane permeability and enhances renal excretion, limiting their application. In an effort to circumvent these problems, glycomimetics and glycoconjugates have been intensively studied during the past decades. It was found that the conjugation of the carbohydrate moiety to a more hydrophobic counterpart enhances their pharmacokinetic properties.³² Furthermore, the conjugation of carbohydrates to therapeutic agents can be exploited as
delivery systems due to their ability to recognise and bind specific receptors in the
membrane known as lectins.33

1.2.1 Carbohydrates Binding Lectins
The term lectin was introduced in 1954 by W.C. Boyd34 to describe proteins of non-
immune origin, with no catalytic activity, which are able to reversibly bind
carbohydrates.27 The function of lectins is to promote cell-cell interactions by binding to
carbohydrates that are displayed on the surface of cells.35 Hence, they have to be
distinguished from carbohydrate-specific immunoglobulin, enzymes that use carbohydrates
as substrates such as glycosidases, or sensor/carrier proteins for carbohydrates.36 In
general, lectins possess multiple binding sites capable of interacting with different
monosaccharides of the same oligosaccharide. Although their specificity can vary, the
vast majority exert a poor affinity for their substrate. Some lectins can recognise only one
substrate whereas others have a lower fidelity and are able to interact with similar
substrates, for example, d-glucose and d-mannose can both be recognised by a multitude
lectins.27 Although this low affinity might not seem very efficient, it is necessary to allow
for the reversible nature of the communication events between cells. The hydroxyl groups
present in carbohydrates also perform an important role in this reversible communication
allowing for a fast and dynamic association/disassociation process at room temperature and
physiological environment, owing to their ability to hydrogen-bond.27

Due to their affinity for carbohydrates, lectins have been used as biomarker tools for
different diseases. They are essential components for biological processes such as
inflammation. It is known that the level of both lectins and cell-surface carbohydrates
become significantly altered in cancer.28,37 For instance, overexpression of (sialyl Lewis X)
sLeα in several carcinomas has been reported on multiple occasions in the literature, as
well as its presence in the activated endothelial cells and platelets promotes the adhesion of
cancer cells, contributing to their metastatic state.37-39

1.3 The Importance of the Glycome
The term glycome covers the totality of glycans and glycoconjugates synthesised by a cell,
tissue or organism.40 Glycans participate in a wide range of biological processes, from
intracellular signalling to tumor growth. Therefore understanding the processes by which
glycans govern these processes is of great interest.1,13
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There are many factors that influence and modify the glycome. Genes and proteins are involved in the synthesis and processing of the glycome but also environmental factors such as nutrition or pH can alter it and modify it. Thus studying the glycome goes beyond exploring the cell surface but instead allows an insight and an understanding of the cell machinery.\(^{41}\)

Importantly, changes in the glycome are associated with malignancies, such as altered N- and O-glycosylation or up-regulation of sialylated antigens. In many cases the reason behind the altered glycan is still unclear, however, these alterations are useful as a disease biomarker.\(^{42}\)

1.3.1 Studying the Glycome

Different techniques have been used to study the glycome, such as Mass Spectrometry (MS), lectin and antibodies arrays or via metabolic and covalent labelling.\(^{41}\) MS allows for the analysis of small samples by multiple rounds. Glycolipids or glycoproteins can be sequenced by separation of the protein or lipid content from the glycan. The most common problem found in MS is the saturation of the detector due the abundance of some glycans, which in turns causes less abundant glycoproteins or glycolipids to be missed, as they are outside of the detection limit. To avoid this, the samples are usually enriched and the proteins or lipid not bearing glycans are discarded.

Using lectins or antibodies allows for a wider throughput. Some lectins are able to recognise single monosacharides in almost any context whereas others can recognise larger glycans.\(^{36}\) A method to detect carbohydrates is by using lectins presented on a glass chip, which is subjected to the sample of interest, previously labelled fluorescently. Further fluorescent detection gives information about the types of glycans presented in the sample, however, no structural information is given.\(^{43}\)

Metabolic and covalent analysis involves treatment of the cell with modified sugars (usually bearing -N\(_3\) functionality) that are recognised by the cells and metabolically incorporated to their glycome as their natural glycome. This allows for covalently labelling with fluorophores via bioorthogonal “click” ligation. This technique does not provide with a wide structural information but allows for the monitoring of metabolic changes in vivo.\(^{44}\)

1.4 Glycosidase Enzymes

Glycosidases are enzymes that catalyse the hydrolysis of glycosidic linkages. They are the best-studied carbohydrate binding enzymes and are highly efficient, possessing the ability
to accelerate the spontaneous hydrolysis by $10^{17}$ fold. They can hydrolyse both terminal and internal glycosides within a chain and can be classified as *exo* or *endo*-glycosidases, respectively. Their mechanism of action, which was described by Koshland in 1953, can proceed via retention or inversion of the configuration, but in both cases the participation of either a Glutamate or Aspartate amino acid in the active side is required and an oxacarbenium ion is formed as intermediate species.

The inversion mechanism is the simplest as it occurs in only one step where two enzymic residues are used as an acid and base, respectively, Scheme 1.4.1a.

The retaining mechanism involves two-steps, each of which resulting in inversion of configuration. Similarly, it also involves two enzymic residues, typically carboxylates, that will act as a nucleophile and as an acid/base, respectively. In the first step, the anomeric centre is attacked by the enzymic carboxylate forming a glycosyl-enzyme covalent intermediate. Then, the second enzymic carboxylate acts as a base and assist a water molecule in the hydrolysis of the glycosyl-enzyme intermediate (Scheme 1.4.1b).

Due to the complex and varying roles of glycans in biology, glycosidases have been associated with important biological processes such as gene markers, and play an important role in the pathology of several disease states.

Scheme 1.4.1. Mechanisms of enzyme-catalyse hydrolysis via inversion of the configuration (a) and retention (b). (c) Intermediate species formed through an oxacarbenium ion.
For example, they are partially responsible for the abnormal glycosylation in cancer while glycosidase enzyme deficiencies are the marker for several pathological conditions, including Gaucher’s and Parkinson’s disease.\textsuperscript{29,50-53}

For the reasons outlined above, the development of glycosidase probes is an attractive area of research and recent reviews have covered the latest advances.\textsuperscript{14,54} However, there still exists the need for developing reliable and fast strategies of sensing the glycosidases activity.

1.4.1 Glycosidases and Cancer

Glycosidase enzymes have been used as markers for certain diseases.\textsuperscript{55} Many examples have been found linking an altered expression of these enzymes with tumours as an elevated level of certain glycosidases in tumour environments compared to healthy tissues has been observed. For instance, Fishman and Alman reported in 1947 an increased level of $\beta$-glucuronidase in breast, lung, ovarian and gastrointestinal track carcinomas\textsuperscript{56} In these tumours, $\beta$-glucuronidase is found extracellularly as is secreted by inflammatory cells. Importantly, $\beta$-glucuronidase is found within the lysosomes of healthy cells, therefore, targeting $\beta$-glucuronidase enzymes is of especial interest as it easily allows for the differentiation between healthy and cancerous cells.

Wielgat \textit{et al.}\textsuperscript{57} found that the presence of \textit{exo}-glycosidases such as $\beta$-galactosidase or $\alpha$-mannosidase, were significantly increased in malignant brain tumours compared to normal brain tissues, and postulated that their activity is dependent on the phase of the tumour development.

Some studies suggest that the levels of \textit{exo}-glycosidases in serum and urinary samples could be used as makers for certain tumours, such as colon or pancreatic cancer, but even for HIV infection. Szajda \textit{et al.} have found a significant increase of the activity of $\beta$-galactosidase, among others, in urinary samples. Using this data they were able to develop a method for the diagnosis of colon cancer using urine samples with a 86% sensitivity and 93% specificity.\textsuperscript{10}

It has been demonstrated that the level of $\beta$-galactosidase is directly related to the ability of cells to enter in senescence.\textsuperscript{58} Senescence is the mechanism by which cells cease to divide due to the cell damage caused by aging. Therefore, when senescence fails tumours arise as damaged cells accumulate and propagate. It has been previously demonstrated that high levels of $\beta$-galactosidase are found in colon cancer environments.
Because of the aforementioned, significant effort has been applied to the development of probes for glycosidase activity. Previous work carried out in a collaborative project between the Scanlan and the Gunnlaugsson group focused on the development of a luminescent glycosidase probe using lanthanides, as discussed in section 1.1.2.24

1.4.2 Glycosidases Used in Drug Delivery Systems
Glycosidase enzymes exert a high selectivity for the different glycosides and, as such, they can be exploited for the activation of glycosylated prodrugs. An important advantage of this strategy is the wide variety of glycosidases available in nature, allowing for the development of many possible glycosylated drugs that could subsequently be activated. However, glycosidases are widespread in the human body performing numerous roles and prodrug release by endogenous enzymes could potentially, lead to toxicity and other undesirable side effects. Therefore, an ideal situation would be one where the enzyme that mediates the activation does not have a human homologue or that the human homologue is not expressed in the targeted area.

The use of glycoconjugates as prodrugs using enzymes was reported in 1984 by Chang et al.56 They developed a sophisticated methodology exploiting endogenous glycosidases presented in the colon to treat bowel inflammatory disease. The coordination of two steroids, dexamethasone and hydrocortisone (Scheme 1.4.2), to glycosides was tested in vivo. The efficacy of the specific delivery system was proven as the oral administration of the glycosylated drug showed that 60% of the drug reached the target region, in contrast

![Scheme 1.4.2](image)

**Scheme 1.4.2.** Glycosylated prodrug of hydrocortisone (compound 4), which releases hydrocortisone (compound 5) in colon intestine as high concentrations of β-galactosidase are found there.
with only a 1% of the non-glycosylated version. Despite these promising results, not many examples using this simple yet innovative strategy have been reported.

Glycosidase enzymes can also be used in drug-delivery by introducing self-immolative units as linkers between the carbohydrate and the drug. In this strategy, the drug is

Scheme 1.4.3 Different prodrugs bearing self-immolative linkers (compounds 6a-d) for drug delivery using glycosidase enzymes. separated from the carbohydrate moiety by a self-immolative linker that spontaneously degrades upon enzymatic treatment (Scheme 1.4.3). This linker is usually non-toxic
although the inclusion of toxic linkers has also been tested as an attempt to increase the cytotoxicity of the drug. This approach has been widely exploited by Papot et al. and an extensive review compiles these examples. This strategy has been used for well-known anticancer drugs such as Paclitaxel, Duocarmycin or Daunurobicin. Many of these examples have used β-glucuronic acid as the β-glucuronidase enzyme is widely over expressed in a range of cancers, however, many examples can also be found exploiting β-galactosidase enzyme.

Antibody-Directed Enzyme Prodrug Therapy (ADEPT) is a methodology that has been widely utilised and consists of the use of antibodies to carry enzymes to tumour sites, where the prodrug is already present awaiting activation (Figure 1.4.1). Thus, the prodrug is only activated where the enzyme is present, after selective delivery by the antibody. Usually, non-endogenous enzymes are used and because the enzyme is specifically delivered the activation process takes place quickly.

Some variants of ADEPT have been developed, such as antibody-guided enzyme nitrile therapy developed by Rowlinson-Busza and co-workers, that activates amygdalin to release cyanide, a powerful metabolic poison. They created a monoclonal antibody linked to a β-glucosidase that was transported to the surface of tumour cells where the enzyme can then exert its activity. Lectin-directed enzyme-activated prodrug therapy developed by

![Figure 1.4.1. Schematic illustration of ADEPT, where antibodies carry enzymes to the tumor site where the prodrug is already present. Illustration by Darren J. Phelps.](image-url)
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Davis et al.\textsuperscript{62} involved the selective delivery of a glycosylated enzyme to the target along with the prodrug. Once the two components are in the cells, the glycosidase releases the drug. To ensure the drug is only activated in the desired area, they used a rhamnose-capped drug, as rhamnosidase is not endogenous in humans. Although selectivity toward some types of cells was achieved, \textit{i.e.} galactose was used as it is recognised for a specific receptors of liver cells, this technique still lacks the sophistication and ability to distinguish between healthy and tumour cells.

Having explained the importance of carbohydrates for different biological purposes and their participation in prodrugs that use glycosidase enzymes, 1,8-naphthalimides will be described in the next sections, by means of their relevance as fluorescence probes as well as their anticancer properties.

1.5 Naphthalimides

1,8-Naphthalimides (benz[de]isoquinolin-1,3-diones) can be readily synthesised from reaction of 1,8-naphthalic anhydride with a primary amine (Scheme 1.5.1), which allows for versatile functionalisation at the imide site.

![Scheme 1.5.1. General procedure for the synthesis of 1,8-naphthalimides from 1,8-naphthalic anhydride and primary amines.](image)

Functionalisation of the aromatic rings is also feasible with common synthetic chemistry such as S\textsubscript{N}Ar, Sonogashira coupling, \textit{etc}. Their ease of synthesis and functionalisation makes them very appealing, as the development of a family of naphthalimide derivative is readily accessible.

The planar structure of naphthalimides has been widely exploited for medicinal purposes, such as DNA intercalation, but also in the development of materials, as they are able to intercalate \textit{via} π-π interactions.\textsuperscript{63,64} Furthermore, they are excellent fluorophores that possess fluorescence quantum yields close to the unity in organic solvents, thus being very attractive as fluorescence probes and sensors.\textsuperscript{65} These applications will be further discussed in the next sections.
1.5.1 Photophysical Relevance of 1,8-Naphthalimides

Due to their excellent photophysical properties, naphthalimides are widely used as chemical probes and in the development of dyes. The absorption and fluorescence emission range of naphthalimides lie within the UV and visible region. However, these photophysical properties can be readily tuned by altering their substitution pattern. For unsubstituted naphthalimides, the lowest excited energy states (S1) correspond to \( \pi-\pi^* \) transitions (Figure 1.5.1), as described by Wintengs et al. An isoenergetic n-\( \pi^* \) triplet state (T2) allows for efficient intersystem crossing (ISC).

Although the functionalisation of the imide does not invoke a dramatic change, the substitution of the naphthalene ring governs the photophysical properties. The introduction of an electron withdrawing group or electron donating substituents induces a polar charge transfer (CT) excited state. The electronic properties of several 4-substituted naphthalimides (compounds 11(a-e), Figure 1.5.2) were reported by Glusac et al. They proved that initial photoexcitation produces the n-\( \pi^* \) excited state (S2), contrary to what was expected. This n-\( \pi^* \) excited state was deactivated to S1 by fast internal conversion (IC).

![Jablonski diagram](image)

**Figure 1.5.1.** Jablonski diagram of unfunctionalised 1,8-naphthalimides.

This example illustrates very well how the different substituents modify the electronic properties. For instance, for compounds 11a-c, S1 was \( \pi-\pi^* \) in nature, being able to undergo ISC to produce T1, whereas for compounds 11d-e, the excited state S1 possess
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Figure 1.5.2. 4-substituted 1,8-naphthalimides with different electronic properties reported by Glusac et al.\textsuperscript{69}

...a polar CT character.

As previously described, naphthalimides containing a nitro group in the 3- or 4-position present high-energy excited states with a broad absorption and short emission wavelength, as a result of the electron withdrawing nature of both the nitro and imide groups causing a CT. In contrast, a 3- or 4-amino substituted naphthalimides possess a large excited-state dipole and therefore the absorption and emission are shifted to a maximum found around 450 and 550 nm, respectively. This bathochromic effect is caused by an Internal Charge Transfer (ICT) excited state or “push-pull” system due to the electron-donating behaviour of the amine and the electron withdrawing nature of the imide (Scheme 1.5.2).

This ICT is highly dependent on the solvent system as it is more stabilised by polar protic solvents, a phenomena known as solvatochromic effect.\textsuperscript{70} Usually, the Stokes shift decreases when moving from a polar to a non-polar solvent, due to ICT character the lone pairs are not available and the n-\(\pi^*\) transition is absent in non-polar solvent.

Scheme 1.5.2. ICT excited state occurring in 4-amino-naphthalimides.

The Stokes’ shift is a combination of vibrational relaxation and solvent re-organisation. As the dipole moment of the molecules changes upon excitation forcing the molecules of solvent to re-align to adjust.\textsuperscript{71}
The solvent polarity may alter the energy levels by enhancing or inhibiting radiationless pathways to the ground state in an effect called “proximity-effect”.

Hydrogen bonding can alter the energy levels more drastically, being able even to reverse the n-π and π-π* states. Although Φ_F of naphthalimides is dramatically lowered when measured in water, it has been demonstrated that they are still suitable candidates for in vitro experiments.

Fluorophores presenting a polar CT are of great interest due to their oxidizing or reducing properties. Dhar and co-workers exploited these properties of the naphthalimide derivatives to synthesise compound 12 (Figure 1.5.3), which is capable of down-regulating the oxidative stress present in carcinogenesis.

Castellano et al. were able to observe for the first time, naphthalimide phosphorescence at rt due to the formation of platinum complex, compound 13 in Figure 1.5.3, via Sonogashira coupling. The strong coupling between the Pt center and the acetylide ligand promotes intersystem crossing S-T in the coordinated cromophore. This rigid bonding provides unrestricted access to long-lived triplet excited states. Excitation of compound 13 at 420 nm gives a luminescence band centred at 620 nm, which can be assigned as intra ligand-based phosphorescence, with excited-state lifetime in the range of 160 nm.

Naphthalimide derivatives have also been used as sensor through photoinduced electron transfer (PET) processes. PET is the phenomenon from which an excited-state electron is donated through space from a donor molecule/group (lumophore) to an accepting one (receptor), with the lumophore and receptor held together by a spacer. PET is usually faster than the luminescence emission process if the lumophore and receptor

---

Figure 1.5.3. Compounds 12, capable of down-regulate the oxidative stress in carcinogenesis, and 13, first naphthalimide derivative exhibiting phosphorescence due to the formation of a Pt complex.
have been picked carefully according to their thermodynamic properties and are close to each other in space.\(^8^0\)

Recently, a PET-naphthalimide based probe for glucoronosyltransferase has been reported by Ling and co-workers (Scheme 1.5.3).\(^8^2\) This probe is formed by a 4-hydroxyphenyl substituted naphthalimide (compound 14), in which the phenol is deprotonated under physiological conditions (pH 7.4). This negatively charged oxygen atom induces weak blue fluorescence, due to a PET processes occurring. However, when glucuronic acid is transferred onto the phenol (compound 15), the oxygen’s lone pair is no longer accessible thereby turning off the PET process, leading to an enhance in the fluorescence (Scheme 1.5.3).\(^8^2\)

![Scheme 1.5.3. 1,8-Naphthalimide probe for glucoronosyltransferase enzyme based on PET developed by Ling et al.\(^8^2\)](image)

1,8-Naphthalimides have been widely exploited for anion sensing, with many anion sensors being reported by the Gunnlaugsson group.\(^8^3\) Some examples of naphthalimides used for the sensing of monovalent anions, such as F\(^-\), OAc\(^-\) and H\(_2\)PO\(_4\)^-, found in the literature are shown in Figure 1.5.4. Compounds 16 and 17 form hydrogen bonding interactions that shift the emission bands of the naphthalimides, allowing for the sensing. Compound 18 on the other hand undergoes fragmentation after displacement of the silyl ether group after binding with fluoride, due to the great affinity of fluoride towards silicon. This triggers the fragmentation of the Si-O bond, ultimately releasing a 4-amino-
naphthalimide derivative, leading to a red shift in the fluorescence emission that produces a visible colour change even with the naked eye.\textsuperscript{84, 85}

\textbf{Figure 1.5.4.} 1,8-Naphthalimide derivatives used for anion sensing.

\textbf{1.5.2 Biological relevance of 1,8-naphthalimides}

Due to their good luminescence properties, 1,8-naphthalic anhydrides have also been used as probes for biological purposes.\textsuperscript{86-96} McCarley and co-workers\textsuperscript{86, 87} have developed naphthalimide probes for cellular imaging of tumorous environments (compounds 19 and 20 in Figure 1.5.5). They contained a self-immolative linker (blue) and a recognition moiety (green) that selectively binds to an enzyme (quinone oxido-reductase isozyme 1), which is up-regulated in many human cancer cells. After cellular uptake, compounds 19 and 20 (Figure 1.5.5) are reduced by this enzyme and undergo fragmentation, thus releasing a 4-aminonaphthalimide derivatives (red), which exhibits a strong fluorescent signal.\textsuperscript{86, 87}

Another interesting property of the use of naphthalimide derivatives as fluorescent probes is their capacity to absorb two photons, enabling for two-photon microscopy imaging. Two-photon microscopy (TPM) offers significant advantages because it allows for tissue penetration of up to 1 cm, and therefore can be used for \textit{in vivo} imaging.\textsuperscript{97} The near infrared window, or therapeutic window, comprises the range of wavelengths between 650 and 1350 nm, which is the range in where the light has the maximum tissue penetration.\textsuperscript{98} At these wavelengths the most dominant interaction between light and tissue is scattering, which increases the distance travelled by photons within tissue, therefore increasing the absorption of photons. However, this window is narrowed by the light absorption of
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melanin and water absorbing at long wavelengths. TPM is based on two-photon absorption and tissue penetration is possible due to the longer excitation wavelengths used. In this process an electron is promoted, usually from the ground state of a molecule, to a higher energy state. Because two photons are being absorbed, lower energies are required.

![Figure 1.5.5. 1,8-Naphthalimide derivatives used for cellular imaging based the reduction by quinone oxidoreductase isozyme 1, causing a fragmentation of the molecule and releasing 4-aminonaphthalimide, which produces a color change.](image)

Figure 1.5.5. 1,8-Naphthalimide derivatives used for cellular imaging based the reduction by quinone oxidoreductase isozyme 1, causing a fragmentation of the molecule and releasing 4-aminonaphthalimide, which produces a color change. 86,87

Usually, two-photon absorption requires excitation wavelengths of about half of the normal excitation process. This is of great interest for compounds that require low excitation wavelengths (lower than 405 nm), as in vitro nor in vivo imaging is possible because of cell damage.

Many examples have been reported in the literature exploiting this behaviour. 88-96 Yang et al. 95 reported the first fluorescent probe for human cytochrome P450 (CYP1A) in real

![Scheme 1.5.4. 1,8-Naphthalimide derivative used as probe for the CYP1A (compound 21) based on TPM.](image)

Scheme 1.5.4. 1,8-Naphthalimide derivative used as probe for the CYP1A (compound 21) based on TPM.
time using compound 21 (Scheme 1.5.4), which upon demetylation by the CYP1A gives the 4-hydroxy-1,8-naphthalimide (compound 22). This hydroxyl group is deprotonated under physiological pH (compound 23) and capable of giving a broad fluorescence band via 2-photon absorption with an excitation wavelength required of 820 nm.

Fan et al.\textsuperscript{92} created a fluorescence probe to monitor the level of thiols in the lysosomes of live cells using TPM. This system was also based on a “turn-on” mechanism, as compound 24 (Scheme 1.5.5), possesses a sulfonamide group able to respond to thiolates, affording an “off-on” signal (Scheme 1.5.5).\textsuperscript{92}

![Scheme 1.5.5](image.png)

**Scheme 1.5.5.** Naphthalimide probe for thiols in lysosomes of live cells using TPM. The sulphonamide group of compound 24 responds to thiolates present in the lysosomes, giving compound 25 which can be imaged by TPM.

Lastly, another example of naphthalimide derivatives using TPM imaging was reported by Peng et al.,\textsuperscript{89} who were able to demonstrate that the levels of methylglyoxal in liver and kidney tissues are higher in diabetic mice than in normal mice.

However, the use of naphthalimides in biological systems is not limited to the imaging field. Naphthalimides are also known for exhibiting good anticancer properties, which will be discussed in the next section.
1.5.2.1 Anticancer properties of 1,8-naphthalimides

Naphthalimides are well known anticancer agents since they were first reported by Braña and co-workers.99-101 Due to their planar structure they have the ability to act as intercalators, thereby disrupting the DNA structure.102 The most significant examples of this family of compounds in terms of biological significance are Amonafide and Mitonafide, 3-amino and 3-nitro-1,8-naphthalimides, respectively, (compounds 26 and 27, respectively, in Figure 1.5.6), both DNA intercalating agents. Amonafide binds to DNA via intercalation and inhibits topoisomerase II activity and has been in clinical trials for the treatment of Acute Myeloid Leukemia (phase III) and for prostate and breast cancer (phase II). Additionally shows toxicity in HeLa cells (human cervical cancer).103-105

![Amonafide (26) and Mitonafide (27)](image)

**Figure 1.5.6.** Most important naphthalimide derivatives with anticancer activity.

Structure-activity relationship (SAR) studies have shown that the basic terminal group in the side chain, separated by 2-3 methylene units from the naphthalene ring, is key for the anticancer properties.104 The importance on the substitution of the naphthalene ring has also been demonstrated. 3-Nitro substituted naphthalimide exhibit better toxicity towards cancerous cell lines compared to 4-nitro analogues due to its stacking properties, as the 3-nitro group can adopt a co-planar structure with the imide moiety, the angular plane between the nitro group and the imide moiety destabilises the DNA intercalation.

Zee-Cheng and co-workers reported derivatives of Amonafide and Mitonafide that exhibit better IC₅₀ values (concentration needed to reduce the cell-growth by 50%) against leukaemia. These derivatives were formed by the 3,6-diamino and 3,6-dinitro-1,8-naphthalimide derivatives, respectively.106 Subsequently, Braña et al. developed a family of 3-amino-6-nitro-1,8-naphthalimide derivatives (compounds 28a-b and 29, Figure 1.5.7) that exhibited higher toxicity levels than the parent compounds Amonafide and Mitonafide against human colon and lung carcinoma cell lines.107
Functionalisation of the 3-amine of Amonafide with ureas, thioureas or carbamates was investigated by Quaquebeke et al. finding that their best results was obtained with a trichloro-acetyl-urea derivative of Amonafide as it showed lower IC\(_{50}\) values in several human cancer cell lines and weaker hematotoxicity in healthy mice than Amonafide.\(^{108}\)

Functionalisation of the 3-amine residue with aromatic groups was investigated showing in some cases an increase in anti-proliferative effect of cancerous cell lines.\(^{109}\) However, derivatives that present an extended aromatic ring such as Azonafide (compound 30, Figure 1.5.7) are of more interest. Azonafide, which was also reported by Braña et al.,\(^{63}\) exhibited good toxicity levels towards leukaemia, breast cancer and melanomas.\(^{110}\) Interestingly, Azonafide is been currently investigated for antibody directed therapy in microgravity by NASA, as in microgravity cancerous cells grow in spherical shapes that resemble the natural form found within the human body, allowing for a better testing than typical in vitro techniques and procedures.\(^{111}\)

![Figure 1.5.7. Naphthalimide derivatives of Amonafide.](image)

Azonafide has inspired the development of extended aromatic rings.\(^{109,112}\) Meng and co-workers have developed triazolonaphthalimide derivatives of Amonafide (compounds 31(a-e), Figure 1.5.8) that exhibit better cytotoxicity than the parent drug Amonafide towards a range of cancer cell lines such as colon (HCT-116) or hepatic (HepG2) carcinomas. Giving average IC\(_{50}\) values of 1.01 µM and 0.73 µM for HCT-116 and HepG2, respectively, in contrast with Amonafide, which IC\(_{50}\) values for those cell lines are of 4.55 µM and 1.41 µM, respectively. SAR revealed that fusing a triazole ring onto the naphthalene core increases the intercalation and thus the cytotoxicity. Furthermore, the presence of basic amino chains at the triazole ring enhances the toxicity, being compound 31e the most toxic of this family.\(^{73}\)
Braña et al. developed a family of naphthalimides containing extended \( \pi \)-excedent aromatic rings, such as furans or thiophene, in two different orientations (compounds 32ab and 33ab, Figure 1.5.8). They also appear to significantly increase the toxicity towards colon and pancreatic carcinoma cell lines compared to Amonafide.\(^7\) Molecular modelling carried out with DNA with compounds 32a and 33a demonstrated that 32a is more stable than compound 33a and the most stable conformation is the one where the furan is stacked between AC bases and the site chain is in the major groove.

\[ \text{Figure 1.5.8. Derivatives of Amonafide containing fused aromatic rings.} \]

Although Amonafide has shown promising results in clinical trials, its applications are limited due to central neurotoxicity side effects.\(^1\) Therefore, substantial efforts have been made to develop derivatives to eradicate these side effects while also improving site selectivity.\(^4,10,11\)

Some naphthalimide derivatives have shown other modes of actions varying from the long-established DNA intercalation. Xie et al.\(^1\) demonstrated that compound 34 (Figure 1.5.9), which utilises polyamine transporter for cell uptake, induced apoptosis in two cell lines by caspase activation via mitochondrial pathway or by formation of autophagosomes increasing lysosomal activation.\(^1\) Similarly, compound 35 (Figure 1.5.9) exhibited good anti-proliferative effect in non-small cell lung cancers by triggering cell apoptosis via p53 signalling.\(^1\)

Another mechanism from which naphthalimide are able to exert cytotoxicity is by photoactivation inducing DNA strand cleavage.\(^1\)\(^,1\) Most commonly, an electron is transferred from guanine to the naphthalimide (as guanine nucleotide is the easiest to oxidise) as demonstrated by Saito and co-workers with compound 36 (Figure 1.5.9).\(^2\)
1.5.3 Recent Naphthalimide Derivatives Developed within the Gunnlaugsson Group

Recent work carried out in the Gunnlaugsson’s group has involved the development of V-shaped Troger’s base naphthalimides for coordination polymers (compounds 37 and 38 in Figure 1.5.10).\textsuperscript{121,122} Compound 37 is able to bind to Zn (II) in water with strong luminescence properties. Addition of phenolic-nitroaromatic compounds as well as picric acid, known to be explosive, produces a quenching response down to a minute threshold, as low as concentrations of 26 parts per billion. Therefore, compound 37 can be used as a probe for explosive agents in water.\textsuperscript{121}
Naphthalimides derivatives bound to Ru(II)-polypyridyl complexes, derivatives of compound 38, have proven to be rapidly uptaken by the cells, localising in the cytoplasm, presenting good luminescence properties in vitro. Antiproliferative effect was assessed and demonstrated to be on the range of the cis-platin value.\textsuperscript{123,124}

Previous work also focussed on the development of naphthalimides for anion sensing.\textsuperscript{83,125} Compound 39 (Figure 1.5.11) is a sensor for Zn(II) based on PET achieving good selectivity over other transition metals.\textsuperscript{83} The 3-urea-1,8-naphthalamide, compound 40, is also a good anion sensor for mono anions, showing its best binding properties towards fluoride anions. In this case, strong changes were observed in the absorption and emission bands, allowing for visible changes to be observed with the naked eye. Therefore making compound 40 a colorimetric chemosensor for fluoride.\textsuperscript{125}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.5.11.png}
\caption{Naphthalimide derivative for anion sensing developed within the Gunnlaugsson group.\textsuperscript{125}}
\end{figure}

Many naphthalimide derivatives have been developed as DNA intercalators and their binding has been proven with a range of techniques such as UV-vis and fluorescence spectroscopy, linear and circular dichroism spectroscopy, thermal denaturalisation and viscosity measurements.\textsuperscript{63} Of great interest is cationic compound 41, which binds to DNA by intercalation but is also capable of inducing DNA photocleavage of supercoiled DNA in the absence of oxygen. Interestingly, the compound binds more strongly to rich sequences of AT, as was demonstrated by spectroscopic titrations with various DNAs. Figure 1.5.12 shows its packing by $\pi-\pi$ interactions, where the naphthalimide orientate in a head-to-tail fashion.\textsuperscript{126}
Chapter 1. Introduction

1.6 Previous Examples of Glycosylated Naphthalimides

Limited examples of glycosylated naphthalimides have been reported in the literature.\textsuperscript{16,127-129} A recent study shows that the glycosylation of naphthalimide-based probes for Zn\textsuperscript{2+} enhances their solubility in water and the selectivity towards cancerous cells, therefore decreasing undesired cytotoxicity.\textsuperscript{128} Glycosylation also appears to increase stability over a wider pH range, which can be advantageous in targeting cancer cells, which typically present more acidic environments. The majority of the previous examples exploit the carbohydrate-lectin interaction as a strategy to induce cellular recognition and uptake. Jia and co-workers installed the oligosaccharide hyaluronic acid to a 4-amino-1,8-naphthalimide based probe for the selective recognition of the CD44 receptor, which is overexpressed in cancerous angiogenesis.\textsuperscript{129}

One of the most exploited carbohydrate receptors is the asialoglycoprotein receptor (ASPG-R), a membrane-bound lectin that is widely expressed in hepatocytes and is capable of recognising D-galactose and mediating endocytosis.\textsuperscript{130-132} Hee-Lee et al. utilised this receptor to promote the endocytosis of a galactosylated disulfide containing naphthalimide for the detection of oxidative stress that can be linked with diseases such as Parkinson’s, Alzheimer’s or cancer.\textsuperscript{133} Hepatocyte cells were targeted, as the liver is the most important organ in maintaining the oxidative-reductive balance in the organism, making it crucial in the early detection of such diseases. The naphthalimide probe was based on a switchable mechanism; becoming activated once inside the cells, upon cleavage of the disulfide bond and spontaneous release of 4-amino-1, 8-naphthalimide.

Naphthalimides have also been used in glycome labelling by Wong et al. by coordinating it \textit{in vivo} through CuAAC.\textsuperscript{16} A 4-substituted-1,8-naphthalimide with inactive
fluorescence was synthesised by installing either an azide or an alkyne functionality in the 4-position. The subsequent reaction with chemically modified azide or alkyne containing glycans, that have been metabolically installed on the surface of cells, leads to the formation of a 4-triazole-1,8-naphthalimide, which is brightly fluorescent due to the electron donating nature of the triazole ring (compound 42, Figure 1.6.1). The toxicity problems induced by copper were overcome by the development of a ligand that facilitates the reaction, allowing for completion within 30 minutes. Although in vivo ligation has become a deeply studied area in the past years, no other example using naphthalimides have been reported.

More recently, Sancenón and co-workers have developed a glycosylated naphthalimide derivative (compound 43, Figure 1.6.1) formed by a per-acetylated-β-D-galactose and an L-hystidine methyl ester linker. This derivative serves as probe for senescence by detection the activity of β-galactosidase enzyme. Due to the high hydrophobicity of the molecule, is able to be uptake by the cells and once inside hydrosylisis of the N-glycosidic bond occurs causing an enhanced fluorescence signal that can be monitor in vivo using TPM.

![Figure 1.6.1. Some examples of previous glycosylated naphthalimide reported in the literature.](image)

1.7 Work Described within this Thesis
This thesis outlines the broad application of glycosylated naphthalimide as molecular probes for relevant biomolecules including proteins and enzymes, as well as their use for drug delivery application and a brief investigation of their self-assembly.

Chapter 2 describes the synthesis of a family of glycosylated naphthalimides including their spectroscopic and biological evaluation. The compounds were evaluated for glycosidase enzyme activity, by means of hydrolysis of the glycosidic linkage. Cellular uptake studies carried out in vitro with a range of mammalian cancerous cell lines
demonstrated that uptake of the compounds did not occur. However, upon exposure to glycosidase enzyme, which releases the naphthalimide core, rapid cellular uptake takes place. The chapter studies this enzyme-dependant cellular uptake as the naphthalimide core can act as a fluorescence probe for the over expression of glycosidases in certain tumours (Figure 1.7.1).

Figure 1.7.1. Illustration of the selective intracellular delivery of a naphthalimide fluorescence probe using glycosidase enzymes.

Chapter 3 outlines our studies into the application of glycoslated naphthalimide derivatives as substrates for glycome labelling. The objective was to exploit the property of these compounds to only exhibit cellular uptake in the presence of enzymes as a strategy for selective labelling of cell-surface glycans. The alkyne functionality enables for CuAAC chemistry with modified sugars containing azide groups. Chapter 3 describes the synthesis of modified azido sugars and the alkyne functionalised naphthalimide, its photophysical and biological evaluation as well as in vitro CuAAC reaction with modified sugars.

In Chapter 4, novel glycosylated naphthalimide derivatives were synthesised with the aim to act as prodrugs of the known drug Amonafide. These derivatives can be activated using glycosidase enzymes as previously described for Chapter 2, however, the naphthalimide core in this case is the known drug Amonafide. Enzymatic activation entails a self-immolative process where Amonafide is released along with a non-toxic linker and the carbohydrate unit. The compounds were fully characterised spectroscopically and in
in vitro studies in a range of mammalian cancerous cell lines demonstrated a successful delivery of Amonafide inside the cells, thus causing cell death.

In Chapter 5 the application of glycosylated naphthalimides and the corresponding Troger’s bases derivatives to function as chemical probes for lectin sensing is discussed. A monovalent naphthalimide was directly compared to a divalent naphthalimide (Figure 1.7.2) to investigate if the multivalent mechanism improves lectin binding. Both ligands proved to bind to Con A (lectin used as a model) and this binding was not affected by the presence of other macromolecules. In vitro studies proved the compound non-toxic as desired.

![Figure 1.7.2. Compounds 44 and 45 used as mono- and divalent ligands, respectively, for Con A binding.](image)

Chapter 6 summarises the varying self-assemblies of the glycosylated naphthalimides prepared in all the previous chapters. Different morphologies were obtained even when the structural changes were minimal, which reflects the capacity of the naphthalimides to aggregate but also the versatility imparted by the carbohydrate moiety. The varying morphologies were analysed using a range of techniques such as confocal microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Chapter 7 gives an overall conclusion on the relevance of glycosylated naphthalimides for medicinal applications based on the results obtained in the previous chapters.
Lastly, Chapter 8 describes the experimental procedure used in all the previous chapters as well as characterisation of the compounds by NMR and IR spectroscopy, mass spectrometry (MS) and physical tests such as melting points or specific rotation.
Chapter 2

Glycosylated Naphthalimides as Enzyme-Activatable Probes
Chapter 2. Glycosylated Naphthalimides as Enzyme-Activatable Probes

2.1 Introduction

2.1.1. Naphthalimides as fluorescent probes used in vitro and in vivo

Naphthalimides have been widely used as biological probes due to their good luminescence properties, their ease of synthesis and tunability. Recently, the use of naphthalimides as specific probes for mammalian cancer cell lines as well as bacteria has been reported. Many naphthalimide probes are formed by a “off-on” mechanism, being the activated form the 4-amino-1,8-naphthalimides due to its high luminescence properties, with marked Stokes shift and high quantum yields. To achieve selective on-switching the amine group can be masked either by a reducible group or with a self-immolative linker that releases the 4-amino-1,8-naphthalimide (Scheme 2.1.1). An example of such design is that of McCarley et al. who developed a naphthalimide base fluorescence probe in which fluorescence is induced in the presence of quinone oxido reductase, an enzyme that is up-regulated in cancer cells at different levels. They were able to demonstrate the probe activation by endogenous enzymes and measured the levels of activation, by means of fluorescence intensity, in a library of cell lines, thus being able to identify certain cell lines.

Scheme 2.1.1. Different naphthalimide derivatives that can form 4-aminonaphthalimide under endogenous stimuli.

As the example developed by McCarley and co-workers, the use of endogenous enzymes allows for the discrimination between different tissues, therefore enabling high selectivity. Some examples of enzyme activatable probes will be discussed in the next section.

2.1.2 Enzyme Activatable probes

As enzymes can be used as biomarkers for certain diseases they have been used previously in the literature for the selective delivery or activation of fluorescence probes. From the selective detection of the endoplasmatic reticulum using trans-amino
peptidases to the detection of cancer cells via thioredoxic reductase which is known to be over expressed in cancerous cells. An example of enzyme-activated probe using naphthalimides was developed by Elmes’ group utilising the nitroreductase enzyme (NTR). Enzymatic activation generates a cascade reaction that leads to the fragmentation of the molecule changing its photophysical properties, releasing a 4-amino-1,8-naphthalimide derivative (compound 47, Scheme 2.1.2), allowing for visualisation with the naked eye acting as a probe for reductive cellular stress. The fragmentation process leading to the release of a fluorophore which emits at longer wavelengths is shown in Scheme 2.1.2.

Enzyme activatable probes can also be used in non-mammalian cell lines. For instance, Yu Hu et al. also exploited NTR in bacteria to switch on the fluorescence upon enzymatic activity due to the reduction of the nitro-imidazol group acting as quencher. This enables the early detection of pathogens as it allowed for in vitro visualisation in a range of bacteria, obtaining different fluorescence intensity values which can be correlated with the presence of NTR in different pathogens, paving the possibility of using this system for detection of pathogens.

Cirillo and co-workers reported the detection of MTb in live mice by a fluorescent probe that was activated upon exposure to β-lactamase, which is expressed in MTb but not in mammalian cell lines. The probe was based on a FRET system in which the quencher is cleaved after enzymatic activity turning on the fluorescence.

2.1.3 Carbohydrates in fluorescent probes
Carbohydrates participate in a range of biological processes such as recognition events between cells with other cells and cells and other molecules. Thus, including glycans in fluorophores allows for cell targeting and cell uptake via ASGPr-mediated endocytosis or

Scheme 2.1.2. Enzyme activatable probe developed by Elme’s group using NTR.
the insulin-independent glucose transporter GLUT-1.\textsuperscript{143} Furthermore, carbohydrates increase hydrophilicity, which is beneficial for \textit{in vitro} and \textit{in vivo} applications.\textsuperscript{128,144}

Some examples of fluorescent probes containing glycans can be rhodamine containing \textit{N}-glycans for the detection of lysosome-dependant apoptosis as the lysosomal membrane is heavily glycosylated with \textit{N}-glycans,\textsuperscript{145} glucosylated coumarin for the identification of the enhanced metabolism in cancer cells,\textsuperscript{146} and glycosylated porphyrins for PDT.\textsuperscript{23}

\subsection*{2.1.4 Previous examples of glycosylated naphthalimides used as fluorescent probes}
Some examples of glycosylated naphthalimides used as probes for lysosomes, intracellular thiols or senescence have been published recently.\textsuperscript{94,127,129} Several groups have exploited the conjugation to a \textit{β-D}-galactose unit that regulates its uptake via ASGP-R receptor, which is over-expressed in HepG2 (hepatic carcinoma cell line) leading to a cellular internalisation.\textsuperscript{127,147} For instance, Kim \textit{et al.} demonstrated that only the \textit{β-D}-galactose conjugated naphthalimide, compound 48 in Figure 2.1.1, was able to undergo cell uptake. As previously described, compound 48 possesses a self-immolative linker as the disulfide bond will be cleaved in the presence of endogenous thiols, which is related to oxidative stress conditions. The cleavage of the disulfide bond releases the 4-amino-1,8-naphthalimide derivative, creating a red-sift in the fluorescence emission. This was demonstrated \textit{in vivo} using rats.\textsuperscript{10a}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures/figure2.1.1.png}
\caption{Examples of previous glycosylated naphthalimides used as fluorescence probes \textit{in vitro}.}
\end{figure}

Jia and co-workers instead exploited the over-expression of CD44 receptor, for hyaluronic acid, in cancer cells to deliver a naphthalimide functionalised with a hyaluronic
acid residue (compound 49, Figure 2.1.1). Once inside the cells, compound 49 can be used as a cancer probe using fluorescence polarisation. Both examples rely on the overexpression of certain carbohydrate receptors to deliver the fluorescent probe inside the cells, however as explained in section 2.1.2, the use of glycosidase enzymes to hydrolyse glycosidic linkages is well-known. Thus, the use of glycosidase enzymes to release and deliver a fluorophore inside cells can be used as an alternative in systems in which the cellular uptake via membrane receptors is not possible. Similarly to the examples previously described that exploited the over-expression of membrane-receptors, these systems would exploit the over-expression of glycosidase enzymes in certain tumours. Importantly, they also offer the possibility of delivering a fluorophore inside the cells as desired by simply adding enzymes, as glycosidase enzymes would not interfere with the normal cell proliferation.

2.1.5 Work described in this chapter
Initially, the aims of this PhD project were to develop a family of glycosylated naphthalimides that could serve as imaging tools for cancer. The glycosylation of naphthalimides would serve to improve their solubility and to increase their specificity towards cancerous cells. Various glycosylation patterns were investigated in order to ascertain the effects of carbohydrates in different cell lines. However, in vitro results on cancer cell lines demonstrated that none of the synthesised compounds were being taken up by the cells, and thus uptake via carbohydrate-mediated receptor was not occurring. No similar results are reported in the literature when using natural unprotected carbohydrates in glycoconjugates. Since previous experiments carried out in the Gunnlaugsson group demonstrated that naphthalimides are rapidly uptaken by the cells, an enzyme-mediated mechanism for the release of the naphthalimide motif using glycosidases, creating not only an activatable probe using enzymes but also achieving cell uptake only in the regions where the enzyme is up-regulated was investigated (Scheme 2.1.3). In this system, the glyconaphthalimide was not able to enter in the cells unless a glycosidase enzyme is present in the media, which would hydrolyse the glycosidic linkage releasing the naphthalimide moiety (compound 50 in Scheme 2.1.3). The naphthalimide unit would be able to penetrate the cells rapidly due to its low molecular weight and high hydrophobicity, giving fluorescence emission inside the cells, which could be monitored using confocal microscopy. Therefore, the selective delivery of a fluorescent probe inside the cells upon
enzymatic addition can be achieved, and opens the possibility of using this system in vivo where endogenous enzymes are overexpressed in certain tumours, leading to fluorescence signal observed only in the tumorous areas. Lastly, the toxicity of these compounds would be analysed, by means of IC\textsubscript{50} values, to validate their application as cancerous fluorescent probes.

Scheme 2.1.3. Schematic illustration of the enzyme-mediated release of naphthalimide derivate 50 capable of cellular uptake.

2.2 Synthesis

2.2.1 Synthesis of compounds 63(a-e) and 50.

Compounds 54(a-d) were synthesised by glycosylation of commercially available peracetylated glycosides using 3-chloro-1-propanol and BF\textsubscript{3} \cdot OEt\textsubscript{2} as acceptor and activator, respectively (Scheme 2.2.1.a). This reaction was followed by the displacement of the chloride using sodium azide in DMF at 80 °C to introduce the azido clickable handle. Deprotection of the glycosides under Zemplén conditions\textsuperscript{148} furnished the desired azido-glycosides in good yields.

The synthesis of 58 began by formation of the lactosyl bromide 56 from peracetylated lactose using HBr in AcOH (Scheme 2.2.1.b). Compound 57 was obtained by the introduction of the azide, followed by deacetylation as described previously.

The 4-amino-1,8-naphthalimide motif was synthesised using conditions described by Peng et al.\textsuperscript{149} by refluxing naphthalic anhydride with propargylamine in ethanol for 6 h to give 61 in 87% yield (Scheme 2.2.2).
Chapter 2. Glycosylated Naphthalimides as Enzyme-Activatable Probes

Reduction of the nitro group was first attempted using Pd/C 10 wt.% under H₂ atmosphere, which afforded poor yields. Subsequent attempts using SnCl₂ as the reducing agent proved to be successful, furnishing 62 in 86% yield. The glycosylated naphthalimides were synthesised using [Cu(MeCN)₄]BF₄ as catalyst in a Huisgen 1,3-dipolar cycloaddition, as previously described in the literature. However, purification issues resulted in poor yields. 1,8-naphthalimides easily form aggregates due to π-π stacking interactions, making compounds that possess different polarities, in this case, the glycosylated naphthalimide vs. the non-glycosylated one, very difficult to separate under normal chromatographic conditions. To circumvent this problem, size exclusion chromatography column was employed.

Following the synthesis of the desired glycosylated naphthalimides conjugates, the glycosidase-catalysed hydrolysis of the glycosidic linkage was investigated as a method for release of the naphthalimide core. Compound 50 was prepared from the galactosyl naphthalimide 63a in an enzymatic reaction by stirring with β-galactosidase in phosphate buffer solution (PBS) at 30 °C (Scheme 2.2.2) giving 50 in 95% yield.
Scheme 2.2.2. Synthesis of compounds 63(a-e) and 50.

All the compounds were characterised with standard techniques such as NMR, IR and MS, amongst others. Figure 2.2.1 shows the $^1$H NMR spectra of compounds 63a (a) and 50 (b) recorded in D$_2$O and CD$_3$OD, respectively. The peak found at 8.00 ppm demonstrated the successful synthesis of compound 63a as it represents the C=CH in the triazole ring (Figure 2.2.1a). Enzymatic hydrolysis giving compound 50 is proven by the lack of the typical carbohydrate peaks found between 5.16 – 3.65 ppm. An accurate mass of m/z = 536.1753 (ESI)$^+$ was obtained for compound 63a, corresponding with C$_{24}$H$_{27}$N$_5$O$_8$ ([M+Na]$^+$).

Although compounds similar to 50 are known for showing anticancer activity, no cytotoxic results were observed with any of the compounds in relevant cell lines (see later discussion in section 2.4.3). Therefore, efforts to develop glycosylated naphthalimide derivatives with interesting anticancer properties were carried out and are described in the next section.
Chapter 2. Glycosylated Naphthalimides as Enzyme-Activatable Probes

2.2.2 Design and Synthesis of compound 64 and 74

As an attempt to create a cytotoxic anticancer drug, compound 64 was designed taking into account the structural features of previous cytotoxic naphthalimides found in the literature.\textsuperscript{109,150-152} The presence of 2-3 methylene units in the naphthalimide linker, analogous to Amonafide (Figure 2.2.2), has been proven to play a pivotal role in anticancer activity.\textsuperscript{104} Therefore many derivatives have been developed based on this observation.\textsuperscript{109,150-152} Meng and co-workers synthesised a family of fused-heterocyclic naphthalimides (compounds 65 in Figure 2.2.2) and studied their influence in HCT-116 and HepG2 cell lines, finding higher IC\textsubscript{50} values than Amonafide (4.55 and 1.41 μM, respectively).\textsuperscript{109} X. Li \textit{et al.} developed a similar strategy using click chemistry to introduce a phenyl ring thereby increasing the planarity and enhancing the intercalation capability (Figure 2.2.2, compound 66).\textsuperscript{150,152} The cytotoxic activity of those compounds was evaluated in HeLa and MCF-7, showing improved activity over Amonafide in MCF-7. Intercalative behavior of the naphthalimide was demonstrated spectroscopically by DNA titrations. Interestingly, the commercial anticancer drug Doxorubicin (Figure 2.2.2), which belongs to the anthracyclines family and it is also an isopoiisomerase II inhibitor, and it
Figure 2.2.2. Comparison between previous compounds reported in literature (65 and 66) and the designed compound 64 and Doxorubicin.

also present a carbohydrate unit.\textsuperscript{153} Bearing this in mind, compound 64 was designed, which retains the tertiary amine side-chain and possesses a similar triazole moiety.

Scheme 2.2.3. Synthesis of compounds 64 (a) and 74 (b).
Chapter 2. Glycosylated Naphthalimides as Enzyme-Activatable Probes

The synthesis for the preparation of the related glycosylated naphthalimide \( \textbf{64} \) is described in Scheme 2.2.3b, peracetylated-\( \beta \)-galactose (\( \textbf{51a} \)) was glycosylated with propargyl alcohol (\( \textbf{67} \)) using BF\(_3\cdot\text{OEt}_2\) as activator (Scheme 2.2.3a) followed by deacetylation (under Zemplén conditions) to obtain compound \( \textbf{69} \).

The modified naphthalimide moiety (Scheme 2.2.3b) was synthesised in a similar manner as previously described for compound \( \textbf{62} \), starting from 4-bromo-1,8-naphthalic anhydride (\( \textbf{70} \)) and reacting it with \( N,N \)-dimethylethylenediamine in EtOH at reflux (Scheme 2.2.3b).

Displacement of the bromide using sodium azide proceeded quantitatively to give compound \( \textbf{73} \). The click reaction using the same conditions previously described for \( \textbf{63a-e} \) gave \( \textbf{64} \) in 29% yield. The yield of this reaction was lowered by the purification issues of the glycosylated naphthalimides. Following this, compound \( \textbf{74} \) was synthesised in a similar enzymatic reaction for the synthesis of compound \( \textbf{50} \), to furnish the product in 87% yield (Scheme 2.2.3b).

As previously explained, compounds \( \textbf{64} \) and \( \textbf{74} \) were fully characterised and their \(^1\text{H}\) NMR can be found in Figure 2.2.3. An accurate mass was obtained for compound \( \textbf{74} \) of m/z = 366.1563 (ESI) for \([\text{M+H}]^+\) (\( \text{C}_{19}\text{H}_{20}\text{N}_5\text{O}_3 \)).

![Figure 2.2.3. \(^1\text{H}\) NMR (600 MHz) of a) compound \( \textbf{64} \) recorded in CD\(_3\)OD and b) \( \textbf{74} \) recorded in D\(_2\)O.](image-url)
An O-acetylated version of compound 64 was also synthesised (compound 75, Scheme 2.2.4), subjecting compound 75 to Zemplén conditions would give 74. Compound 75 was synthesised by the click reaction of 68 and 73, using the same Cu catalyst previously used for compound 74, in this case however, the reaction was carried out at lower temperature (40 °C) to avoid decomposition of the carbohydrate moiety 68, as they are more prone to decompose when they are protected. In an attempt to ensure completion a longer reaction time of 3 h was used. The product 75 was obtained in 15% yield.

The low yield obtained was due to the fact that the azide group was reduced to the amine, giving compound 76 in 24% yield. This reduction was quite unexpected as no reducing species were present in solution. However, the reduction of aromatic azides in the presence of Cu(I) has been reported in the literature by Wang et al., who reported the reduction of an arylazide to an amine while also attempting a CuAAC reaction. Their investigations into this phenomenon demonstrated that the presence of Cu(I) promoted the reaction in various polar solvents such as DMF, DMSO, and H₂O. Interestingly, the reaction was proven to be temperature dependant, with full conversion observed at 80 °C.

However, the reduced product 76 was not observed in the previous CuAAC carried out for the synthesis of 74, which was performed at higher temperature (115 °C). It could be postulated that the side product 76 was formed in this case as the CuACC was not as promoted due to the lower temperature and longer reaction time (3 h).

The synthesis of compound 75 was done in parallel as an alternative route to the synthesis of 74, however, as the reaction yield was lower this route was not pursued. The spectroscopic characteristic and biological evaluation of compound 75 will be discussed in Chapter 4, as comparison with similar compounds described there.

Scheme 2.2.4. Synthesis of compound 75 as an O-acetylated version of compound 74, and compound 76 obtained as side-product.
Prior to photophysical and biological evaluation, the affinity of the glycosylated naphthalimides as substrate for the glycosidase enzymes was evaluated using β-galactosidase and β-glucosidase enzymes. Compounds 63a and 63b were treated with 1 U of β-galactosidase and β-glucosidase, respectively. Enzymes are measured by units (U), which corresponds to the amount of enzyme that is able to catalyse the conversion of 1 μmole of substrate per minute.\textsuperscript{155} Lactose-containing naphthalimides (63d and 63e) were treated first with β-galactosidase followed by β-glucosidase (Scheme 2.2.5). Both enzymes exerted their activity on compounds 63(a-d), readily hydrolysing the glycosidic bond and releasing 50. Mass spectrometry confirmed that the reaction had occurred and that naphthalimide 50 had been released (Figure A.2.1). Compound 63e was designed to be a negative control, as there is no glycosidic linkage between the glucose unit and the triazole therefore the enzyme would be unable to exert its activity. As predicted, compound 63e only showed activity of the β-galactosidase enzyme and therefore compound 50 was not released (Scheme 2.2.5).

Scheme 2.2.5. a) Release of the naphthalimide core (compound 50) from compound 63d by addition of β-galactosidase and β-glucosidase, respectively. b) Release of compound 79 from compound 63e by β-galactosidase.
Chapter 2. Glycosylated Naphthalimides as Enzyme-Activatable Probes

After synthesising and characterising a range of glycosylated naphthalimide derivatives, their photophysical properties were evaluated. Section 2.3 describes the absorption, fluorescence and excitation spectra recorded in solvents of different polarity.

2.3. Photophysical Evaluation

The UV-vis and fluorescence emission spectra of 63a (1 × 10^{-5} M) in 10 mM PBS pH 7.2 are shown in Figure 2.3.1. A broad absorption band is observed at ca. 432 nm (ε = 6,500 M^{-1} cm^{-1}) due to the ICT between the electron-donating amino group in the 4-position of the naphthalene ring, and the electron withdrawing carbonyl group.

Figure 2.3.1. Compound 63a and UV-vis absorption and fluorescence spectra (λ_{max} = 430 nm) of compound 63a (1 × 10^{-5} M) in 10 mM PBS pH 7.2 recorded at rt.

Higher energy transitions (π-π*) can also be observed at ca. 250 nm. As can be seen in Figure 2.3.1, the excitation spectrum (red line) matches the absorption spectra. The excitation of 63a at 430 nm gives a broad emission band centred at 550 nm (Figure 2.3.1).

Absorbance and fluorescence spectra of 63a were recorded vs. concentration. The results demonstrated that, in aqueous solution up to 0.6 mM, molecules behave as monomers following Beer-Lambert law (Figure 2.3.2a). However, this effect does not apply to concentrations above 0.6 mM, which could be the result of aggregation processes or inner-filter effect.
Figure 2.3.2. Changes in a) absorbance and b) fluorescence spectra ($\lambda_{\text{max}} = 430$ nm) of compound 63a in 10 mM PBS pH 7.2 with respect to concentration. All the measurements were recorded at rt. Experiment carried out twice.

As a result of this aggregation, fluorescence emission is quenched, showing its maxima efficiency at 0.1 mM (Figure 2.3.2b). A similar aggregation effect has been observed for all the other compounds. The ability of naphthalimides molecules to aggregate due to $\pi-\pi$ stacking interactions and their dipole nature has been widely described in literature. X-ray diffraction studies performed previously in the Gunnlaugsson group with similar compounds demonstrated that head-to-tail arrangements of the naphthalimides took place (Figure 2.3.3). In compound 80, the ethylene group adopted an anti-conformation and the pyridinium ring was not co-planar to the naphthalimide ring, with an angle of ca. 35° out of the naphthalimide plane. The distance between the naphthalimide units stacked by $\pi-\pi$ interaction was of 3.606 Å.$^{156}$

Figure 2.3.3. Chemical structure of a compound previously described (80) and the X-ray crystal structure of showing $\pi-\pi$ interactions.
A crystal structure of 50 was obtained in water. The crystal structure was resolved by Dr. Chris Hawes. As it can be seen in Figure 2.3.4 naphthalimides are forming $\pi-\pi$ stacking interaction, being arranged in a head-to-tail fashion with a 120° orientation between one tail and the next head, which was not seen in the previous structure (compound 80). They are forming columns where the chains are bent to the internal phase of the columns, interacting with the triazole groups and stabilising them. There are two types of hydrogen bonds (donor and acceptor) between the triazole groups and the chain, with the CH$_2$ and the alcohol, respectively. Therefore, the triazole groups are held in place.

The molecules are interacting with a molecule of water, shared between four molecules, forming hydrogen-bonding interactions with the terminal alcohol. The naphthalimides can be orientated in two positions (Figure 2.3.4b), without a favoured conformation with a distribution 3:1, approximately, therefore the molecule presents a high degree of disorder.

**Figure 2.3.4.** a) Chemical structure of compound 50 and its X-ray crystal structure formed in H$_2$O showing its $\pi-\pi$ interactions (b) and two different possible positions of the molecules (c).

UV-vis absorption and fluorescence studies of compounds 64 and 74 showed a blue shift occurring for the ICT band (ca. 347 nm) of 64 in comparison to 63a (Figure 2.3.5), due to the influence of the triazole ring in the 4-position, which connection through $N$-1 makes conjugation hindered due to its sp$^3$ hybridisation. This influence has been reported in literature for similar molecules.¹⁵⁷ Excitation of compounds 64 and 74 at 347 nm results in broad emission at 443 nm (Figure 2.3.5a). Concentration studies performed in PBS
solution showed that aggregation could occur at concentrations of 0.15 mM or higher (Figure 2.3.5b).

![Figure 2.3.5](image)

**Figure 2.3.5.** a) UV-vis absorbption and the emission spectrum ($\lambda_{exc} = 347$ nm) of 20 (0.1 mM) in 10 mM PBS pH 7.2 recorded at rt. b) Changes in absorbance of 64 in 10 mM PBS pH 7.2 with respect to concentration. All measurements were recorded at rt.

A summary of the absorption and fluorescence emission properties, along with their respective molar extinction coefficients ($\varepsilon$) is given in Table 2.1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{max}$ (nm)</th>
<th>ICT ($\varepsilon$ (M$^{-1}$ cm$^{-1}$) $\pm$10%)</th>
<th>$\Phi_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>63a</td>
<td>254</td>
<td>433 (6,500)</td>
<td>12.7</td>
</tr>
<tr>
<td>63b</td>
<td>254</td>
<td>435 (6,400)</td>
<td>11.6</td>
</tr>
<tr>
<td>63c</td>
<td>254</td>
<td>434 (12,000)</td>
<td>13.1</td>
</tr>
<tr>
<td>63d</td>
<td>254</td>
<td>433 (12,000)</td>
<td>11.5</td>
</tr>
<tr>
<td>63d'</td>
<td>253</td>
<td>432 (3,700)</td>
<td>11.8</td>
</tr>
<tr>
<td>50</td>
<td>254</td>
<td>434 (8,800)</td>
<td>11.0</td>
</tr>
<tr>
<td>64</td>
<td>a</td>
<td>347 (19,000)</td>
<td>a</td>
</tr>
<tr>
<td>74</td>
<td>a</td>
<td>347 (4,900)</td>
<td>a</td>
</tr>
</tbody>
</table>

*a Data could not be registered.

The fluorescence quantum yields ($\Phi_F$) of emission of compounds 63(a-e) were measured in PBS at pH 7.2 using fluorescein ($\Phi_F = 0.920$ in 0.1 N NaOH) as the primary
reference standard, at the excitation wavelength of 436 nm using equation 8.1 described in Chapter 8, and are presented in Table 2.1. The data was collected in the range of 436 – 700 nm. $\Phi_F$ of compounds 64 and 74 could not be measured as they do not absorb in the same region as the standard fluorescein.

**2.3.1 Effect of Solvent Polarity on the Photophysical Properties**

As was previously mentioned, the photophysical properties of naphthalimides are governed by an ICT system, which is highly dependent on the solvent, phenomena known as solvatochromic effect. The large excited state dipole moment that arises from the ICT can vary depending on the solvent polarity and its hydrogen-bond donor or acceptor capacity. When polar protic solvents are used, proton exchange of the 4-amino moiety may occur, leading to a less energetic excited state and ICT bands shifted towards the red can be observed, as well as lower fluorescence intensity.

As a representative example, the absorption and emission spectra of 63a and 64 were recorded in different solvents to investigate the effect of solvent polarity and are shown in Figure 2.3.6.

Although the optical density (OD) remains relatively unchanged, higher $\varepsilon$ values were obtained for both cases in MeOH. A moderate hypsochromic shift (ca. 6-15 nm) was observed for both compounds when less polar solvents were used. Although MeOH and MeCN posses similar polarity value, proton exchange from the amino nitrogen cannot occur in MeCN, leading to a blue shift (ca. 7-13 nm).

In contrast to the absorption spectra, a quenching of the fluorescence intensity was observed in aqueous solvents, due to the stabilisation of the ICT. However, the blue-shifted behaviour persists for the more apolar solvents. The absorption properties of 63a and 64 for the different solvents investigated are summarised in Table 2.2.
Figure 2.3.6. a) and c) UV-vis absorption of 63a and 64 (20 μM), respectively, in different solvents. b) and d) fluorescence ($\lambda_{\text{max}} = 430$ nm) spectra of 63a and 64 (20 μM) respectively in different solvents. All measurements were recorded at rt.

Table 2.2. Variation in the ICT band (nm) of the UV-vis spectra of 63A and 12 in different solvents.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CHCl$_3$</th>
<th>MeCN</th>
<th>MeOH</th>
<th>H$_2$O</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>63a</td>
<td>425</td>
<td>418</td>
<td>431</td>
<td>434</td>
<td>433</td>
</tr>
<tr>
<td>64</td>
<td>a</td>
<td>427</td>
<td>434</td>
<td>434</td>
<td>433</td>
</tr>
</tbody>
</table>

a Data could not be recorded due to solubility issues.

After evaluation of the characteristic bands of the glycosylated naphthalimides, the pH stability of compounds 64 and 74, possessing a tertiary amine susceptible to protonation was then evaluated, to investigate if this protonation affects their photophysical properties.
2.3.2 pH evaluation and pKa determination for compounds 64 and 74
As the activity of compounds 64 and 74 should be within the physiological pH window, they were subjected to a spectroscopic pH titration, where both the ground and the excited states were monitored (Figure 2.3.7).

![Figure 2.3.7](image)

Figure 2.3.7. a) and c) Changes in the UV-vis absorption of compound 64 (1 \( \times \) 10^{-5} M) and compound 74 (1 \( \times \) 10^{-5} M), respectively, with the plot of maximum absorbance values at 298 nm and 347 nm vs. pH for graphs as inserts. b) and d) changes in fluorescence emission spectra (\( \lambda_{exc} = 298 \) nm, 314 nm and 347 nm) of compound 64 (1 \( \times \) 10^{-5} M) and compound 74 (1 \( \times \) 10^{-5} M), respectively, in PBS with different pH. All measurements were recorded at rt. Representative example of three independent experiments.

The maximum absorption band, ICT band, for compounds 64 and 74 was centred at 347 nm, and as shown in Figure 2.3.7a and c, upon basification a decrease in the absorbance at \( \lambda_{max} \) 347 nm was observed, whereas a second band was seen at 298 nm above pH 10. An isosbestic point at 314 nm was observed. The fluorescence emission spectra were also recorded upon excitation at 298, 314 and 347 nm, respectively. As shown in Figure 2.3.7b and d, a significant decrease in the fluorescence emission occurred above pH 8. Unfortunately, these changes were not reversible, although a similar trend was observed for compound 64 and 74 when a back titration was carried out. An increase of the band found \( ca. \) 347 nm upon acidification was observed whilst the band found at 314 nm
decreased. However, although the OD and fluorescence intensity values in the back titrations (base-acid) followed the same trend, their magnitudes were not similar enough to the corresponding initial values (acid-base) to be able to consider compounds 64 and 74 as pH sensors.

From these luminescence changes pKa values for compounds 64 and 74 were determined in both H2O and PBS solutions (Table 2.3) and correspond to their conjugated acid. These experiments were carried out twice and pKa values were determined using non-linear regression analysis using the program ReactLab™. These pKa values of ca. 11.4 are in agreement with the ones found in the literature for tertiary amines.158

<table>
<thead>
<tr>
<th>Compound</th>
<th>64</th>
<th>74</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKα</td>
<td>H2O</td>
<td>PBS</td>
</tr>
<tr>
<td>11.47 (± 0.01)</td>
<td>11.35 (± 0.02)</td>
<td>11.39 (± 0.01)</td>
</tr>
</tbody>
</table>

Having investigated the photophysical properties of the compounds and their pH stability, their changes in the luminescence upon enzymatic cleavage, releasing compounds 50 and 74, respectively, was next evaluated.

2.3.5 Enzymatic Activity Evaluation

The changes in the absorption and emission spectra of 63a and 64 over time after the addition of β-galactosidase were next examined at pH 7.2 and 30 °C (optimal conditions for this enzyme). For compound 63a, enzymatic hydrolysis did not lead to a significant change in the ICT absorption or fluorescence emission over time (Figure 2.3.8a and b). Different concentrations of the enzyme (0.01, 0.1 and 1.0 U) were added to the solution, and the same behaviour was observed, however, the treatment of 64 with 0.1 U of the enzyme led to a significant reduction in fluorescence intensity (Figure 2.3.8c and d) with no shift in the λmax = 550 nm. Importantly, no significant changes were seen in the absorption spectra of 74 and 64 upon addition of enzymes, which rules out any non-specific or strong association between the enzyme and the naphthalimide substrate.
Figure 2.3.8. Study of β-galactosidase activity with 63a (1 × 10⁻⁵ M) and 12 (1 × 10⁻⁵ M), respectively. a) and c) UV-vis absorption and b) and d) fluorescence spectra of 63a (λ<sub>max</sub> = 550 nm) and 64 (λ<sub>max</sub> = 440 nm), respectively. All the measurements were recorded at 37 °C. Representative image of two independent experiments.

Since no noticeable changes were observed for the enzymatic release of compound 50, no further studies were carried out with them. However, time-dependent studies were carried out with compound 64 and β-galactosidase at different temperatures, as can be seen in Figure 2.3.9. Prior to incubation with the enzyme, the changes in the fluorescence emission of compound 64 were monitored over time at 25 and 37 °C (Figure 2.3.9a). Although no changes were observed in the emission intensity at 25 °C, a significant decrease occurred when the compound was incubated at 37 °C. Temperature could cause changes in the fluorescence emission due to the disruption of aggregation, as higher temperatures would inhibit the formation of aggregates. However, the aggregation of naphthalimides leads to self-quenching, as previously discussed, therefore an increase in fluorescence emission was expected. Thus, this decrease in the fluorescence emission must be owed to the increase with the temperature of non-radiative processes (or the increase of...
their rate-constant) affecting the excited state. Unfortunately, no examples supporting this hypothesis can be found in the literature for naphthalimides.

After 10 min, the fluorescence intensity remained stable and no further changes were observed. Therefore, compound 64 was pre-incubated at the desired temperature for 15 min before the addition of the enzyme, to ensure that the changes observed in the emission intensity were solely due to the enzymatic hydrolysis. Following this experiment, compound 64 was incubated at different temperatures with 1, 0.1 and 0.01 U of β-galactosidase enzyme (Figure 2.3.9b-d), respectively. Although the optimal temperature specified for this enzyme is 30 °C, in all cases the enzymatic reaction proceeded quicker when carried out at 37 °C.

![Graphs showing emission intensity over time at different temperatures and with different enzyme concentrations.](image)

**Figure 2.3.9.** Changes in the emission spectra of 64 (1 × 10^{-5} M, λ_{em} = 440 nm) at different temperatures (a) and upon the addition of β-galactosidase enzyme b) 1 U, c) 0.1 U and d) 0.01 U recorded in PBS.

Importantly, the reaction at low concentrations of enzyme (0.01 U, Figure 2.3.9d) also proceeded at relatively short times. As can be seen in Figure 2.3.9d the hydrolysis had
almost completely finished in 1 h. These results demonstrated that this class of compounds could be successfully used for the enzymatic cleavage in short periods of time.

Unfortunately, attempts to fit this data in order to obtain the rate constant were not successful, indicating that the kinetics are more complex than a first order rate, which is common in enzymatic reactions as a mixture of substrate, substrate + Enzyme, Enzyme + product and product are present in the reaction mixture. Therefore, enzymatic reactions are usually studied using the Michaelis-Menten model. This model assumes that the product formation is the limiting reaction step and therefore the binding and dissociation occurred much faster. The Michaelis-Menten constant ($K_M$) can be obtained experimentally by measuring the changes in the absorption spectrum and plotting them against the substrate concentration. Unfortunately, no changes were observed in our system and this method could not be employed.

A control experiment was carried out with compound 64 ($1 \times 10^{-5} \text{ M}$) and β-D-galactose β-galactosidase (1 U) and β-glucosidase, respectively, to prove that only the right enzyme (β-galactosidase) was able to release compound 74, inducing a decrease in the fluorescence emission. As seen in Figure 2.3.10a, the treatment of 64 with 1 U of β-glucosidase did not provoke any changes in the fluorescence emission.

A second control experiment was carried out by combining the previously isolated compound 74 ($1 \times 10^{-5} \text{ M}$), with β-D-galactose (1 U) and β-galactosidase (1 U), respectively. The results, shown in Figure 2.3.10b, proved that the combination of compound 74 with β-D-galactose or the enzyme did not lead to any change in the fluorescence emission.

![Figure 2.3.10](image-url)
fluorescence, proving that the decreased in emission intensity previously observed for the enzymatic hydrolysis of compound 64 is due to the release of compound 74, which fluorescence emission is lower, and not to an interaction between compound 64 with the release unit β-D-galactose or the enzyme β-galactosidase.

After demonstrating the enzymatic hydrolysis, the capacity of compounds 50 and 63a to act as DNA intercalator was then investigated.

### 2.3.6 stDNA Binding Evaluation
As will be discussed in section 2.4.1, only compounds 50 and 74 were internalised by the cells, hence their affinity for stDNA was evaluated by carrying out stDNA titrations in PBS pH 7.2 at 37 °C. As can be observed in Figure 2.3.11a and c, absorbance values decreased with increased DNA concentration. Changes in the $\lambda_{\text{max}}$ were plotted against the stDNA equivalents (Figure 2.3.11b and d) and a linear correlation is observed between the OD and stDNA concentration, suggesting that compound 50 binds to stDNA, however with very low affinity. Although compound 74 was expected to have a higher affinity for stDNA, due to the presence of the basic tertiary amine having an affinity for the acidic stDNA, the same behaviour as for 50 was observed. No further experiments to determine the binding constant values were performed, as the binding process was too weak to be relevant in biological processes. Changes in the $\varepsilon$ were also plotted against the stDNA equivalents in order to correct for the dilution factor (Figure A.2.2), however the same linear correlation was observed.

Following spectroscopic characterisation the biological evaluation of compounds 63(a-e), 50, 64 and 74 was carried out in three different cancerous mammalian cell lines (HeLa, HepG2 and HCT-116). Cell uptake studies, anti-proliferative effect as well as subcellular localisation of the compounds is described in section 2.4.
Figure 2.3.11. Study of stDNA binding activity of 50 and 74, respectively. a) and c) UV/Vis absorption spectra and b) and d) changes in the $\lambda_{\text{max}}$ 430 (for compound 50) and 347 nm (for 74), respectively, vs. DNA equivalents. All the measurements were recorded at 37 ºC. Experiments carried out in duplicates.

2.4 Biological Evaluation
2.4.1 Cellular Uptake of Compounds 63(a-e) and 64.
HeLa cells were incubated with each of the compounds 63(b-e) (0.1 mM) for 3 h, after which time they were imaged as live cells by using confocal fluorescence microscopy, upon excitation at 405 nm. Glycosylation typically enhances tumor cell endocytosis, either through ASGPr-mediated endocytosis or via the insulin-independent glucose transporter GLUT-1 pathway. As shown in Figure 2.4.1, cellular uptake did not occur and thus the compounds remained outside the cells. The recorded green-channel fluorescence only arising from compounds within the extracellular media excited with a high laser power (the cells appearing black with the nucleus stained by DRAQ5 appearing as red emission).
Chapter 2. Glycosylated Naphthalimides as Enzyme-Activatable Probes

Figure 2.4.1. Incubation of compounds 63(b-e) (0.1 mM) in HeLa cells for 3 h. Representative image of three independent experiments.

Longer incubation times up to 24 h did not show any change in the cellular uptake processes. The effect on the uptake of the released naphthalimides core upon enzymatic activation in cancer cells was next investigated. A 3 h incubation of HeLa cells with compound 63a (0.1 mM) was followed by the subsequent addition of the β-galactosidase enzyme (1 U) 1.5 h later (Figure 2.4.2). Results showed that the naphthalimide moiety had been taken up by the cells upon hydrolysis of the glycosidic linkage by the

Figure 2.4.2. Incubation of compound 63a at different concentrations in HeLa cells for 3 h (a) and in the presence of β-galactosidase (1 U) for further 1.5 h (b). Image representative of three independent experiments.
glycosidase enzyme, as can be seen in figure 2.4.2 by the green fluorescence emitted by compound 50 inside the cytoplasm of the cells. The released carbohydrate counterpart may also undergo endocytosis, however its visualization is not possible.

To prove that this uptake process was not concentration dependent, HeLa cells were also incubated with compound 63a at varying concentrations, followed by the subsequent addition of the β-galactosidase enzyme (1 U) 1.5 h later. Importantly, the results showed that the naphthalimide 50 had been released and had been taken up by the cells (Figure 2.4.2), thus demonstrating that the process is not concentration dependent. However, cell uptake of the released naphthalimide is more difficult to image at 0.01 mM concentration and required higher laser power to be visualised.

Carbohydrate-mediated endocytosis is widely used as a selective way to transport macromolecules. For instance, hepatocytes express the ASGP-R allowing them to bind with β-D-galactose residues and assimilate large molecules. In order to further investigate the generality of the enzyme release approach, compound 63a was tested with two other different cell lines; HCT-116 (colon carcinoma) and HepG2 (hepatocellular carcinoma), the latter known to express ASGP-R. As shown in Figure 2.4.3, 63a did not undergo the uptake process in any case without the presence of the enzyme, concluding the enzyme-mediated process is independent of the specific-receptor interaction. Crucially, this finding demonstrates that the presence of the β-D-galactose binding receptor protein does not inhibit activity of the β-galactosidase enzyme and that the β-D-galactose moiety is available for hydrolysis.

Unfortunately, in vitro studies of 63b with the β-glucosidase enzyme were not successful, despite the growth of the cells in a glucose-free media, to prevent saturation of the enzyme. Different concentrations of the enzyme were also investigated (0.01, 0.1 and 1 U), however no improvement was seen. Previous experiments showed positive activity of the β-glucosidase, but unfortunately β-glucosidase appeared inactive in vitro. Nonetheless, all the compounds (63a-d) showed a requirement for enzymatic activation to facilitate cellular entry, indicating that cell uptake appears independent of the carbohydrate receptors.

In vitro studies with 64 showed the same behavior as to that previously described; the compound required the presence of β-galactosidase enzyme to be internalised by the cells. Figure 2.4.4 shows the confocal images of different mammalian cancer cell lines incubated
with compound 64 when no β-galactosidase was present, and no fluorescence signal was observed inside the cells (Figure 2.4.4a).

![Figure 2.4.3](image)

**Figure 2.4.3.** Confocal microscopy image of different cell lines incubated with a) compound 63a (0.1 mM) for 3 h, b) compound 63a (0.1 mM) in the presence of 0.1 U of β-galactosidase for 1.5 h further and c) compound 50 (0.1 mM) for 3 h. Image representative of three independent experiments.

However, when 0.1 U of β-galactosidase were present, the carbohydrate moiety was cleaved, releasing 74, which was able to penetrate the cells, as proved by the fluorescence signal observed inside them in Figure 2.4.4b. Compound 74 was also incubated into the cells (Figure 2.4.4c), however, in this case no fluorescence signal was observed under the same microscopy settings (laser power, pinhole, *etc*).
Figure 2.4.4. Confocal microscopy image of different cell lines incubated with a) compound 64 (0.1 mM) for 3 h, b) compound 64 (0.1 mM) in the presence of 0.1 U of β-galactosidase for 1.5 h and c) Compound 74 (0.1 mM) for 3 h. Image representative of three independent experiments.

After investigating the cellular uptake of compounds 63a-d and 64, and demonstrating that the naphthalimide moiety (compounds 50 and 74) were capable to penetrate the cells, their effect in the cell proliferation was calculated in HeLa cells.
**2.4.2 Cytotoxicity studies in HeLa cells**

The effect on the cell proliferation of HeLa cells of compounds 63a-d, 64 and 74 was investigated via AlamarBlue viability assay for 24 h in a range of concentrations of 100 – 1 μM. The results were obtained as IC\textsubscript{50} values (μM) from three independent experiments, and demonstrated that none of the compounds were toxic in HeLa cells after 24 h incubation. Therefore their IC\textsubscript{50} values were of > 100 μM, which is considered non-toxic.

Having demonstrated that all compounds are non-toxic, including 50 and 74, which are capable of undergoing cell uptake, the subcellular localisation of these compounds was next investigated.

**2.4.3 Subcellular localisation**

Previous *in vitro* studies showed the possibility of a selective localisation of the compounds in the mitochondria.\textsuperscript{116,160} As the compounds did not exhibit any effect in the cell proliferation of HeLa cells, some efforts were made to understand the sublocalisation of compounds 50 and 74 in order to investigated if the delivery of an specific cellular organel was possible using the enzyme-dependent approach.

HeLa cells were transfected with a DsRed plasmid, which selectively labels the mitochondria (\(\lambda_{\text{exc}} = 558\) nm, \(\lambda_{\text{em}} = 583\) nm), followed by the treatment with compounds 63a and 64 (in the presence of 1 U β-galactosidase) and 50 and 74, which were incubated for 2 h prior to confocal imaging. As can be seen in Figure 2.4.5, merged images of the compounds with DsRed demonstrated that the compounds localised mainly in the mitochondria, however, other regions of the cells also presented green fluorescence, indicating that these compounds do not localise selectively in the mitochondria.

Unfortunately, the compounds cannot be used as specific probes for mitochondrial localisation but this opens the possibility of using the enzyme-mediated mechanism for selective mitochondrial probes. A possibility could be to introduce mitochondrial directing group, such as a cationic group, to promote selective localisation as it will be attracted by the negatively charged mitochondrial membrane.
Figure 2.4.5. Confocal image of HeLa cells transfected with DsRed incubated with a) 63a (50 μM, in the presence of 1 U β-galactosidase), b) 50 (50 μM), c) 64 (50 μM, in the presence of 1 U β-galactosidase) and d) 74 (50 μM) for 2 h. Image representative of three independent experiments.
2.5. Conclusions
A family of glycosylated naphthalimides have been synthesised and spectroscopically characterised. The effect on the photophysical properties upon enzymatic treatment was investigated. For compounds 63a-e no changes in the photophysical properties were observed when the hydrolysis of the glycosidic linkage takes place, whereas for compound 63a, which presents the carbohydrate moiety closer to the naphthalimide core, enzymatic cleavage caused a decrease in the fluorescence emission, demonstrating that the substitution pattern affects the photophysical properties of the probe.

The photophysical properties of the compounds were studied under physiological conditions, proving that they are suitable for in vitro and in vivo studies.

Cell uptake studies in a range of mammalian cancer cell lines demonstrated that the glycosylated naphthalimides are not able to undergo cellular uptake, instead remaining outside the cells. However, when glycosidase enzymes were present the naphthalimide core (compound 50 and 74) was released and taken up into the cells. Since compound 50 and 74 are smaller and more hydrophobic they were able to be internalised rapidly by the cells, similarly to the known drug Amonafide. Therefore, a controlled release of fluorescent probes, which can either be activated by the endogenous over-expression of glycosidase enzymes or by applying the enzyme as desired have been developed. This class of compounds can be called pro-probe, as analogy to the term pro-drug.

Efforts were made to understand the sub-cellular localisation of the compounds. Co-localisation studies demonstrated that the compounds localised mainly in the mitochondria. As cell viability assays proved the compounds non-toxic, they can be safely utilised as sub-cellular probes or as probes for the over-expression of glycosidase enzymes in certain tumour regions.
Chapter 3

Glycosylated Naphthalimides for Cellular Labeling
3.1 Introduction
3.1.1 Modified Carbohydrates for Labeling
Molecular imaging of post-translational modifications (PTMs) such as glycosylation offers an important tool for probing the biological role of PTMs.\textsuperscript{161,162} Glycome imaging through metabolic labeling with chemical reporters has emerged as an important technique for molecular imaging \textit{in vivo}.\textsuperscript{163} Small structural modifications in carbohydrates, such as the incorporation of azido or alkyne functionalities (Figure 3.1.1), can be tolerated by cells, being recognised and incorporated into their glycome, glycoproteins and glycolipids.\textsuperscript{164} These modifications need to be small so that the natural biomachinery is still capable of processing these molecules as enzyme substrates. These modifications enable covalently labeling with fluorophores, allowing for the monitoring of metabolic changes \textit{in vitro} and \textit{in vivo}.

\begin{center}
\includegraphics[width=\textwidth]{figure311.png}
\end{center}

\textbf{Figure 3.1.1.} Example of modified sugars that can be used for metabolic cellular labeling.\textsuperscript{165-168}

For instance, \textit{d}-mannosamine is a substrate of the salvage pathway for sialic acid.\textsuperscript{169} Bertozzi and co-workers demonstrated that small modifications in \textit{d}-mannosamine are tolerated. These can include the incorporation of an azido or alkyne group at the \textit{N}-acyl group (compounds 81 and 82).\textsuperscript{167} It estimated that when \textit{d}-mannosamine is incubated in mammalian cell lines between 4-40\% of the natural sialic acids are modified.\textsuperscript{166} These modified mannose derivatives have been used for visualising sialic acids in cell lines, mice and zebrafish, for instance.\textsuperscript{165,166,170}

Compound 83 (Figure 3.1.1), a galactosamine derivative bearing an azido functionality, can be used for the labeling of mucin-type \textit{O}-linked glycans as 83 links glycans to a serine or threonine residue of proteins. 83 can function as an efficient substrate of the GalNAc salvage pathway and is recognised by GalNAc transferase enzymes in the Golgi apparatus.\textsuperscript{168} Similarly, compound 84, Figure 3.1.1, has been used to label proteins located in the cytosol and nucleus.\textsuperscript{171}
3.1.2 Glycome Labeling Through ‘Click’ Chemistry

The term glycome covers the totality of glycans and glycoconjugates synthesised by a cell, tissue or organism. Glycans participate in a wide range of biological processes, from intracellular signalling to tumor growth. Therefore, understanding how glycans modulate these processes is of great interest. Importantly, changes in the glycome are associated with malignancies, such as altered N- and O-glycosylation or up-regulation of sialylated antigens. In many cases the reason behind the altered glycan is still unclear, however, these alterations are useful as bio-markers for disease.

The most common method for covalent glycome labeling is conducted using a “click” reaction (CuAAC) that take place between modified glycosamine residues and alkyne-containing fluorophores. Covalently labeling via click reactions have been widely used for glycome labeling. The process is illustrated in Scheme 3.1.1 using modified mannosamine (compound 81) as an example.

Scheme 3.1.1. Schematic illustration of methabolic and covalent labeling via click reaction between an alkyne probe and an azido-modified sugar.

3.1.3 Intracellular Labeling using ‘Click’ Chemistry

Bioorthogonal click reactions have enabled the selective labeling of proteins. Chi-Huey Wong et al. used naphthalimides for labeling glycoproteins in vivo by CuAAC reaction between a non-fluorescent naphthalimide, bearing azido or alkyne groups at the 4-position (compound 85(a-b) in Scheme 3.1.2), with a modified fucosyl derivative that was incorporated into proteins via the fucose salvage pathway. They exploited the rapid cell uptake of their probe, which was a consequence of the probe’s small size and high hydrophobicity (Scheme 3.1.2). Once the click reaction had taken place, forming a triazole ring (compounds 86(a-b)), the fluorescence signal was enhanced and the process could be monitored by confocal microscopy.
A similar strategy can be applied for labeling the DNA using modified nucleobases (Figure 3.1.2) that can be incorporated into the DNA metabolically. Subsequent click reaction with the corresponding azido- or alkyne-containing fluorescent probes allows for DNA and RNA visualisation and monitoring of chromosome formation. It is important to note that to label DNA \textit{in vitro} the fluorescent probes need to be able to enter the cellular nuclei without damaging it, which can be challenging.

**Figure 3.1.2.** Modified nucleobases bearing alkyne or azido functionality for CuAAC.

### 3.1.4 Copper-free Alternatives for Click Labeling

One of the main drawbacks of \textit{in vitro} and \textit{in vivo} labeling using CuAAC resides in the use of Cu(I) as catalyst due to the toxicity associated with it and problems with protein coordination resulting in loss of catalytic activity. To overcome these issues, “copper-free” techniques were designed by Bertozzi and co-workers in 2004 by utilising strained alkynes. These systems are based on work originally reported by Wittig \textit{et al.} in 1961 in which the reaction between an azide and a cyclooctyne proceeded exothermically to form a regioisomeric mixture of triazole rings (Scheme 3.1.3). The reaction is driven by the release of enthalpy in the transformation from a strained ring to a fused ring, as the bond
angles are more favorable for the sp$^2$-hybridisation of the carbon atoms in the triazole formed.$^{182}$

\[ \text{Scheme 3.1.3. Click reaction between an cyclooctyne and an azide to give a regioisomeric mixture of products.} \]

The use of strained alkynes as a copper-free alternative is termed as Strain Promoted Azide Alkyne Cycloadditions or SPAAC, and since it was first reported there have been many examples of its use for DNA, proteins and glycome labeling.$^{44,183,184}$

### 3.1.5. Design of Glycosylated Naphthalimides for Cellular Labeling

Taking into account previous results discussed in Chapter 2, it was hypothesised that an alkyne containing glycosylated naphthalimide, compound 90 (Scheme 3.1.4a), could function as an effective probe for glycome labeling, as it will remain outside the cell. Compound 90 presents a β-D-galactose unit linked to the naphthalimide core by a triazole ring and three-carbon chain. The naphthalimide displays an alkyne at the 4-position, which changes the photophysical properties, leading to an increase in the fluorescence emission, as was also described by Wong et al.$^{16}$

Interestingly, incubation of compound 90 with β-galactosidase would hydrolyse the glycosidic linkage of compound 90 releasing compound 91, which is more hydrophobic and capable to penetrate the cellular membrane. Once inside the cells, compound 91 could be used to label proteins or DNA (Scheme 3.1.4b).

Therefore, this project aimed for the development of a single probe that can be used for extracellular or intracellular labeling as desired by enzymatic activation.
Scheme 3.1.4. a) Glycome labeling using compound 90 and modified sugars. b) Intracellular labeling using compound 90 incubated with β-galactosidase, releasing compound 91, which could undergo rapid cell uptake, being able to undergo CuAAC with modified nucleobases in the DNA.

3.2 Synthesis

3.2.1 Synthesis of the modified carbohydrates

The synthesis of the modified carbohydrates, compounds 84 and 81, was based on previous syntheses described in the literature. 185, 186 The synthesis of compound 84 (Scheme 3.2.1) started with commercially available d-glucosamine hydrochloride, compound 92, which underwent a condensation reaction with p-anisaldehyde in the presence of base. This furnished compound 93 where the amine is protected as an imine (or Schiff base) in a 45% reaction yield in 2 h. Due to the bulkiness of the p-anisaldehyde only the β-anomer was obtained. This stereochemistry was confirmed by 1H NMR which revealed a coupling constant of $J_{1,2} = 8.0$ Hz for the axial anomic proton interaction with the adjacent axial C-2 proton. It is important to note that access to a single anomer is not essential since the cells have the ability to recognise and incorporate both anomers equally, however, having only one anomer facilitated the purification and characterisation of the following products.

Compound 93 was protected using standard conditions with acetic anhydride and pyridine to yield the per-acetylated sugar 94. Compound 94 was obtained in lower yields than expected (70%), due to some imine hydrolysis occurring as a result of AcOH being
generated *in situ*. This hypothesis was supported by the isolation of \textbf{Ac$_4$GlucNAc} as a side product. To avoid this unwanted side-product, the reaction mixture was carefully monitored by TLC and immediately worked-up upon complete consumption of the starting material (6 h).

**Scheme 3.2.1.** Synthesis of compound 84.

Subsequently, compound 94 underwent amine deprotection *via* imine hydrolysis using HCl (5 M) in acetone furnishing the hydrochloride salt of the tetra-acetylated-\(\beta\)-D-glucosamine. Purification of this material only required filtration and therefore the desired product 95 was obtained in high yield of 92%. Analysis of the coupling constant for the anomeric proton doublet signal revealed that the \(\beta\)-anomer was retained \((J_{1,2} = 9.0 \text{ Hz})\). Compound 95 was treated with chloroacetic anhydride in the presence of triethylamine to yield the acetylated material 96 after 18 h in 86% yield. Finally, compound 96 was treated with \(\text{NaN}_3\) to displace the chloride *via* \(\text{S}_\text{N}2\) reaction. This was achieved by stirring compound 96 in DMF in an excess of \(\text{NaN}_3\) for 16 h at 60 °C, yielding final compound 84 in 45% yield.

Figure 3.2.1 shows the $^1$H NMR spectra of the final compound 84 recorded in CDCl$_3$. The formation of only the \(\beta\)-anomer is confirmed by the doublet found at 5.77 ppm with a coupling constant of \(J_{1,2} = 7.8 \text{ Hz}\). The amide proton appears as doublet (chemical shift 6.36 ppm) as is coupled to H-2 with a coupling constant of \(J_{NH,2} = 9.5 \text{ Hz}\).
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Figure 3.2.1. $^1$H NMR (400 MHz) of compound 84 recorded in CDCl$_3$.

For the synthesis of 81, a similar strategy was employed (Scheme 3.2.2). However, the initial imine formation step using $p$-anisaldehyde and NaOH was not successful. Precipitation of the product did not occur after 2 h as was observed with the glucose equivalent, so the reaction was run for extended periods of time (up to 48 h), but unfortunately no product was observed.

Scheme 3.2.2. Attempts to synthesise compound 81 following Scheme 3.2.1.

Attempts to precipitate the product through addition of hexane to the reaction mixture also failed. Although similar protecting groups have been successfully employed in the literature, no product was observed using $p$-anisaldehyde. Since the commercially
available mannosamine was prohibitively expensive, it was decided to abandon this route and follow one previously described by Sampathkumar et al. (Scheme 3.2.3). \(^{188}\)

The synthesis commenced with the protection of commercially available D-mannosamine hydrochloride (compound 97) upon treatment with 2-hydroxynaphthaldehyde (99) in a 6 M solution of sodium acetate. This lead to isolation of the protected compound 100 in an 89% yield following reaction overnight. As observed previously for the glucosamine derivative, the bulky group directed the configuration, in this case furnishing the α-anomer as the major isolated product, which was proved by \(^1\)H NMR in which the predominant anomer exhibited a coupling constant of \(J_{1,2} = 1.0\) Hz. However, a ratio of 7:3 α/β was obtained. To simplify assignment of this compound, the experimental section was based solely on the predominant α-anomer.

The rest of synthesis proceeded in an identical manner to that described above for the glucosamine derivative. Compound 101 was formed in 65% yield in 18 h from compound 100 by global protection using acetic anhydride dissolved in pyridine. A catalytic amount of DMAP was added to increase the yield of the reaction, as DMAP is a stronger nucleophile than pyridine due to the inductive effect of the dimethylamino substituent.

Selective deprotection of the amine was achieved upon treatment with oxalic acid in acetone to furnish compound 102 as the oxalate salt. Oxalic acid was used as it furnished the most stable and pure salt product in comparison to other trapping agents. \(^{187}\) Treatment with chloroacetic anhydride in the presence of TEA gave compound 103 in 76% yield, followed by the chloride displacement using NaN\(_3\) in DMF led to the final compound 81 in a 15% yield over 5 steps. The synthesis of compound 81 was carried out in collaboration with Stephen McLaughlin, as part of his undergraduate project.
3.2.2 Synthesis of the alkyne probe compound 90
The synthesis of the alkyne-functionalised naphthalimide probe 90 started with a condensation reaction of commercially available 4-bromo-1,8-naphthalic anhydride and propargyl amine (Scheme 3.2.4). After 6 h refluxing in EtOH compound 105 was obtained in 97% yield. Compound 105 was subjected to CuAAC ligation with galactosyl derivative 55a (the synthesis of which has been previously described in Chapter 2) using Cu(BF₄)(MeCN₄) as catalyst in DMF in a microwave reactor for 1 h to furnish compound 106 in 44% yield. The low yield is due to the challenging purification caused by aggregation of the glycosylated naphthalimide (see Chapter 6 for further discussion). Compound 106 was reacted with the protected alkyne trimethylsilylacetylene in a Sonogashira coupling reaction, leading to compound 107, which was used without purification. Finally, deprotection of the alkyne using potassium carbonate in a THF/MeOH mixture furnished the final alkyne derivative 90 in 38% yield over the two steps (Scheme 3.2.4).

Scheme 3.2.4. Synthesis of the alkyne functionalised naphthalimide probe.
The formation of compound 90 was confirmed by \textsuperscript{1}H NMR by the disappearance of the singlet found at 0.43 ppm in 107 integrating for nine protons corresponding to the SiMe\textsubscript{3} protecting group (Figure 3.2.2a) and a singlet with a chemical shift of 4.40 ppm, integrating by one proton corresponding with the alkyne proton (Figure 3.2.2b), confirming the de-protection of the silyl group furnishing compound 90. An accurate mass of m/z = 545.1632 ([M+Na]\textsuperscript{+}) was obtained for compound 90 by MS (ESI)\textsuperscript{+} for C\textsubscript{26}H\textsubscript{26}N\textsubscript{4}NaO\textsubscript{8}.

![Figure 3.2.2](image)

**Figure 3.2.2.** \textsuperscript{1}H NMR (400 MHz) of a) compound 107 and b) compound 90, both recorded in CD\textsubscript{3}OD.

To test that compound 90 could successfully undergo click reaction with the modified sugars, compound 108 was synthesised (Scheme 3.2.5) from reaction with compound 84, using the same CuAAC conditions previously described. Compound 108 was obtained in quantitative yield and was used as a model substrate for spectroscopic characterisation only.

Due to the small reaction scale compound 108 was not fully characterised by NMR spectroscopy but its formation was confirmed by the disappearance of a peak integrating by one at 4.38 ppm, corresponding with the alkyne peak, and by MS with an accurate mass obtained for [M+Na]\textsuperscript{+} by MALDI of m/z = 975.2982 for C\textsubscript{24}H\textsubscript{48}N\textsubscript{8}O\textsubscript{18}Na.
Having successfully synthesised and characterised the modified carbohydrates, a novel naphthalimide probe and demonstrated that CuAAC ligation between them is successful, the novel glycosylated naphthalimide probe was spectroscopically characterised. Hence, next section will describe the absorption, luminescence and excitation properties of compound 90 in aqueous solution as well as comparison of its characteristic bands with compound 108.

### 3.3 Photophysical Evaluation

The absorption, fluorescence emission and excitation spectra of 90 were recorded in PBS buffer at 25 °C and are shown in Figure 3.3.1. Compound 90 posses a π-π* transition band at 238 nm and an ICT band at 355 nm. A broad fluorescence emission band is observed ca. 414 nm when excited at 355 nm.

**Figure 3.3.1** Absorption, excitation and fluorescence emission of 90 (1 x 10^{-5} M) recorded in PBS at 25 °C.
The fluorescence quantum yield was calculated using quinine sulphate as reference (Φ_F = 54%),189 following equation 8.1 (described in Chapter 8), with an obtained value of 35%, which is quite high taking into account that it was measured in H2O.

Concentrations studies carried out in PBS showed that aggregation occurred above approximately 3 × 10^-5 M. This was observed by plotting fluorescence emission intensity vs. concentration. After 3 × 10^-5 M the linear trend is lost and the emission intensity plateaus due to π-π stacking interaction of the naphthalimide cores, leading to a quench in fluorescence intensity (Figure 3.3.2b). However, when the absorption was plotted vs. concentration no plateau was observed, proving that aggregation of the molecules does not interfere with its absorption capacity.

![Figure 3.3.2](image)

**Figure 3.3.2.** Changes in the absorption (a) and fluorescence emission (b) spectra upon increasing concentration of 90. Inserts represent the maximum absorption (λ_max = 355 nm) and fluorescence emission (λ_max = 415 nm) bands plotted against the concentration, respectively. All the studies were carried out in PBS buffer pH 7.2 at 25 °C.

Photophysical characterisation of compound 108 was also carried out to understand the spectroscopic changes of the probe 90 following click reaction with modified carbohydrates. The absorption and fluorescence spectra were recorded in PBS buffer pH 7.2 at 25 °C (Figure 3.3.3a). Similar to compound 90, compound 108 possesses a broad absorption band at 355 nm corresponding to the ICT system and a higher intensity band at 236 nm corresponding to a π-π* transition band.

As can be seen in the normalised fluorescence spectra of 108 (Figure 3.3.3b) excitation at 355 nm leads to a broad emission band with a λ_max ca. 461 nm. This corresponds to a red shift in the fluorescence emission following the click reaction of approximately 50 nm.
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Figure 3.3.3. a) Absorption spectra of compound 108, and 90 for comparison. b) Normalised fluorescence emission spectra of 108 (λ_{exc} = 355 nm), and 90 for comparison. All spectra recorded in PBS buffer pH 7.2 at 25 °C.

This was quite satisfactory as a red shift in fluorescence emission could potentially be used as a marker of a successful click reaction.

Table 3.1 highlights the most representative bands of compound 90 and 108. Importantly, the quantum yield of 108, which was measured under identical conditions to 90 and using quinine sulphate as standard, is significantly higher than 90 (55% instead of 35%).

Table 3.1. Summary of characteristic bands, ε and \( \phi_F \) of compounds 90 and 108 recorded in PBS buffer pH 7.2 at 25 °C from three independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \lambda_{max} ) (nm)</th>
<th>( \pi-\pi^* )</th>
<th>ICT (ε (M(^{-1}) cm(^{-1})) ±10%)</th>
<th>( \phi_F ) (±SEM %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>238</td>
<td>355 (10,200)</td>
<td>373</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>108</td>
<td>236</td>
<td>355</td>
<td></td>
<td>54.9 ± 0.7</td>
</tr>
</tbody>
</table>

After spectroscopical characterisation of compound 90 and comparison with compound 108, compound 90 was then evaluated in vitro with HeLa cells. Next section describes cellular uptake studies of compound 90 using confocal and two-photon microscopy, the effect in the cell proliferation of compound 90 and CuAAC reaction in vitro.
3.4 Biological Evaluation
3.4.1 Previous *in vitro* click labeling studies carried out with modified sugars and commercially available Alexa Fluorophore.

In order to demonstrate that the modified sugars 81 and 84, were able to be internalised by the cells and metabolically expressed in their glycome, some experiments were carried out with different cell lines. These experiments were carried out in collaboration with Dr. Jerrard Hayes and Prof. Gavin Davies in the, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute. They were able to demonstrate that the two modified glycans are metabolically incorporated by HeLa cells (Figure 3.4.2). These findings are in agreement with previously reported findings in the literature.

The procedure for the click labeling was optimised following procedures reported in the literature,\textsuperscript{190} and the procedure used for the *in vitro* click labeling was as follows: HeLa cells were grown on microscope cover slips until approximately 50% confluent. Azido derivatives of the monosaccharides 81 and 84 were added to cell culture media (at 1 μM) followed by incubation at 37 °C, 5 % CO\textsubscript{2} for 24 h. Cells were then washed 3 times with PBS in preparation for the click chemistry reaction. Each coverslip was gently washed with 500 μL PBS followed by addition of 250 μL of the click chemistry solution containing the alkyne fluorophore (5 μM Alexa-488, Figure 3.4.1), 1 mM amino guanidine, 2.5 mM sodium ascorbate, 5 μM copper sulphate, and 25 μM tris(3-hydroxypropyl-triazoylmethyl)amine (THPTA).

![Figure 3.4.1. Commercially available fluorophore Alexa-488 containing an alkyne moiety.](image)

Cells were incubated at room temperature for 5 min followed by washing three times with PBS. Cells were then fixed using a 6% solution of paraformaldehyde (PFA) and incubation for 15 min at room temperature. The cells were then washed using PBS three
times and a final wash with ultra filtered H$_2$O. Coverslips were mounted with Prolong Gold for visualisation by confocal microscopy.

As can be seen in Figure 3.4.2, the modified glycans 81 and 84 were able to function as substrates for the *in vitro* click reaction. However, better results were obtained with 81, as it is a substrate of the sialyl acid pathway and is more highly expressed. Therefore, 81 was further used for glycome labeling experiments in another two different cell types; breast cancer cells, from cell line SKBR-3, and fully differentiated neurons (primary cells from mice).

It can be seen in Figure 3.4.2 that the external glycome of the cell has been successfully labelled, as it appears can be observed by the fluorescence of the Alexa-488 in green ($\lambda_{\text{exc}} = 488$ nm, $\lambda_{\text{em}} = 500 - 550$ nm) upon CuAAC with the modified sugars expressed in the cell glycome. Cell nuclei were stained using DAPI (4′,6-diamidino-2-phenylindole, with $\lambda_{\text{exc}} = 405$ nm and $\lambda_{\text{em}} = 400 - 460$ nm).

These experiments proved that the azido-modified sugars are, as predicted by previous reported studies, able to be metabolically incorporated into the lipids and proteins in the glycocalix, in both, healthy and tumorous cells. After demonstrating this property, the *in vitro* behaviour of the naphthalimide probe and its applications for *in vitro* labeling were studied.

### 3.4.2 Cellular uptake of compound 90

HeLa cells were incubated with compound 90 (50 μM) for different lengths of time (1 and 24 h) and the confocal images are shown in Figure 3.4.3. As can be seen, the compound was not uptake by the cells. In the case of 1 h incubation, no difference in fluorescence between the cells and the cell media was observed, whereas after 24 h incubation green
fluorescence in the cell media was seen, with the cells contrasting as a black space. This observation could be explained as the compound could precipitate over time and deposit at the bottom of the well, making it visible when using confocal microscopy, as it will be at the same level as the cells. A 6 h incubation point was also carried out but it did not show any significant difference with 1 h time point.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DRAQ 5</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="a" alt="Image" /></td>
<td><img src="a" alt="Image" /></td>
<td><img src="a" alt="Image" /></td>
</tr>
<tr>
<td><img src="b" alt="Image" /></td>
<td><img src="b" alt="Image" /></td>
<td><img src="b" alt="Image" /></td>
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<td><img src="c" alt="Image" /></td>
<td><img src="c" alt="Image" /></td>
<td><img src="c" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 3.4.3.* Confocal microscopy images of HeLa cells incubated with 90 (50 µM) for 1 h (a) and 24 h (b), respectively, and 90 (50 µM) in the presence of β-galactosidase (1 U) for 1 h (c). Representative image of three independent experiments.

Compound 90 was also incubated in the presence of β-galactosidase (1 U), and in this case after only 1 h incubation the cells presented a strong fluorescent signal, proving that the compound had been successfully uptake by the cells (Figure 3.4.3). Interestingly, it was
possible to observe that the compound was also localised in the nuclei of the cells, which had not been observed with similar compounds synthesised in Chapter 2. Z-stack confocal imaging proved that the compounds were distributed throughout the entire cell (Figure A.3.1).

As *In vitro* studies with compound 90 demonstrated its suitability as fluorescence probe for extra- and intracellular processes, respectively, by activation with β-galactosidase. Subsequently, two-photon microscopy studies of compound 90 were carried out to investigate whether it could be used for *in vivo* imaging.

**3.4.3 Two-Photon Microscopy Studies**

In two-photon microscopy (TPM), two different photons are absorbed to promote an electron from, generally, the ground state of the molecule to a higher excited state. Because two photons are being absorbed, the energy required to promote the electron is lower than in a typical fluorescence process. Leading to excitation wavelengths longer than the emission wavelengths. This allows to the use of near infrared microscopy, which is advantageous in the imaging of living tissue as it enables up to 1 mm penetration, due to the reduced scattering and absorption by endogenous chromophores.\(^97\)

The quality of this technique however is not as accurate as confocal imaging. Nonetheless, it is especially useful for imaging compounds that absorb at shorter wavelengths than 405 nm and cannot be imaged using confocal microscopy because it will lead to cell damage, allowing instead for the use of a softer laser power that does not damage the cells. This experiments were carried out in collaboration with Dr. Gavin McManus, form the School of Biochemistry, who took the TPM images.

Compound 90 was incubated in HeLa cells for 1 h prior imaging. TPM images were recorded at an excitation wavelength of 840 nm (Figure 3.4.4). Compound 90 showed great fluorescence between 455-638 nm. Interestingly, the nuclei of the cells presented a stronger fluorescent signal at longer wavelengths (red channel) than at shorter ones (green channel) (Figure 3.4.4). This is of considerable interest as it could potentially be used as a cell marker.
3.4.4 Cell Viability studies of 90

The effect of 90 on the cell proliferation was assessed via Alamar Blue assay in HeLa cells using a range of concentration (100 - 1 μM) after 24 h incubation. The values are expressed as IC₅₀ (μM) and were obtained from three independent results. As incubation of 90 with β-galactosidase enzyme leads to the cell uptake of the released compound 91, the cell viability of 90 in the presence of β-galactosidase enzyme (1 U) was also investigated. The results can be found in Table 3.2.

Table 3.2. Cell viability of compound 90 in the presence and absence of β-galactosidase (1 U) in HeLa cells after 24 h incubation. Values obtained from three independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>&gt;100</td>
</tr>
<tr>
<td>90 + Enzyme</td>
<td>65 ± 7</td>
</tr>
</tbody>
</table>

Gratifyingly, compound 90 proved to be non-toxic for HeLa cells after 24 h incubation (IC₅₀ >100 μM), demonstrating that it could be a suitable probe for cell imaging. However,
the incubation of compound 90 in the presence of β-galactosidase presented an IC₅₀ of 65 μM, being slightly toxic, nonetheless it could still be used as probe as the incubation time will be shorter than 24 h.

3.4.5 Click reaction carried out in vitro with 90.

Initial attempts to perform the click reaction in vitro were carried out using a click solution prepared in the lab, as reported in the literature,¹⁹⁰ and as was previously demonstrated by Dr. Jerrard Hayes. The click solution consisted of CuSO₄ (2.0 mM), amino guanidine (1 mM), ascorbic acid (2.5 mM), THPTA ligand (50 μM) and alkyne 90 (5 mM) or Alexa-488 (5 mM) in H₂O. The fluorophore Alexa-488 was used as control experiment.

HeLa cells were treated with the modified sugars 81 or 84 (50 μM) 24 h prior labeling. After 24 h incubation, enough sugars have been able to be metabolized by the cells and incorporated into their glycome. HeLa cells were then washed with PBS three times followed by H₂O, after which the click solution was added into the cells and left for 5 min. Longer incubation times with the click solution might lead to cell damaging due the presence of the Cu(I) and DMSO from the THPTA ligand solution. After 5 min incubation, the cells were washed with PBS three times and H₂O and fixed using PFA (4%, v/v).

After fixation, the cells were stained with DRAQ5, for cells labelled with 90, and DAPI for cells labelled with Alexa-488. Cells were imaged using confocal microscopy (λ exc = 405 nm, λ em = 500-550 nm). Unfortunately, this procedure proved unsuccessful even for the control experiment with Alexa-488, with no glycome labeling being observed in the confocal fluorescence images. Several attempts increasing the concentration of alkyne and the modified sugars incubation time (up to 48 h) were conducted but unfortunately were also unfruitful.

Following these experiments, the click reaction was attempted in vitro by using instead a commercial solution for the click labeling, which can be obtained from Sigma Aldrich. To this commercial solution CuSO₄ (5 mM) was added, as well as the alkyne (5 mM) to HeLa cells previously incubated with 81 (50 μM) for 24 h. Cells were incubated with the click solution followed by washing and fixation as previously described and reported in the literature (Figure 3.4.5).⁴⁴ This was attempted using the commercially available Alexa and compound 90. Compound 90 was also incubated in the presence of β-galactoside, to compare the labeling process in the presence and absence of the enzyme. However, although this method gave successful glycome labeling with Alexa fluorophore (Figure
3.4.4.a), no successful reaction was observed with the naphthalimide alkyne compound 90 (Figure 3.4.5.b and c).

Since the CuAAC reaction was successfully carried out previously (synthesis of 108), it was postulated that what was hindering this reaction was the short reaction time (5 min). In order to find out whether this hypothesis was correct the click reaction was carried out using identical conditions as used in vitro, and monitored it spectroscopically. The differences in the emission bands of product and starting material would allow to quantify
Chapter 3. Glycosylated Naphthalimides for Cellular Labeling

the reaction time. Figure 3.4.6 represents the emission bands of 90 while the click reaction is proceeding over time. This was carried out in PBS buffer pH 7.2 at 37 °C and the click solution containing 81 was set as blank for the emission. The reaction was conducted at a 10-fold dilution of the one carried in vitro in order to avoid saturation of the detector. Normalised emission spectra of 90 (1 × 10⁻⁵ M) and 108 (1× 10⁻⁵ M) can also be found in Figure 3.4.6b for comparison.

As can be seen in Figure 3.4.6, after 5 min reaction time (red line) resulted in no formation of the desired product, as there is no apparent shift in the fluorescent band. The reaction was left for longer incubation times with no noticeable reaction occurring. After overnight incubation (16 h, blue line) a small red shift was observed, indicating that the reaction was proceeding, however, starting material remained. This experiment demonstrated that under these conditions the click reaction with 90 requires long incubation times that are not suitable for in vitro experiments.

A plausible explanation of why the click reaction proceed at a low rate is that as demonstrated in section 3.3, compound 90 aggregates at concentrations higher than 3 × 10⁻⁵ M (Figure 3.3.2), therefore these aggregates would be present at the concentration chosen for the in vitro labeling (5 mM). This aggregation may hinder the click reaction by making the alkyne group inaccessible.

![Figure 3.4.6](image)

**Figure 3.4.6.** a) Normalised emission spectra of click reaction of 90 (5 × 10⁻⁴ M) in PBS in the presence of CuSO₄ (0.2 mM), ascorbic acid (0.25 mM), THPTA (5 μM) and 81 (5 μM) at different times. b) Normalised emission of 90 (1 × 10⁻⁵ M) and 108 (1 × 10⁻⁵ M) in PBS for comparison.
3.4.6 Design of a new probe and *in vitro* click reaction with compound 110

In order to circumvent the aforementioned problems, compound 109 was designed possessing a propargyl substituted amine in the 4-position of the naphthalimide (compound 109 in Figure 3.4.7). In this compound, the alkyne group is separated from the naphthalimide core by an amine and a methylene unit thereby increasing its exposure in the case of aggregation. However, the installation of the propargylamine onto the aromatic ring of the naphthalimide is quite challenging. Fortunately, Dr. Emma Veale had previously synthesised compound 110, which possesses the desired functionality and ethyl chain at the imide side (Figure 3.4.7). It was decided to first attempt the *in vitro* click reaction using compound 110 as a test substrate.

Commercial click solution was used to conduct the *in vitro* reaction using 110, as it allowed for successful labeling using Alexa-488 with it. Since compound 110 is very small and hydrophobic, rapid cellular uptake was expected. This was confirmed by the incubation of HeLa cells with 110 (50 μM) for 1 h (Figure 3.4.8).

![Figure 3.4.7. Designed compound 109 and 110 for *in vitro* labeling.](image-url)

![Figure 3.4.8. Confocal image of HeLa cells incubated with 110 (1 × 10^−5 M) for 1 h.](image-url)
This rapid uptake by the cells makes these types of probes undesirable for click labeling, as it will lead to a mixture of fluorescence signal of the labelled glycome and internalised compound. This emphasises the importance of the glycan unit in this system to prevent the uptake and allowing for controlled delivery by glycosidases. Unfortunately, glycome labeling following the previously described procedure with compound 110 also resulted unsuccessful, as the only fluorescence signal observed in the confocal microscopy images (Figure 3.4.9b) was resulted from the internalisation of the compound. Control experiments carried out with Alexa-488 fluorophore (Figure 3.4.9a) demonstrated that the modified sugar 81 had been successfully metabolised by the cells as the *in vitro* click reaction worked and the glycome was labelled successfully, as seen in Figure 3.4.9a by the fluorescence signal at the outer membrane layer of the cell. A control experiment carried out where HeLa cells were not treated with 81 beforehand (Figure 3.4.9c) demonstrated that the fluorescence signal observed with compound 110 is only due to the internalisation of the compound, as the fluorescence signal obtained was identical in both cases, where HeLa cells had been previously treated with 81 and where they had not.

In this case, spectroscopic monitoring was not possible as the alkyne group is further away from the naphthalimide core and thus, the click reaction would not alter the ICT character of the naphthalimide. Therefore, the clicked product would possess identical emission bands to the started material.

Since the results obtained with compound 110 for the glycome labeling were not promising, and time was limited, it was decided to concentrate the efforts in other projects, which are described in Chapters 4-6.
Figure 3.4.9. Confocal image of fixed HeLa cells after click labeling with a) Alexa-488 (10 μM) DAPI used as nuclear stain, b) 110 (10 μM) and c) 110 (10 μM) without S1 treatment. DRAQ 5 used as nuclear stain in c and d.

3.5 Conclusions
The synthesis of several modified sugars that could be used for glycome labeling was carried out. These modified sugars were internalised and metabolised by the cells allowing for glycome labeling, as the experiments carried out in Dr. Gavin Davies group proved.

The design, synthesis, spectroscopic and biological characterisation of a glycosylated probe for cellular labeling was attempted. Spectroscopic analysis of compound 90 was
promising as a red shift in the fluorescence emission wavelengths as well as an increase in the fluorescence quantum yield in H$_2$O occurred after click reaction.

*In vitro* studies with HeLa cells demonstrated that compound 90 was not internalised by the cells, which is desirable for glycome labeling. In the presence of β-galactosidase enzyme compound 90 was transformed to 91, which underwent rapid cell uptake, including localisation in the cell nuclei. This could be exploited for the labeling of different cell contents such as modified proteins or DNA. These encouraging results as well as cell proliferation studies carried out in HeLa cells, which deemed compound 90 non-toxic in HeLa cells after 24 h incubation, led to believe that compound 90 could be a good candidate for cellular labeling.

Unfortunately, *in vitro* click labeling studies proved unfruitful. It was demonstrated that long incubation times that are not compatible with physiological conditions were needed. Attempts made to increase the reaction rate by modifying the alkyne unit by using instead a N-propargyl group were not successful.

Although the initial idea behind this project was not successful, the results obtained from the cell viability studies with 90 in the presence of β-galactosidase releasing a slightly toxic agent (IC$_{50}$ = 65 ± 7 μM), led to focus in the use of glycosidase enzymes for the release of cytotoxic agents, which will be discussed in Chapter 4.
Chapter 4

Glycosylated Naphthalimides as Prodrugs of Amonafide
4.1 Introduction
4.1.1 Cytotoxic naphthalimides
1,8-Naphthalimides are known to intercalate DNA due to the planarity of their aromatic moiety.63,100 The substitution pattern at the imide site plays an important role in DNA binding.65 For example, SAR studies have shown the importance of a basic terminal group (such as the dimethylamine in Amonafide) separated by 2-3 methylene units.63 However, substitution at the aromatic moiety is also crucial for their DNA binding and subsequent anticancer activities. Naphthalimides containing an amino group possess greater DNA intercalating ability than the nitro corresponding substituted derivatives. At the same time, 3-nitro substituted naphthalimides are better DNA binders than 4-nitro substituents, due to the angular orientation found between the nitro group and the imide site disrupting the DNA intercalation, whereas in the case of the 3-nitro substitutes the imide and the nitro groups are coplanar to each other.191

DNA intercalation is not the only mechanism through which naphthalimides can induce cytotoxicity. Some poly-amine naphthalimide derivatives have been found to induce cytotoxicity by other mechanisms such as caspase activation inducing apoptosis via mitochondrial pathway or inducing autophagosomes to increase lysosomal activity, ultimately leading to cell death.116 Nonetheless, most of the cytotoxic naphthalimide derivatives developed within the Gunnlaugsson group owe their cytotoxicity to DNA intercalation.74,192,193

4.1.2 Amonafide derivatives
Amonafide, although a promising anticancer drug, was withdrawn from clinical trials due to undesired side effects. Two metabolites of Amonafide are mainly responsible for these side effects; N’-hydroxy- and N-acetyl-Amonafide (Scheme 4.1.1). N-acetyl transferase 2 (NAT2), responsible for the acetylation of Amonafide, exhibits varying activity amongst individuals, thus leading to two different categories, slow and fast acetylators, respectively, depending on the acetylation rate.108 Fast acetylators present higher side effects, as N-acetyl-Amonafide competes with the metabolic inactivation of the drug via CYP1A2, which leads to accumulation of high concentration of Amonafide causing toxicity.194 Efforts have been made to individualise the dose of Amonafide depending on the acetylation rate of the individual patient.194 However, this is tedious and logistically difficult, preventing the use of Amonafide as an effective cancer treatment.
To overcome these aforementioned issues, Amonafide derivatives that maintain their cytotoxic potency where the amino residue cannot be acetylated have been developed. For instance, Mitonafide and Azonafide (Figure 4.1.1) possess a nitro group and anthracene ring, respectively, instead of the amine group. They have also been tested in a range of cancerous cell lines and exhibit similar or better cytotoxicity than Amonafide.

Quaquebeke et al. conducted an extensive study on Amonafide derivatives in which the amine had been substituted by an amide, urea, imine, or thiourea and evaluated their cytotoxicity and DNA binding affinity. Their most promising results were found for a urea-modified Amonafide derivative (113, Figure 4.1.1), which showed higher cytotoxicity in vitro and in vivo. Although its properties as DNA intercalator were lower than Amonafide, compound 113 was able to induce senescence and autophagy.

Meng and co-workers developed a family of triazol-fused Amonafide derivatives which exert higher cytotoxicity levels than Amonafide in five different cancer cell lines. They also developed an N'-oxide derivative as a prodrug derivative, which possessed potent anticancer activity in in vivo assays conducted in mice, signifying this derivative as
a potential anticancer agent. The use of \( N' \)-oxide functionality as prodrugs will be discussed in the next section.

### 4.1.3 Amonafide prodrugs

To avoid the side-effects that Amonafide presents, several prodrugs have been developed. Hong and co-workers\textsuperscript{196} designed a prodrug of Amonafide in which the tertiary amine had been oxidised to an \( N' \)-oxide (compound 114 in Scheme 4.1.2), which disrupts the DNA binding affinity thereby diminishing toxicity. The \( N' \)-oxide could be selectively reduced by cytochrome CP450 reductase in hypoxic tumour cells. Disappointingly, low selectivity for cancer cells was observed in \textit{in vitro} assays.

![Scheme 4.1.2. Prodrug of Amonafide using an \( N' \)-oxide developed by Hong et al.\textsuperscript{196}](image)

Xiao et al.\textsuperscript{197} have recently pioneered a nano-diamond glycopolymer as a drug-delivery system for the release of Amonafide, in which Amonafide has been conjugated onto the surface of a nano-diamond through an imine bond, which is stable at physiological pH but degrades under acidic conditions, which is characteristic of tumorous environment. \textit{In vitro} studies in a mammalian breast cancer cell line demonstrated that the nano-diamond glycopolimers carrying Amonafide were capable of inhibiting cell growth even at lower doses than Amonafide.\textsuperscript{197}

Other Amonafide prodrugs reported involve its conjugation onto antibodies that recognise gamma-glutamyl transpeptidase (GGT) enzyme specifically expressed in tumour cells, imparting specificity. Subsequently, GGT cleaves a gamma-glutamyl group conjugated to Amonafide, thus releasing the active species.\textsuperscript{198}
4.1.4 Prodrug Activation Using Glycosidase Enzymes

Prodrugs containing glycosides have been extensively studied as conjugation of drugs to glycosides usually diminishes toxicity of the drug by inhibiting the cellular passive-diffusion due to the hydrophilicity imparted by the glycoside moiety. These types of prodrugs consist of three components (Scheme 4.1.3): a glycoside unit, a self-immolative linker, and the active drug. The drug is usually conjugated to the glycosylated self-immolative linker through a heteroatom such as O, N, or S.

The linker plays an important role in the therapeutic activity, as it is needs to be cleaved directly after enzymatic activation to avoid cellular diffusion of the linker-drug conjugate. The most common linkers are either carbamates, which decompose to give CO₂, or a 3-nitro-4-acetoaldehyde linker (compound 115 in Scheme 4.1.3), which decomposes spontaneously after enzymatic cleavage of the glycosidic linkage, releasing the drug. The presence of the nitro group in the ortho position decreases the pKₐ of the phenol formed, enhancing the rate of self-immolation.¹⁹⁹

![Scheme 4.1.3. Activation of glycosylated prodrugs using glycosidase enzymes.](image-url)

Different glycoside units such as β-galactose or β-glucose have been employed in these systems, however, β-glucuronic acid containing prodrugs are of significant importance. It has been known since the 1950’s that higher concentrations of glycosidases, and in particular β-glucuronidase, are found in cancerous tissues such as breast, lung, ovarian, gastrointestinal cancers and melanomas. Most importantly, the concentration and the dispersion of β-glucuronidase varies between healthy and tumour environments. In cancerous tissue, β-glucuronidase is secreted by inflammatory cells extracellularly, whereas in a healthy cellular environment β-glucuronidase can be found exclusively within the lysosomes.
It is therefore not surprising that β-glucuronidase has been exploited for the release of anti cancer drugs since it confers intrinsic selectivity. The first example of a prodrug containing glucuronic acid was developed by Tietze in 1988 and since then many examples of prodrugs using β-glucuronidase have been developed for well-known anti-cancer drugs such as paclitaxel, duorcamycin or doxorubicin.

With this in mind, the development of a prodrug of Amonafide was attempted, by introducing an enzymatic cleavable moiety as described above.

4.1.5 Design of Amonafide Prodrugs.
Initially, a glycosylated prodrug of Amonafide that contained a carbamate linker between the naphthalimide moiety and the glycoside unit was designed, as outlined in Scheme 4.1.4a. The synthesis started using a β-galactose derivative as previous work (described in Chapter 2) gave good results in vitro using β-galactosidase enzyme. However, the ultimate goal was to develop a prodrug that could be activated upon exposure to β-glucuronidase, therefore glucuronic acid was employed in the design (Scheme 4.1.4a).

Scheme 4.1.4. Release of Amonafide upon enzymatic activation using prodrugs containing (a) carbamate linkers and (b) self-immolative linker, respectively.
However, this type of compounds containing a carbamate linker proved very challenging to synthesise due to the poor nucleophilicity of the hemiacetal as well as the undesired formation of the two anomers (α/β) from which only one of them would be recognised by the enzyme. To circumvent these undesired problems, compounds 119 and 120 (Scheme 4.4b) that possess a self-immolative linker between both moieties were designed instead, leading to a more facile synthetic route.

### 4.2 Synthesis

#### 4.2.1 Synthesis of Amonafide Moiety.

The Amonafide moiety was synthesised in two high yielding steps starting from 3-nitro-1,8-naphthalic anhydride in a condensation reaction using N′,N′-dimethylethylene diamine refluxing in ethanol for 4 h to give compound 27 in 97% yield (Scheme 4.2.2). Reduction of the nitro group was achieved via hydrogenation using Pd/C (wt. 10%) in a mixture of MeOH/EtOH (1:1) to give Amonafide in quantitative yield overnight (Scheme 4.2.2).

![Scheme 4.2.2. Synthesis of Amonafide.](image)

#### 4.2.2 Synthesis of the carbohydrate moieties

The synthesis of the carbohydrate units was first approached with the β-galactose derivative, due to its relative ease of synthetic manipulation. To synthesise the hemiacetal galactose derivative, 1,2,3,4,6-penta-O-acetylated-D-galactose was reacted with benzylamine in THF for 16 h at rt, giving the desired product 123 in 70% yield (Scheme 4.2.3).

![Scheme 4.2.3. Synthesis of compound 123.](image)
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This compound was used in the attempted conjugation with Amonafide, discussed in the next section.

The galactosyl bromide donor was synthesised by reaction of 1,2,3,4,6-penta-O-acetylated-D-galactose with HBr (33 wt. % in acetic acid) in CH₂Cl₂ to form a galactosyl-bromide, compound 124, in 72% yield after work up (Scheme 4.2.4). From this, a

Koenigs-Knorr glycosylation was carried out using silver carbonate as a promoter and water as the acceptor in acetone as described by Allen et al.200 The reaction was performed at rt for 18 h, giving the desired 2,3,4,6-tetra-O-acetylated-β-D-galactose, compound 123α, in 66% yield after work up.

The synthesis of the galactoside containing a self-immolative linker started from the reaction of compound 124 with 4-hydroxy-3-nitro-benzaldehyde using a Michael glycosylation in a phase transfer reaction illustrated in Scheme 4.2.5a.199 Followed by the reduction of the aldehyde upon treatment with sodium borohydride, leading to compound 127 in 83% yield.

Scheme 4.2.5. Synthesis of the glycoside moieties containing self-immolative linkers; a) galactose (127) and b) glucuronic acid (131).
Silica gel was added to the reaction mixture to prevent the migration of the acetate groups, as described by Tietze et al.\textsuperscript{199}

The glucuronic acid derivative containing a self-immolative linker was synthesized according to the same route as previously described for compound 127 (Scheme 4.2.5b).

As can be seen in Figure 4.2.1, the $^1$H NMR of compound 131 demonstrates the reduction of the aldehyde by the disappearance of its characteristic peak at ca. 10 ppm, and the presence of a doublet ($J = 5.8$ Hz) at 4.73 ppm, which integrates by 2 H, corresponding to the CH$_2$. The product formation was also confirmed by MS, which an accurate m/z obtained of 508.10685, for [M$^+$-Na] C$_{20}$H$_{23}$NNaO$_{13}$.

![Figure 4.2.1. $^1$H NMR (400 MHz) of compound 131 recorded in CDCl$_3$.](image)

Having synthesised and fully characterised the carbohydrate-moiety, the coupling reaction with Amonafide was next carried out.

### 4.2.3 Coupling Reaction between Amonafide and Carbohydrate units

Compound 51a was reacted with N,N'-carbonyldiimidazole (CDI) in acetonitrile at rt for 16 h, following a procedure described in the literature for glucose derivatives (Scheme 4.2.6).\textsuperscript{201} However, this reaction resulted in a poor yield of only 30% and the desired
product was obtained as a mixture of both anomers, with the α-anomer being the predominant isomer (β/α (1:3)), which could be explained by a combination of steric hindering and anomeric effect. However, the glycosidase enzyme would only recognise the β-anomer so the reaction was repeated with compound 123β in order to obtain the β-anomer exclusively.

This reaction was carried out in toluene at rt (Scheme 4.2.6). Starting material consumption was observed by TLC after 1.5 h, however, the desired product 123β was not observed. The reaction was attempted using anhydrous diethylether as the solvent, as proposed in the literature.202

Scheme 4.2.6. Synthesis of compound 133.

Under these conditions, complete consumption of the starting material was observed by TLC after 3 h, giving the desired product in 84% yield. However, the subsequent reaction between compound 132β and Amonafide using DBU catalytically in acetonitrile at rt proved unsuccessful and only starting material was isolated (Scheme 4.2.6).

The coupling reaction was also attempted by first activation of the amine followed by reaction with compound 127. Amonafide was reacted with CDI in DMF in an effort to furnish compound 133, using a procedure previously described in the literature for aromatic amines containing electron-withdrawing groups.203 The solvent was changed to DMF in order to increase the solubility of Amonafide. Unfortunately, under these modified
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Scheme 4.2.7. Attempted synthesis of compound 133.

conditions no product was formed (scheme 4.2.7), and once again only the starting material, compound 123β, was isolated. This is most likely due to the ICT character of 26, while being less flat than the 4-amino-1,8-naphthalimide is also significant, which reduces the nucleophilicity of the amine.

Due to the extremely challenging nature of the synthesis of compound 40, it was decided to concentrate the synthetic efforts towards the formation of glycosylate prodrugs containing a self-immolative linker (compounds 119 and 120). The benzylic alcohol compound 127 was synthesised using p-nitrophenylchloroformate (134) in CH$_2$Cl$_2$ and catalytic amount of pyridine as previously described by Kamal et al., this yielded compound 135 in 79% after 18 h (Scheme 4.2.8).

Scheme 4.2.8. Synthesis of compound 135.

The coupling reaction with Amonafide was attempted using a catalytic amount of pyridine in DMF as described by Fernandes et al. however, no formation of compound 136 was observed, even following extended reaction times (scheme 4.2.9). Hydrolysis of the carbonate bond was observed for compound 135, leading to the formation of compound 127.
Hydroxybenzotriazole (HOBt) was used instead of pyridine in an attempt to increase the reactivity of the amine. However, the reaction with Amonafide failed, thus the reaction conditions were optimized with other commercially available amines as model substrates.

The reaction was attempted with benzenamine and 4-nitroaniline and, disappointingly, neither of these approaches resulted in the successful formation of a carbamate linker (Scheme 4.2.10). In contrast, when the reaction was conducted under identical conditions with diethylamine, 50% yield was observed after 3 h (Table 4.1). Taking into account that diethylamine is more nucleophilic than Amonafide and yet only 50% yield was observed, it was clear that this reaction was very challenging and consequently it was decided to abandon this synthetic route.

The different amines used to optimised the synthesis of compound 136 are summarised in Table 4.1.

### Table 4.1. Different amines used as test reaction with compound 135.

<table>
<thead>
<tr>
<th>Amine</th>
<th>t (h)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amonafide</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>Benzenamine</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>4-Nitroaniline</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>Diethylamine</td>
<td>3</td>
<td>50%</td>
</tr>
</tbody>
</table>
As it was clear that the coupling reaction with Amonafide required harsher conditions, it was decided to use triphosgene or bis(trichloromethyl)carbonate (BTC) to effect the desired transformation. BTC is a derivative of phosgene that is easier and safer to handle as it is a solid at room temperature. However, its toxicity is the same to phosgene as it decomposes to phosgene on heating and upon reaction with nucleophiles (Scheme 4.2.11).\(^{206}\)

The reaction between compound 127 and Amonafide was carried out several times (Scheme 4.2.12) and the results are summarised in Table 4.2. Initially, the reaction was attempted using toluene as the solvent, at low temperature (\(-42^\circ C\)), and using 0.3 equivalents of BTC, which lead to stoichiometric amount of the reactive species phosgene (Scheme 4.2.12), to avoid the formation of carbonates or ureas (entry 1, Table 4.3).

Compound 127 was reacted initially with BTC until complete starting material consumption was observed by TLC (1 h), and then Amonafide was added to the reaction mixture, with a total reaction time of 3 h. This reaction resulted in no product formation, which could be due to the poor solubility of Amonafide in Toluene.

DMF was chosen as the preferred solvent and the reaction was repeated. In this case, the reaction mixture was allowed to warm up to 0 °C after each addition. Traces of the desired product were obtained in this case, which was encouraging, however, a dimer of Amonafide (compound 140, scheme 4.2.12) was also obtained in 15% yield.

In parallel, the reaction was attempted under identical conditions but inverting the order of addition, Amonafide was reacted with BTC first in this case. It was observed that...
alcohol consumption was not occurring after several hours so the reaction was left to stir overnight. However, this only led to more formation of the dimer 140 (89% yield) and only trace amount of the desired compound. Finally, THF was chosen as the solvent and the alcohol was added first into the reaction mixture. Full starting material consumption was observed after 3 h. A small amount of the dimer side product was also observed but analysis of the crude material by $^1$H NMR showed a 53% formation of the desired product.

Scheme 4.2.12. Synthesis of 136 by reaction with BTC, giving urea 140 as a side product.

However, following purification using SiO$_2$ column chromatography only 8% of compound 136 was isolated pure. The desired compound and side-product 140 aggregate by π-π staking interactions, which made their purification very challenging.

The different conditions used for the synthesis of compound 136 are summarised in Table 4.2.

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Amine</th>
<th>Temp. (°C)</th>
<th>t (h)</th>
<th>Solvent</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^{st}$</td>
<td>2$^{nd}$</td>
<td>-42</td>
<td>3</td>
<td>Toluene</td>
<td>-</td>
</tr>
<tr>
<td>1$^{st}$</td>
<td>2$^{nd}$</td>
<td>-42 – 0</td>
<td>3</td>
<td>DMF</td>
<td>Traces</td>
</tr>
<tr>
<td>2$^{nd}$</td>
<td>1$^{st}$</td>
<td>-42 – 0</td>
<td>16</td>
<td>DMF</td>
<td>Traces</td>
</tr>
<tr>
<td>1$^{st}$</td>
<td>2$^{nd}$</td>
<td>-42 – 0</td>
<td>3</td>
<td>THF</td>
<td>8%</td>
</tr>
</tbody>
</table>

The formation of the urea side-product was confirmed by MS and NMR, as can be seen in Figure 4.2.2 the aromatic protons close to the amino group increased their chemical shift due to the deshielding effect of the electron withdrawing urea. Urea 140 has previously described in the literature and it possesses similar toxicity to Amonafide.$^{108}$

Since the isolated yield obtained was very low, the reaction was ultimately carried out in collaboration with Dr. Robert Elmes group (University of Maynooth) using phosgene.
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(15 wt. % in toluene), which proved to be a lot more successful than BTC. Dr. Robert Elmes had previously reported a similar coupling using phosgene with 4-amino-naphthalimide in high yields.\textsuperscript{139}

\textbf{Figure 4.2.2}. Partial $^1$H NMR (400 MHz) of Amonafide (blue) and the side-product 140 formed (red), recorded in CD$_3$OD.

Elmes’ group carried out the reaction by reacting Amonafide with phosgene in anhydrous CH$_2$Cl$_2$ for 4 h (Scheme 4.2.13) followed by the addition of the alcohol (compounds 127 or 131) in dry CH$_2$Cl$_2$. The reaction was successful yielding both compounds 136 and 141 after 18 h in 32\% and 49\% yield, respectively, after C18 HPLC purification.
Scheme 4.2.13. Coupling reaction between Amonafide and compounds 127 or 131 carried out by Dr. Robert Elmes using phosgene.

As an example, Figure 4.2.3 shows the UV chromatogram showing relative purity of 75.1% ($\lambda_{abs} = 254$ nm) with a retention time of ca. 24 min, and in the bottom the MS analysis with a $m/z = 795.4$, corresponding to [M+H]$^+$. 

Figure 4.2.3. LC-MS spectrum of compound 141.

Compounds 136 and 141 were fully characterised by NMR and IR spectroscopy and MS spectrometry. Figure 4.2.4 shows the $^1$H NMR of compound 141 recorded in DMSO-$d_6$, the presence of a singlet found at 5.20 ppm integrating for two protons, corresponding to the CH$_2$ adjacent to the carbamate linker, is characteristic of this molecule as in the starting material, compound 131, these protons were coupled to the OH, thus appearing as a doublet with a coupling constant of 5.8 Hz (Figure 4.2.4). MS of compound 141 gave an accurate $m/z$ of 793.219124 for [M-H]$^-$ (C$_{37}$H$_{37}$N$_4$O$_{16}$).
Figure 4.2.4. a) $^1$H NMR (400 MHz) of compound 141 recorded in DMSO-$d_6$.

Having synthesised and characterised compounds 136 and 141, the deprotection reactions were carried out to synthesise the final compounds as prodrugs of Amonafide.

4.2.4 Deprotection of Compounds 136 and 141

The synthesis of the final deprotected compounds was achieved using Zemplén conditions involved cat. NaOMe in MeOH for the galactosyl derivative (compound 136) and NaOH in a mixture of MeOH/H$_2$O (1:1) was used for the glucuronyl derivative 141 to achieve complete ester hydrolysis (Scheme 4.2.14), being both hydrolysis achieved in almost quantitative yield. Compounds 119 and 120 were fully characterised by IR, MS and NMR techniques. The HRMS (ESI) of compounds 119 and 120 gave an accurate mass for [M+H]$^+$, which in the case of 120 is m/z = 655.1890, for C$_{30}$H$_{31}$N$_4$O$_{13}$. As can be seen in the $^1$H NMR of 120 (Figure 4.2.5), the O-Ac peaks and the methyl ester peak from compound 141 were no longer present, indicating the successful hydrolysis and formation of the product 120. Unfortunately, compound 120 was found to be insoluble in CD$_3$OD or D$_2$O (Figure A.4.1), thus DMSO-$d_6$ was used as solvent. Unfortunately, many of the characteristic peaks from the carbohydrate moiety lie underneath the solvent peak. However, 2D NMR experiments such as HSQC (Figure 4.2.5, inserts) allowed for full characterisation of the compound.
Scheme 4.2.14. Deprotection of compounds 136 and 141 using Zemplén conditions to give compounds 119 and 120.

Figure 4.2.5. $^1$H NMR (600 MHz) of compound 120 recorded in DMSO-$d_6$. 
These experiments also demonstrated that the singlet found at 6.54 ppm integrating by one, corresponds to a hydroxyl group. $^{15}$N NMR determined that the peak found at 10.51 ppm corresponded to the amino proton at the naphthalimide ring. As can be observed in Figure 4.2.5, some peaks in the aromatic region appeared doubled, which was also observed for compound 119. This was due to the presence of different conformers (rotamers) present, possibly due to rotation around the CH$_2$ adjacent to the carbamate linker or to the rotation of the carbamate linker (Scheme 4.2.15). Unfortunately, high temperature NMR experiments did not cause the peaks to coalesce, however, HSQC experiments demonstrate that there is only one compound present and this was further supported by the MS analysis as mentioned above.

Scheme 4.2.15. Possible rotation occurring around the CH$_2$ linker or carbamate linker present in compound, 120, leading to rotamers.

Having successfully synthesised and characterised compounds 119 and 120, their photophysical properties were evaluated, by means of UV-vis and luminescence spectroscopy. Therefore, the following section will present the UV-vis absorption and fluorescence spectra of 119 and 120, and their precursors 136 and 141 in PBS pH 7.2.

4.3 Photophysical Evaluation
4.3.1 Photophysical properties of 119 and 120
The UV-vis absorption, fluorescence and excitation spectra of compounds 119 (1 $\times$ 10$^{-5}$ M) and 120 (1 $\times$ 10$^{-5}$ M) were recorded in 10 mM PBS pH 7.2 (25 °C) and are shown in Figure 4.3.1. In both cases two absorption bands are centred at ca. 240 and 345 nm corresponding, respectively, to $\pi$-$\pi^*$ and ICT transitions (Figure 4.3.1, blue lines). The ICT absorption band presents a shoulder at ca. 383 nm. Excitation spectra, red lines in Figure 4.3.1, matches the absorption spectra, as expected. Excitation at 345 nm gives broad emission bands at 450 (for 119) and 490 nm (for 120), Figure 4.3.1, black lines.
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Figure 4.3.1. Absorption, emission and excitation spectra of a) 119 (1 × 10^{-5} M) and b) 120 (1 × 10^{-5} M), measured in PBS at 25 °C.

Table 4.3 summarises the characteristic bands of 119 and 120, as well as their extinction coefficients and photoluminiscence quantum yields. The extinction coefficients were measured in PBS pH 7.2 at 25 °C whereas quantum yields were measured in H2O. Fluorescence quantum yields have been calculated using equation 8.1, described in Chapter 8, using quinine as a reference standard. The quantum yield for quinine sulphate in 0.1 N H2SO4 solution is 54%.189

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ_{max} (nm)</th>
<th>π-π*</th>
<th>ICT (ε (M^{-1} cm^{-1}) ±10%)</th>
<th>ϕ_F (±SEM %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>119</td>
<td>246</td>
<td>343</td>
<td>387</td>
<td>12.5 (± 0.7)</td>
</tr>
<tr>
<td>120</td>
<td>245</td>
<td>343</td>
<td>377</td>
<td>15.3 (± 0.8)</td>
</tr>
</tbody>
</table>

The fluorescence quantum yield of compound 120 appeared to be 15.3%, whereas the fluorescence quantum yield of compound 119 was a bit lower, (12.5%). This could be explained by the increased hydrophilicity due to the presence of the carboxylic acid, which would influence its solubility in H2O.

Concentration studies of compound 119 and compound 120 were carried out in PBS pH 7.2 solution at 25 °C (Figure 4.3.2), with the view of determining if aggregation was occurring in this media. At concentrations above 4 × 10^{-5} M it became clear that the molecules aggregate, and quenching was observed in the fluorescence intensity (Figure 4.3.2b and d), however, no shift in the λ_{max} was observed. This aggregation does not have a significant effect in the ground state as it can be seen in Figure 4.3.2a and c, where the
absorption follows a linear trend, and as such obey the Beer-Lambert law even at high concentrations. These results suggest that the fluorescence decrease observed could be due to self-quenching caused by the aggregation occurring or by the PET effect, from the lone-pair in the nitrogen to the imide site of the naphthalimide, suddenly becoming more pronounced due to the self-assembly occurring in a head-to-tail fashion.

After photophysical evaluation of compounds 119 and 120, the changes in their photophysical properties upon treatment with glycosidase enzymes were examined.

**4.3.2 Changes in the Photophysical Properties of 119 and 120 Upon Enzymatic Activation**

The enzymatic activity of both probes developed above was evaluated. First, Amonafide (1 \( \times \) 10\(^{-5} \) M) was analysed spectroscopically, by means of absorption, excitation and fluorescence emission in PBS at 25 °C (Figure A.4.2). Next, compound analysis of 119 by MS (Figure A.4.3) and UV-vis spectroscopy (Figure 4.3.3) proved that Amonafide had
been released, as illustrated in Scheme 4.1.4. Changes in the absorption and fluorescence spectra following enzymatic cleavage allowed the reaction to be monitored spectroscopically. As can be seen in Figure 4.3.3a, a broader absorption band (410 nm) is observed after treatment with the enzyme, which corresponds to the Amonafide absorption band (410 nm) as can be seen in Figure 4.3.3c in the normalised absorption spectra, giving good evidence of its enzymatic release. However, the absorption band found at 410 nm is broader than the one found in Amonafide, as it is a superposition of Amonafide and the released linker (compound 116).

![Figure 4.3.3](image)

**Figure 4.3.3** Changes in the photophysical properties (a) absorption and b) emission) of 119 ($1 \times 10^{-5}$ M, $\lambda_{exc} = 347$ nm) before and after the addition of the enzyme ($\beta$-galactosidase, 1 U) recorded in PBS at 30 °C and c) Normalised absorption spectra and d) normalised emission fluorescence spectra ($\lambda_{exc} = 347$ nm) of Amonafide ($1 \times 10^{-5}$ M) and 119 ($1 \times 10^{-5}$ M) after treatment with 1 U $\beta$-galactosidase, recorded in PBS at rt. Figure representative of three independent experiments.

The fluorescence emission intensity ($\lambda_{exc} = 347$ nm) dropped dramatically after treatment with the 1 U of enzyme for 30 min. This decrease in the fluorescent intensity could be owed to the lower quantum yield of Amonafide. According to Qian et al.,207
Amonafide’s $\Phi_F$ in H$_2$O is 7.5%, in contrast with compound 119 which is almost double at 14%. Furthermore, a new band appeared at longer wavelength (590 nm), which corresponds to the characteristic band of Amonafide. This is evidenced in the normalised fluorescence emission spectra in Figure 4.3.3d, proving that treatment of compound 119 with 1 U of enzyme releases Amonafide and this release can be monitored spectroscopically.

In order to investigate the kinetics of the enzymatic process, a time-dependant experiment was carried out with 119 $(1 \times 10^{-5} \text{ M})$ using 1 U of β-galactosidase from Aspergillus Oryzae in PBS (Figure 4.3.4). The experiment was also conducted at three different temperatures (25, 30 and 37 °C), in order to understand the effect of the temperature on the enzyme and the resulting kinetics.

Compound 119 was incubated in PBS at 25, 30 and 37 °C, respectively, prior to the experiment in a thermostat fitted spectrometer, to identify if the temperature has any effect in the emission that could interfere with the emission changes observed in the presence of the enzyme.

Figure 4.3.4a shows how the fluorescence emission response of compound 119 $(1 \times 10^{-5} \text{ M})$ increases with temperature and time, with the highest overall intensity observed at 37 °C. A plausible explanation for this could be that higher temperature disrupts the aggregation of the compounds. Naphthalimides are well-known to form aggregates in solution that self-quench the fluorescence emission. However, as shown in Figure 4.3.2, concentrations studies previously carried out indicated that at the concentration used $(1 \times 10^{-5} \text{ M})$ no aggregation should be occurring. As previously explained, the photophysical properties of naphthalimides are highly dependant on the environment and can be affected by pH, polarity or temperature. However, not many examples can be found in the literature where the photophysical properties of naphthalimides can be modified by the temperature.

As can be seen in Figure 4.3.4, after 5 min incubation the emission stabilises and did not increase further, reaching a plateau. Therefore, before enzyme treatment compound 119 $(1 \times 10^{-5} \text{ M})$ was pre-incubated at the chosen temperature for 15 min. Even though the optimal temperature of β-galactosidase from Aspergillus oryzae was 30 °C, the reaction worked successfully at the three temperatures, with 37 °C found to be the fastest time point. As seen in Figure 4.3.4b, when using 1 U of the enzyme the hydrolysis had finished after 15 min when it was carried out at 37 °C. By contrast, it took 30 min to finish when carried out at 25 °C. However, it is important to note that even at low concentration of the
enzyme (0.1 U), the reaction still proceeded in a relatively short period of time (two hours).

Enzymes are measured by units (U), which corresponds to the amount of enzyme that is able to catalyse the conversion of 1 μmole of substrate per minute. However, this activity is quantified by using a particular substrate at certain temperature and pH. Therefore, the rate of an enzymatic reaction can change depending on the substrate, pH and temperature used, even when U is kept constant.

**Figure 4.3.4.** Emission intensity versus time of 119 (1 × 10⁻⁵ M) at different temperatures in PBS solution when incubated with a) no enzyme, b) 1 U of β-galactosidase, c) 0.5 U of β-galactosidase and d) 0.1 U of β-galactosidase. Figure representative of two independent experiments.

Enzyme kinetics are complex as there are several species competing in the reaction. As represented in scheme 4.3.1, in an enzymatic reaction the rate constant is a combination of the rate constants of formation of enzyme-substrate complex (ES) and the formation of product (P) from this complex.
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Scheme 4.3.1. Different species present in an enzymatic reaction.

The most common model used to study enzymatic reactions is the one developed by Michaelis and Menten. In this model, the rate constant (\(K_M\)) represents the concentration of substrate at which the rate of the reaction is half of the maximum reached by the system (when saturation of the enzyme occurs).

To spectroscopically determine the rate constant using this model, the rate of the reactions had to be determined using the absorbance, as a means of establishing the concentration of product. However, in our system both the substrate and product present similar absorption spectra, and therefore the concentration of the product formed alone could not be calculated. The values found in the literature for this enzyme shown \(K_M\) values of \(7.2 \times 10^{-4} \text{ M}\) and \(1.8 \times 10^{-2} \text{ M}\) when ortho-nitrophenyl-\(\beta\)-galactoside and lactose are used as substrates, respectively, and when the enzymatic reaction took place at 30 °C and pH 4.5 and pH 4.8, respectively, as these are the specified optimal conditions for this enzyme and these substrates. Therefore, the rate constant depends strongly in the environment (pH and T) but also in the substrate used, therefore limiting the possibility of comparing the values obtained with these compounds and the literature.

However, it was found that all the curves in these experiments could be fitted to a mono-exponential function, which corresponds to a first-order reaction. In a first-order reaction the rate constant only depends on the concentration of one species. Therefore the experimental rate constants could be calculated by fitting the curves to a monoexponential function. This implies that the limiting step of the reaction is the formation of product from ES (in bold in Scheme 4.3.2), omitting the reversible formation of the complex ES.

\[
\begin{align*}
E + S &\underset{K_{-1}}{\overset{K_1}{\rightarrow}} ES & K_2 &\rightarrow E + P \\
\end{align*}
\]

**Scheme 4.3.2.** Enzymatic reaction simplified to a first-order reaction and the equation to calculate the rate of a first-order reaction.

With this data, the half-life time of a first-order reaction was also calculated according to equation 4.1.
Equation 4.1

$$t_{1/2} = \frac{\ln 2}{k}$$

The experimental rate constant and their corresponding half-live time are summarised in Table 4.4.

Table 4.4. Rate constants (K) and $t_{1/2}$ of the enzymatic hydrolysis of compound 119 with different U of enzyme.

<table>
<thead>
<tr>
<th>Units Enzyme</th>
<th>T (°C)</th>
<th>$K$ (min$^{-1}$)</th>
<th>$t_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>0.0927</td>
<td>7.47</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.1374</td>
<td>5.04</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.1528</td>
<td>4.53</td>
</tr>
<tr>
<td>0.5</td>
<td>25</td>
<td>0.0424</td>
<td>16.34</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0632</td>
<td>10.96</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.1039</td>
<td>6.67</td>
</tr>
<tr>
<td>0.1</td>
<td>25</td>
<td>0.0097</td>
<td>71.45</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0130</td>
<td>53.31</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.0150</td>
<td>46.20</td>
</tr>
</tbody>
</table>

The same experiments were conducted with compound 120 (1 × 10$^{-5}$ M) using β-glucuronidase from Escherichia Coli. As seen in Figure 4.3.5, both the absorption and emission spectra after 1 h treatment with 1 U of the enzyme indicated the release of Amonafide, which was also confirmed by MS.

Figure 4.3.5. a) Absorption and b) fluorescence emission spectra of 120 (1 × 10$^{-5}$ M) before (black lines) and after (red line) treatment with β-glucuronidase (1 U) for 1 h at 37 °C. Figure representative of three independent experiments.
In contrast with compound 119, incubation of compound 120 at different temperature did not seem to have any effect on its emission intensity (Figure 4.3.5a). Therefore, only 5 min of pre-incubation were needed before adding the enzyme.

Time-dependant studies of 120 upon treatment of the enzyme indicated a much faster reaction than the one for compound 119. The enzymatic reaction appeared to conclude after only 5 min when 1 or 0.5 U of enzyme were used (Figure 4.3.6b and c). However, the reaction slowed down significantly when only 0.1 U of the enzyme were present, taking up to 40 min to conclude at 25 °C (Figure 4.3.6d, black line).

![Figure 4.3.6](image)

Figure 4.3.6. Emission intensity of compound 120 ($1 \times 10^{-5}$ M, $\lambda_{exc} = 350$ nm and $\lambda_{em} = 460$ nm) over time at different temperatures after a) no enzyme treatment, b) 1 U β-glucuronidase, c) 0.5 U β-glucuronidase and d) 0.1 U β-glucuronidase. Figure representative of two independent experiments.

A summary with the experimental rate constants and their half-life time are summarised in Table 4.5.
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The ICT absorption bands of compounds 136 and 141 also exhibit a shoulder at ca. 383 nm. The excitation spectra (red line) match the absorption spectra, indicating the purity of the sample. Excitation at 347 nm gives broad emission bands at 460 nm in both cases (Figure 4.3.7, black line).

<table>
<thead>
<tr>
<th>Units</th>
<th>Enzyme</th>
<th>T (ºC)</th>
<th>K (min⁻¹)</th>
<th>t½ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>25</td>
<td>0.0704</td>
<td>9.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0727</td>
<td>9.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.1445</td>
<td>4.79</td>
<td></td>
</tr>
</tbody>
</table>

Having already studied the effect of enzymatic hydrolysis in the photophysical properties of compounds 119 and 120, and demonstrated that the enzymatic reaction takes place within minutes and that can be monitored spectroscopically, the photophysical properties of the O-acetylated derivatives 136 and 141 were investigated, as they could also present interesting in vitro properties, which will be further discussed in sections 4.4.5 – 4.4.8.

### 4.3.3 Photophysical properties of 136, 141 and 75

The UV-vis absorption, fluorescence emission and excitation spectra of compounds 136 (1 × 10⁻⁵ M) and 141 (1 × 10⁻⁵ M) were recorded in 10 mM PBS pH 7.2 (25 ºC) and are shown in Figure 4.3.7. Two absorption bands are found at ca. 240 and 345 nm, respectively, corresponding to π-π* and ICT transitions (Figure 4.3.7, blue line). The ICT

![Figure 4.3.7](image-url)

**Figure 4.3.7.** Absorption, fluorescence emission and excitation spectra of a) 136 (1 × 10⁻⁵ M) and b) 141 (1 × 10⁻⁵ M) measured in PBS at 25 ºC.
absorption bands of compounds 136 and 141 also exhibit a shoulder at ca. 383 nm. The excitation spectra (red line) match the absorption spectra, indicating the purity of the sample. Excitation at 347 nm gives broad emission bands at 460 nm in both cases (Figure 4.3.7, black line).

In order to investigate the effect of the protected carbohydrate moiety in the photophysical properties of this class of compounds, compound 75, which synthesis has been previously described in Chapter 2, was also analysed. The absorption, fluorescence emission and excitation spectra can be found in Figure 4.3.8 when recorded in PBS buffer pH 7.2 at 25 °C. As can be seen in Figure 4.3.8, the excitation spectrum (red line) matching perfectly the absorption spectrum (blue line). Excitation at 347 nm gives a broad emission band centred at 415 nm, which is approximately 50 nm blue-shifted compared to compounds 136 and 141.

![Figure 4.3.8](image)

**Figure 4.3.8.** Absorption, fluorescence emission and excitation spectra of compound 75 (1 × 10⁻⁵ M) measured in PBS pH 7.2 at 25 °C.

Table 4.6 summarises the absorption and emission properties of compounds 136, 141 and 75. The quantum yields were also measured using quinine sulphate as reference, as previously discussed. Interestingly, the fluorescence quantum yield of compounds 136 and 141 are quite similar, in the range of 7%, whereas compound 75 is a lot higher (18%).
Table 4.6. Summary of characteristic bands, ε and ϕ_F of compounds 136, 141 and 75. Values obtained from three independant experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>π-π*</th>
<th>λ_{max} (nm)</th>
<th>ICT (ε (M^{-1} cm^{-1}) ±10%)</th>
<th>ϕ_F (±SEM %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>136</td>
<td>246</td>
<td>343 (1,800)</td>
<td>383</td>
<td>7 (± 3)</td>
</tr>
<tr>
<td>141</td>
<td>237</td>
<td>343 (3,800)</td>
<td>392</td>
<td>7.4 (± 0.7)</td>
</tr>
<tr>
<td>75</td>
<td>234</td>
<td>347 (8,100)</td>
<td>-</td>
<td>18 (± 4)</td>
</tr>
</tbody>
</table>

Concentration studies carried out with compounds 136 and 141 in PBS indicated that the molecules aggregate at concentrations higher than 2 × 10^{-5} M as seen in Figure 4.3.8b by the quenching in the fluorescence emission intensity above this concentration reaching a plateau. However, no shift in the λ_{max} occurred. The absorption spectrum of compound 136 follows a linear trend up to 3 × 10^{-5} M when plotted versus the concentration; however, there is a big increase in the optical density above these levels, losing this linear trend (Figure 4.3.9a).

Figure 4.3.9. Changes in the absorption (a and c) and fluorescence emission spectra (b and d) of 136 (a and b) and 141 (d and d), respectively, at different concentrations measured in PBS pH 7.2 at 25 °C. The inserts represent the variation of absorption or emission maxima, respectively, vs. concentration. Figure representative of three independent experiments.
For compound 141, both the absorption and the emission spectra follow a linear trend when increasing the concentration that is lost above $2 \times 10^{-5}$ M. However, above $3 \times 10^{-5}$ M a new linear trend is formed. This could be an indication of more than two species present in solution. A typical aggregation process would contain a reversible mixture of free molecules and aggregates up to a critical concentration upon which all the molecules are present in an aggregated state. However, in the case of compound 141, it seems that after all the aggregates are formed, a new species, which has higher optical density and higher fluorescent signal, appears in solution. This could be an indication of aggregation induced emission (AIE), however further experiments would need to be carried out to confirm this, such as DLS to confirm the aggregate formation or lifetime measures to obtain an indication of the number of emissive species in solution.

Concentration studies of 75 carried out in PBS buffer pH 7.2 at 25 °C showed similar aggregation occurring than compounds 136 and 141. However, as can be seen in Figure 4.3.10, the optical density of compound 75 increased linearly, even up to concentrations above $1 \times 10^{-4}$ M, whereas the fluorescence emission started to quench at concentrations higher than $6 \times 10^{-5}$, therefore, compound 75 showed less tendency towards aggregation. This could be explained by carbohydrate moiety being closed to the naphthalimide unit, therefore inhibiting the π-π staking interactions. Furthermore, due to the triazole ring being directly attached to the naphthalimide ring, the ICT effect is not as pronounced as for compounds 136 and 141, which possess a secondary amine in the aromatic site.

![Figure 4.3.10](image-url)

**Figure 4.3.10.** Changes in the absorption (a) and fluorescence emission (b) spectra of compound 75 at different concentrations. Recorded in PBS pH 7.2 at 25 °C. The inserts represent the variation of absorption or emission maxima, respectively, vs. concentration. Figure representative of three independent experiments.
Chapter 4. Glycosylated Naphthalimides as Prodrugs of Amonafide

The ICT is responsible for the dipole moment formed in naphthalimide derivatives, with a δ⁻ at the imide site and a δ⁺ at the aromatic ring (see section 1.5.1). The aggregation of naphthalimide is also caused and stabilised by these dipole moments, therefore, it is expected that a lower ICT leads to lower aggregation.

4.3.4 Effect of the pH in the Photophysical Properties of 136 and 141

The fluorescence emission of this type of compound presenting a tertiary amine separated by 2-3 methylene units from the imide site can be strongly influenced by the pH due to a PET process (Scheme 4.3.3). This PET process is prohibited upon protonation of the amine, as the lone pair of the nitrogen is no longer free to undergo electron transfer into the imide and impede emission back to the ground state, leading to a strong fluorescent signal observed. This phenomenon has been previously described in the literature for similar naphthalimide derivatives.

A pH titration of compounds 136 and 141 was carried out to check their stability at physiological pH. The titrations were carried out using a $1 \times 10^{-5}$ M concentration of compound and PBS as solvent to diminish the ionic strength.

As can be seen in Figure 4.3.11, both compounds exhibit higher a fluorescence signal at lower pH (red line), while the emission is turned off at basic pH (blue line). The quenching of the emission at basic pH is due to photo-induced electron transfer (PET) occurring from the tertiary amine to the imide (Scheme 4.3.1).

As can be observed, the OD and emission intensity did not match the initial values once the initial neutral pH was reached. This could be due to not waiting long enough between each addition, as small additions of sample were made therefore dilution should not have an effect. Back titration (from basic pH to acidic) was also performed to prove if the
compounds could be used as pH sensors; however, the initial emission intensity was not recovered even after waiting long periods of time (Figure A.4.4). This could also be due to de-acetylation occurring at pH > 9. Even though the carbohydrate moiety should not impart a strong effect in the photophysical properties of the molecules, the presence in the media of acetate ions might change their luminescence properties. Importantly, this experiment proved that even though these compounds were more emissive under acidic pH, they were stable a wide pH range, including physiological pH.

Figure 4.3.11. Changes in the absorption (a and c) and fluorescence emission (b and d) spectra of compounds 136 (1 × 10⁻⁵ M, a and b) and 141 (1 × 10⁻⁵ M, c and d) at different pH, carried out in PBS at 25 °C. Inserts represent λ_max vs. pH. Figure representative of two independent experiments.

Having spectroscopically characterised all the compounds, the in vitro properties using three cancerous cell lines; HeLa, HepG2 and HCT-116, previously described, was next investigated. The results are described in the following sections.
4.4 Biological Evaluation

4.4.1 Cellular uptake of compounds 119 and 120

The most critical aspect of these novel glycosylated naphthalimide derivatives was to investigate their ability to function as prodrugs of Amonafide in the presence of the relevant glycosidase enzyme. To investigate how compounds 119 and 120 behaved in vitro before and after enzymatic activation, HeLa cells were incubated with compound 119 (50 μM) and 120 (50 μM) by themselves and in the presence of 1 U of glycosidase enzymes, respectively. HeLa cells were also incubated with Amonafide (50 μM) for comparison. The results are depicted in Figures 4.4.1 and Figure 4.4.2. It can be observed that some

![Figure 4.4.1. Incubation of HeLa cells for 1 h with a) Amonafide (50 μM), b) 119 (50 μM) and c) 119 (50 μM) with β-galactosidase (1 U, 1 h further). Images representative of three independent experiments.](image-url)
sub-cellular localisation of compounds 119 and 120 after 1 h, however, there was a significant increase in the fluorescent signal observed inside the cells after treatment with the enzyme. This fluorescent signal matches the images obtained with Amonafide, supporting the hypothesis that enzymatic release of Amonafide had occurred.

To ascertain that Amonafide was being released successfully, the emission signal inside the cells was measured by confocal microscopy, examining a range of 410 – 780 nm (λ_{exc} = 405 nm) at 5 nm intervals, creating an emission spectrum that could be compared to Amonafide (Figure 4.4.3).

For both compounds 119 and 120 the fluorescent band was centred _ca._ 495 nm when excitation occurred at 405 nm (black line in Figure 4.4.3ab). Amonafide in contrast presented a band at longer wavelengths, _ca._ 550 nm (blue line in Figure 4.4.3).
A red shift was observed in compounds 119 and 120 when they were incubated in the presence of the enzymes (red line in Figure 4.4.3). This red shift consisted of a new band centred at 530 nm, which was indicative of the compounds releasing Amonafide. Since the new band formed was quite broad, it could be postulated that there was still some starting material left. However, this experiment confirmed that the enzymatic reaction was taking place in vitro successfully releasing Amonafide.

These experiments were also performed in HCT-116 and HepG2 cell lines, obtaining identical results. These images can be found in the Appendix (Figure A.4.4).

Further evidence supporting the release of Amonafide was obtained upon incubation of HeLa cells for 1 h with 119 (50 μM) and 120 (50 μM) and glycosidase enzymes (1 U), respectively, for 1 h further. The cell media was removed and the cells were washed with PBS three times followed by their incubation in H2O. After 20 min of incubation with H2O, the cell membrane had been disrupted due to osmotic pressure, allowing for the analysis of the compound found within the cells. The cells were centrifuged to remove cell contents and the supernatant was analysed by MS. Gratifyingly, in both cases, a signal corresponding to Amonafide was found by MS, strongly supporting successful enzymatic release (Figure A.4.5 and A.4.6).

### 4.4.2 Cell Viability of compounds 119 and 120

After demonstrating that enzymatic activation of 119 and 120 successfully released Amonafide in three different cell lines, the effect upon cell growth was measured via
Alamar Blue assay for a range of concentrations (100 – 0.01 μM). The toxicity is represented as the 50% inhibition of the normal cell growth (IC$_{50}$) and is measured in μM at three time points, 24, 48 and 72 h, respectively. The IC$_{50}$ values were calculated using a non-linear regression fitting with Graphpad Prism using data from three independent experiments. Some of the graphs can be found in the Appendix (Figure A.4.11) as representative examples.

The toxicity of Amonafide and the released linker 116 was also evaluated for comparison. Table 4.6 summarises the results.

It has been previously reported in the literature that the toxicity of Amonafide increases over time. For instance the IC$_{50}$ value of Amonafide incubated in HeLa cells for 24 h is of 10.67 μM, whereas after 72 h incubation there is a ca. 5-fold increase, to 2.73 μM. As can be seen in Table 4.7, an identical trend was found here, where the toxicity of Amonafide increased in HeLa and HepG2 cell lines significantly, with IC$_{50}$ values of 14 and 9 μM, respectively, at 24 h to IC$_{50}$ = 0.26 and 0.34 μM after 72 h, which corresponds to a 53 and 26 fold increase, respectively.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>t (h)</th>
<th>26</th>
<th>116</th>
<th>119</th>
<th>119 + E</th>
<th>120</th>
<th>120 + E</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>24</td>
<td>14 ± 1</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>29 ± 6</td>
<td>&gt;100</td>
<td>20 ± 8</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.57 ± 0.07</td>
<td>&gt;100</td>
<td>39 ± 5</td>
<td>18 ± 3</td>
<td>75 ± 1</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.26 ± 0.06</td>
<td>&gt;100</td>
<td>22 ± 2</td>
<td>11 ± 3</td>
<td>69 ± 6</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>HCT-116</td>
<td>24</td>
<td>0.5 ± 0.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>34 ± 6</td>
<td>&gt;100</td>
<td>14.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.26 ± 0.02</td>
<td>&gt;100</td>
<td>5 ± 1</td>
<td>5.4 ± 0.4</td>
<td>34 ± 5</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.30 ± 0.03</td>
<td>&gt;100</td>
<td>11 ± 1</td>
<td>5.2 ± 0.4</td>
<td>25 ± 3</td>
<td>1.65 ± 0.09</td>
</tr>
<tr>
<td>HepG2</td>
<td>24</td>
<td>9 ± 2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>30 ± 4</td>
<td>&gt;100</td>
<td>19 ± 3</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.36 ± 0.02</td>
<td>&gt;100</td>
<td>5 ± 1</td>
<td>3.1 ± 0.9</td>
<td>31 ± 6</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.34 ± 0.03</td>
<td>&gt;100</td>
<td>4.4 ± 0.6</td>
<td>1.5 ± 0.7</td>
<td>9 ± 2</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

The linker 116 proved non toxic (IC$_{50}$ >100 μM) at any time point in any cell line, proving that the antiproliferative effect is exclusively due to the release of Amonafide.
Chapter 4. Glycosylated Naphthalimides as Prodrugs of Amonafide

The toxicity values obtained from these compounds 119 and 120 furnished promising results. Neither of the compounds demonstrated toxicity at 24 h (\(IC_{50} >100 \, \mu M\)) in any cell line when incubated alone. However, when the glycosidase enzymes were introduced, the \(IC_{50}\) appeared to be ca. 30 \(\mu M\) for 119 and ca. 20 \(\mu M\) for 120 at 24 h. When the incubation time increased to 48 and 72 h, the results obtained from each compounds were different. In the case of 119, the compound became slightly toxic even when the enzyme was not present, with an average \(IC_{50}\) between the three cell lines of 16 \(\mu M\). In HeLa cells, the activation with the enzyme led to an increase in the toxicity, from an \(IC_{50}\) of 39 to 18 \(\mu M\). Nonetheless, the difference in toxicity in HCT-116 and HepG2 after activation with the enzyme was negligible. This behaviour remained for the 72 h time point.

In contrast, compound 120 did not show an increase in the cytotoxicity when incubated alone at 48 or 72 h, with the obtained \(IC_{50}\) values ca. 70 \(\mu M\), 30 \(\mu M\) and 30 \(\mu M\) for HeLa, HCT-116 and HepG2, respectively. Even allowing for the small variation in cytotoxicity between each cell line, the enzyme activation was still very effective, as the toxicity imparted by the enzymatic activation was ca. 15 fold higher for each cell line.

To understand the large difference observed in toxicity between the two compounds, further confocal microscopy studies were conducted and will be discussed in the next section.

4.4.3 Cell stability of compounds 119 and 120
To investigate if the compounds were being uptake by the cells in greater concentration over time producing the observed increase in toxicity, incubation of HeLa, HepG2 and HCT-116 cell lines with 119 (50 \(\mu M\)) and 120 (50 \(\mu M\)) for 24 h were carried out. As a representation, the results of this incubation in HeLa cells can be found in Figure 4.4.4, the incubation in HepG2 and HCT-116 are found in the appendix (Figures A.4.7-10).
Figure 4.4.4. Confocal microscopy of Hela cells incubated for 24 h with a) 119 (50 μM) and b) 120 50 μM). Images representative of three independent experiments.

Confocal images of 24 h time incubation show a stronger fluorescent signal inside the cells than the 1 h time point, which could explain the increased toxicity over time. Importantly, for compound 119 the fluorescence spectra recorded inside the cells using confocal microscopy (Figure 4.4.5) showed that its fluorescent band had been red-shifted, matching the typical band from Amonafide. This result indicated that when HeLa cells were treated with 119, endogenous glycosidases were able to cleave the β-D-galactose unit and release Amonafide, which explains why the toxicity at 48 and 72 h time points were very similar in both the presence and absence of the enzyme. This result highlighted the significant potential of this system as prodrugs of Amonafide as it demonstrated that endogenous glycosidases are able to activate the release of a cytotoxic agent. Importantly, this behaviour also remained in HCT-116 and HepG2 cell lines (Figure 4.4.5).
Chapter 4. Glycosylated Naphthalimides as Prodrugs of Amonafide

4.4.4 Two-Photon Microscopy Images of 119 and 120

Being able to monitor the release of Amonafide in vitro is of great importance, as it will allow to monitor where the selective delivery is occurring. As previously explained in Chapter 3, TPM microscopy allows for in vivo imaging as tissue penetration (up to 1 mm) is possible.\textsuperscript{97} TPM was used to prove that this new class of glycosylated naphthalimides absorbed two-photon allowing for in vivo imaging. This experiments were carried out in collaboration with Dr. Gavin McManus, form the School of Biochemistry, who took the TPM images. HeLa cells were incubated with 119 (50 μM) and 120 (50 μM) with and without the presence of glycosidase enzymes (Figure 4.4.6) and the excitation wavelength

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**Figure 4.4.5.** Emission spectra of a) 119 (50 μM) and b) 120 (50 μM) inside the cells after 24 h incubation using confocal microscopy in three different cell lines. Figures representative of three independent experiments.

On the contrary, this was not observed for compound 120, although more compound had been able to undergo cell uptake, the fluorescence spectra did not show a shift in any cell lines, proving that in this case no endogenous glycosidases were able to cleave β-D-glucuronic acid and release Amonafide. This matches the toxicity results obtained, as there is a significant increase in the cytotoxicity when the activation with the enzyme occurs in all the time points. Gratifyingly, this result also highlighted the importance of this system, as only very specific enzymes (β-glucuronidases) that are over-expressed in certain cancers would engender release of the therapeutic payload.

Having demonstrated the capability of compounds 119 and 120 to undergo cell uptake, release Amonafide in vitro thus causing cell death, their capacity to be imaged using two-photon microscopy was investigated, as this could allow for in vivo monitoring.
was 850 nm. Nuclear stain DRAQ5 was not used for this experiment, as it would also be excited at this wavelength. Using TPM four different emission channels are recorded at the same time. Blue range ($\lambda_{em} = 397 – 420$ nm), Green ($\lambda_{em} = 455 – 490$ nm), yellow ($\lambda_{em} = 525 – 557$ nm) and red ($\lambda_{em} = 580 – 638$ nm). These colours are arbitrary and are only assigned for labelling simplification. These ranges are fixed and cannot be modified, as it is possible in confocal microscopy.

The blue channel was not shown as no relevant fluorescent signal was observed. When imaging with TPM a noticeable difference between before and after the addition of the enzyme was observed for both compounds, 119 and 120, in the red channel. However, this difference was less apparent at shorter wavelengths. Better images were obtained with compounds 119 and 120 when $\lambda_{exc} = 750$ nm, and the images can be found in the Appendix (Figure A.4.12). However, it is important to note that excitation at 850 is more suitable for in vivo imaging, which is the ultimate goal of this experiment.

After demonstrating that compounds 119 and 120 successfully release Amonafide in vitro and therefore induce cytotoxicity in a range of cancerous cell lines, compounds 136, 141 and 75, described in Chapter 2, were investigated for similar behaviour, as their higher lipophilicity could change completely their cellular uptake and hence their biological properties.
Figure 4.4.6. Two-Photon Microscopy of HeLa cells incubated with a) 119 (50 μM), b) 119 (50 μM) with β-galactosidase (1 U), c) 120 (50 μM) and d) 119 (50 μM) with β-glucuronidase (1 U) when λ_{exc} = 850 nm. Images representative of three independent experiments.
4.4.5 Cellular uptake of compounds 136, 141 and 75.
Compounds 136, 141 and 75, described in Chapter 2, present a higher hydrophobicity due to the acetylated carbohydrate. An increased hydrophobicity can improve the cellular uptake, and in fact similar compounds have been found in the literature to be able to enter in the cells. Lozano-Torres et al.\textsuperscript{94} synthesised compound 142 and used it as probe for senescence cells (Scheme 4.4.1). Compound 142 was able to be uptake by the cells and once inside the carbohydrate moiety was cleaved, altering the photophysical properties of the molecule and turning on a fluorescent signal. However, no thorough discussion was made on the mechanism by which the glycoside was released inside the cells.

![Scheme 4.4.1. Compounds 142 reported for Lozano-Torres et al. as a senescence probe.\textsuperscript{94}](image)

To investigate whether a similar process could occur with these compounds, 136, 141 and 75 were incubated in HeLa cells for varying periods of time and imaged using confocal laser microscopy exciting at 405 nm.

As can be observed in Figure 4.4.7, after incubation of compound 136 (50 μM) and 141 (50 μM) for 1 h, cells had already incorporated the compounds into their cytoplasm. The cell media was not replaced before imaging therefore it can be assumed that most of the compounds had been uptake by the cells. HeLa cells were treated with DRAQ5 nuclear stain and by comparison it can be concluded that the compounds did not pass the nuclear membrane and remained in the cytoplasm.

In contrast, the incubation of HeLa cells with Amonafide (50 μM) led also to the incorporation of the compound also in the nuclei.
Figure 4.4.7. Incubation of HeLa cells for 1 h with a) Amonafide (50 μM), b) Compound 136 (50 μM), c) compound 141 (50 μM) and d) 75 (50 μM). Images representative of three independent experiments.

It is known in the literature that some Amonafide derivatives can be uptake by the cells via passive diffusion. Taking into account that only after 1 h the cells have incorporated...
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the compounds in great amount, it can be assumed that they also undergo passive diffusion. However, further test would be necessary to corroborate this hypothesis.

4.4.6 Cell Viability of Compounds 136, 141 and 75.
The cell viability of compounds 136, 141 and 75 were evaluated in HeLa, HepG2 and HCT-116 using an Alamar Blue assay and expressed as IC₅₀ (μM), the results are summarised in Table 4.7. Similarly to Amonafide, the toxicity of 136 and 75 increase over time, with a 6 fold increase for 136 and 9 fold for 75 when the compounds are incubated for 72 h instead of 24 h. However, the toxicity of compound 141 remained almost unchanged, with values close to 3 μM in HeLa and HCT-116 and 20 μM in HepG2.

Compounds 136 and 141 have toxicity values approaching those of Amonafide, Amonafide itself being of the range of 10 times more toxic. In contrast, compound 75 is significantly less potent than Amonafide (260 times less for HeLa cells and 46 times less for HCT-116). Suggesting than the linker between the glycoside unit and the Amonafide moiety plays an important role for the toxicity.

Table 4.7. IC₅₀ values of compounds 136, 141 and 75 after incubation for 24, 48 and 72 h, respectively, in HeLa, HCT-116 and HepG2 cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>t</th>
<th>26</th>
<th>136</th>
<th>141</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>24 h</td>
<td>14 ± 1</td>
<td>10.9 ± 0.7</td>
<td>2.6 ± 0.3</td>
<td>65 ± 9</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.57 ± 0.07</td>
<td>11.7 ± 0.7</td>
<td>2.9 ± 0.7</td>
<td>55 ± 3</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>0.26 ± 0.06</td>
<td>7 ± 1</td>
<td>2.5 ± 0.2</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>HCT-116</td>
<td>24 h</td>
<td>0.5 ± 0.2</td>
<td>13 ± 2</td>
<td>2.6 ± 0.6</td>
<td>94 ± 1</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.26 ± 0.02</td>
<td>5.4 ± 0.8</td>
<td>4.3 ± 0.6</td>
<td>10.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>0.30 ± 0.03</td>
<td>2.6 ± 0.6</td>
<td>1.6 ± 0.3</td>
<td>14.3 ± 0.7</td>
</tr>
<tr>
<td>HepG2</td>
<td>24 h</td>
<td>9 ± 2</td>
<td>19 ± 3</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.36 ± 0.02</td>
<td>5 ± 1</td>
<td>17 ± 2</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>0.34 ± 0.03</td>
<td>4.4 ± 0.5</td>
<td>23 ± 2</td>
<td>33 ± 5</td>
</tr>
</tbody>
</table>

After proving that compounds 136, 141 and 75 were rapidly uptake by the cells and that they also exhibited cytotoxicity for a range of cancerous cell lines, some efforts were made in order to understand the mechanism by which they induced toxicity. The following section describes subcellular localisation studies.
4.4.7 Cell stability and cellular localisation of compounds 136, 141 and 75. As the compounds have rapid uptake and good cytotoxicity profiles, some efforts were made in order to understand the mechanism by which the compounds are inducing cell death. As explained in Chapter 3, unnatural acetylated glycosides can be fed into cells and be rapidly internalised. Once inside the cells, cytosolic esterases are able to release the deprotected modified glycosides than are subsequently processed by the cells.\textsuperscript{212} After deprotection, the compounds could be susceptible to the enzymatic release of Amonafide by endogenous enzymes, as previous results demonstrated. This mechanism is depicted in Scheme 4.4.2 using 136 as an example.

\begin{scheme}
\centering
\includegraphics[width=\textwidth]{scheme_4.4.2.png}
\caption{Different species that could be found inside the cells due to deprotection of acetyl groups by cytosolic esterases and release of Amonafide by endogenous glycosidases.}
\end{scheme}

An experiment was performed to investigate if these glyconaphthalimide compounds were compatible with deacetylation by cytolosic esterases. HeLa cells were incubated with compounds 136 (50 μM) and 141 (50 μM), respectively for 2 h, followed by the replacement of the cell media by water to cause cell death and membrane disruption due to osmotic pressure. After 30 min, the suspension was taken and centrifuged to remove the cellular material and the supernadant was analysed using MS. Analysis of the sample using
APCI showed that only acetylated derivatives 136 and 141 were found (Figures A.4.11-12).

To further prove that Amonafide had not been released inside the cells, HeLa cells were incubated for 1 h with 136 (50 μM), 141 (50 μM), and Amonafide (50 μM), respectively, and fluorescence spectra of the compounds inside the cells were recorded using confocal microscopy (Figure 4.4.8).

As it can be observed in Figure 4.4.8, the fluorescence emission spectra of the compounds localised inside the cells possess a band centred at 460 nm, which is ~50 nm blue shifted than the corresponding for Amonafide. Therefore proving that compounds 136 and 141 did not release Amonafide inside the cells.

Because of these very positive results that clearly show that this design principle worked satisfactory, HeLa cells were next incubated with the two compounds at longer time points. However 6 h was the longest incubation time possible to be recorded, as the cells were starting to show high levels of toxicity and after 24 h of incubation no cells were alive. Figure 4.4.9 is a representative image of the incubation of HeLa cells with compound 136 (50 μM), 141 (50 μM), 75 (50 μM) and Amonafide (50 μM) incubated for 6 h.

For compounds 136, 141 and 75 there was no remarkable difference between 1 h and 6 h incubation, however in the case of Amonafide it can be said that there is a higher content.
of compound within the nucleus. This difference indicated that compounds 136 and 141 are not releasing Amonafide.

After proving that the toxicity induced by 136 and 141 was not due to the release of Amonafide, a cellular localisation study was carried out to understand the mechanism of toxicity. Figure 4.4.10 represents an enlarged image of the incubation of compounds 136 (50 μM) and 141 (50 μM) respectively, in HeLa cells for 1 h. It can be noted that the compounds were localising mainly in two specific regions.
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<table>
<thead>
<tr>
<th>Compound</th>
<th>DRAQ 5</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Image](99x162 to 538x771)</td>
<td><img src="240x797" alt="Image" /></td>
<td><img src="520x69" alt="Image" /></td>
</tr>
<tr>
<td><img src="240x797" alt="Image" /></td>
<td><img src="520x69" alt="Image" /></td>
<td><img src="99x129" alt="Image" /></td>
</tr>
<tr>
<td><img src="240x797" alt="Image" /></td>
<td><img src="520x69" alt="Image" /></td>
<td><img src="99x117" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 4.4.9.** Incubation of HeLa cells for 6 h with a) Amonafide (50 μM), b) Compound 136 (50 μM), c) compound 141 (50 μM) and d) 75 (50 μM). Images representative of three independent experiments.
In one hand, it was postulated that the areas showing fiber-like emission was due to mitochondrial localisation. On the other hand, the were areas showing clusters that possessed higher fluorescence intensity and were located right beside the nucleus, causing it to curve around it adopting a bean shape. It was hypothesised that the later could correspond to lysosomal localisation, as they possess lower pH values (ca. pH 4-5) and as demonstrated in section 4.3.4, the fluorescence intensity of these compounds is higher at lower pH. Interestingly, compound 75 (50 μM) exhibited similar subcellular localisation (Figure 4.4.10c).

Figure 4.4.10. Enlarged image of HeLa cells incubated with a) 136 (50 μM), b) 141 (50 μM) and c) 75 (50 μM) for 1 h. Images representative of three independent experiments.
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To demonstrate that the compounds were mainly localised in the mitochondria, HeLa cells were transfected with a plasmid that contains a red fluorescent protein (DsRed). DsRed possesses an excitation and emission band of 557 and 592 nm, respectively, making it suitable for its visualisation along with these compounds. The transfection was done using Lipofectamine as transfection reagent.

The successfully transfected HeLa cells were incubated with compounds 136 (50 μM), 141 (50 μM) and 75 (50 μM) for different periods of time (Figure 4.4.11). As can be seen in Figure 4.4.11. The overlapped images with these compounds and DsRed

![Confocal image of HeLa cells transfected with DsRed and incubated for 6 h with a) 136 (50 μM), b) 141 (50 μM) and c) 75 (50 μM). Images representative of three independent experiments.](image)

**Figure 4.4.11.** Confocal image of HeLa cells transfected with DsRed and incubated for 6 h with a) 136 (50 μM), b) 141 (50 μM) and c) 75 (50 μM). Images representative of three independent experiments.
matched, proving that these compound localised in the mitochondria. It is feasible that the localisation of the compounds in the mitochondria damage the cells over time, thus causing cell death. However, no detailed experiments on the cell death were carried out.

A transfection carried out with Lysosomes-RFP, lysosomal labelling protein ($\lambda_{\text{exc}} = 555$ nm, $\lambda_{\text{em}} = 584$ nm), was also attempted, to prove the localisation of these compounds in the lysosome. Unfortunately, this transfection did not work, possibly due to an old plasmid batch being used. However, due to time constraints no further transfection was attempted with a new batch.

### 4.4.8 Two-photon Images of Compounds 136, 141 and 75.

HeLa cells were incubated with 136 (50 $\mu$M), 141 (50 $\mu$M) and 75 (50 $\mu$M) and were imaged after 1 h incubation (Figure 4.4.12). The best images were obtained when compounds 136 and 141 were excited at 780 nm and 75 at 840 nm.

As previously explained for compounds 119 and 120 the emission between 397-420 nm (defined as blue channel) is not shown, as there was no signal for any of the compounds. Interestingly, the results obtained with compound 136 and 141 were slightly different, while 136 was emissive in the three channels (green, yellow and red), 141 only showed significant signal in the green and yellow channel. Compound 75 showed an intense signal in the yellow channel and weaker emission in the red one.
Figure 4.4.12. Two-photon excitation microscopy image of HeLa cells incubated for 1 h with a) 136 (50 μM, λ_{exc} = 780 nm) b) 141 (50 μM, λ_{exc} = 780 nm) and c) 75 (50 μM, λ_{exc} = 840 nm). Images representative of three independent experiments.

4.5 Conclusions and Future Work
Two glycosylated naphthalimides that could potentially act as prodrugs of Amonafide have been synthesised and spectroscopically characterised, by means of absorption, fluorescence emission and excitation spectra and extinction coefficient and fluorescence quantum yield determination. Time-dependant studies of the enzymatic release of Amonafide demonstrated that the enzymatic reactions occur rapidly under physiological conditions.
The cellular uptake and cell viability of the two compounds evaluated in three cancer cell lines demonstrated that after enzymatic treatment, Amonafide was released and undergoes cellular uptake faster than its precursors. The IC$_{50}$ values obtained correlate to this effect, obtaining an enhanced toxicity after enzymatic activation.

Importantly, it has been demonstrated that endogenous enzymes can activate this class of compounds releasing Amonafide, as seen with the β-galactoside derivative compound 119, and verified by confocal microscopy. This was not observed for the β-glucuronic acid derivative compound 120, showing the relevance of the carbohydrate moiety as it bestows specificity.

Future work with these compounds will involve their evaluation in non-cancerous cell lines and, and if low or non levels of toxicity are obtained, in vivo and pharmacokinetics studies should be carried out to investigate the potential of these compounds as prodrugs of Amonafide.

The acetylated compounds 136 and 141 also demonstrated the importance of the carbohydrate moiety, in this case increasing the hydrophobicity of the molecules making them susceptible for passive diffusion. However, further experiments that prove the mechanism of uptake should be conducted.

These compounds were able to induce cytotoxicity at similar levels than Amonafide; however, it can be postulated that its mechanism of action is different as Amonafide is capable of entering the nucleus whereas these compounds were not. It was demonstrated that the compounds were localising mainly in the mitochondria, perhaps inhibiting its function and causing cell death. Nonetheless, more exhaustive studies should be performed to better understand their mode of action followed by evaluation in non-cancerous cell lines and in vivo studies.
Chapter 5

Glycosylated Naphthalimides for Lectin Detection
5.1 Introduction

5.1.1 Carbohydrates binding lectins

The term lectin was introduced in 1954 by W.C. Boyd to describe proteins of non-immune origin, with no catalytic activity, which are able to reversibly bind carbohydrates. The function of lectins is to promote cell-cell interactions through binding to carbohydrates that are displayed on the surface of cells. In general, lectins possess multiple binding sites capable of interacting with different monosaccharides of the same oligosaccharide. Some lectins can recognise only one substrate, while others have a lower fidelity and are able to interact with similar substrates. Although this low affinity might not appear very efficient, it is necessary to allow for the reversible nature of the communication events between cells. Using multivalent substrates i.e. a multitude sites of interaction simultaneously can lead to enhanced interaction/activity.

Due to their affinity for carbohydrates, lectins have been used as biomarker tools for different diseases. Many lectins show potential as biomarkers for early detection of malignant growth, or as autophagy inducers, and they all have varying degrees of interaction with the immune system.

Different glycoconjugates have been developed to monitor lectins using the multivalency mechanism, such as dendrimers. Different biophysical and biochemical techniques have been used to determine and monitor these carbohydrate-protein interactions, such as X-ray crystallography, NMR spectroscopy, isothermal titrations calorimetry or fluorescence spectroscopy.

5.1.2 Concanavalin A lectin

Con A is a lectin belonging to the legume-type family. It binds preferentially to α-linked mannoses, but it is also able to bind α-glucosides. Con A exists as a tetramer at pH 7 or higher, with each subunit ca. 72 Å apart, while at pH 4.5 – 5.5, it exists as a dimeric structure. However, regardless of the pH, each monomer contains two metal binding sites for Ca\(^{2+}\) and Mn\(^{2+}\) ions, which need to be bound for the sugar binding to take place.

Due to the presence of four subunits making up Con A, the multivalency approach has been widely used for binding. For example, Matsui et al. developed a trigonal DNA–carbohydrate conjugate consisting of a three-way junction piece of DNA containing multiple copies of maltose. Using a fluorescence binding assay they demonstrated that the substrate containing more sugars presented a 760-fold increase in $K_D$ values compared to...
the conjugates containing less sugars. This is due to the binding of 3-subunits of Con A at the same time, which is called the ‘bridging binding mechanism’.

5.1.3 Multivalent binding
Multivalency relies on the formation of non-covalent bonds and enhances affinity at a greater rate than the sum of monovalent interactions. It differs from cooperativity interactions as in this case the binding of one ligand does not facilitate the binding of the subsequent ligands. Multivalent interactions between the epitope and the corresponding ligand can occur simultaneously or independently, depending on the shape and size of both of them (Figure 5.1.1). Sliding binding (Figure 5.1.1. a) is high affinity binding mechanism due to internal diffusion of the lectin along the polymeric chain. Multivalency can also lead to the crosslinking of multimeric lectins (Figure 5.1.1.c). However, one of the largest stabilisation affinities in lectin binding is found when a cluster binding takes place (Figure 5.1.1 b), due to a low entropic cost due to the rigidity of the system once a bond is formed, favouring the binding of a second ligand due to its proximity.
This process may compete with a chelate mechanism in which a ligand, of sufficient length, can bind to several lectins and cross-linked them. In contrast, when the binding sites of a lectin are located far apart, an enhancement in the binding potency can be achieved through a mechanism called statistical rebinding. Here, the bound ligand is replaced by a free ligand in close proximity, leading to reduced off-rates.\(^{215}\)

However, the development of multivalent probes often presents significant synthetic challenges, and thus monovalent probes with high binding affinities are nevertheless still desirable, due to requiring generally less challenging synthetic efforts, which is desirable for commercial applications as complex multi-step syntheses increase development cost.
5.1.4 Tröger’s bases Naphthalimides

Tröger’s bases are cleft shaped molecules, which were synthesised for the first time in 1887 by Julius Tröger, by reacting \( p \)-toluidine and formaldehyde in the presence of acid (Scheme 5.1.1).\(^{221}\)

![Scheme 5.1.1. Synthesis of Tröger’s base from \( p \)-toluidine in acidic media.]

They exhibit a \( C_2 \) axis of symmetry due to the presence of two stereogenic nitrogen atoms, giving a mixture of enantiomers, \( (S,S) \) and \( (R,R) \), respectively.\(^{222}\) The presence of the bridgeheaded nitrogen atoms creates a twist in the molecule, with dihedral angles ranging from 82-104°.\(^{223}\) Because of their right-angle structure, Tröger’s bases have been extensively used in the field of molecular recognition and supramolecular chemistry.\(^{224}\)

The cleft shape of the Tröger’s bases has also been exploited for DNA binding interactions, due to its capacity to mimic the helical shape of the DNA.\(^{225}\) Previous work carried out within the Gunnlaugsson group has investigated the capability of Tröger’s base naphthalimides (TB-Naps) to interact with DNA.\(^{74}\) They synthesised compound 146 (Figure 5.1.2), and using X-Ray crystallography determined that its dihedral angle (112°) was beneficial in achieving a high binding affinity of the TB-Nap towards the DNA groove (\( K \sim 10^6 \text{ M}^{-1} \)). Despite its large size, in vitro studies carried out in HeLa cells demonstrated that compound 146 was rapidly internalised by the cells.
Figure 5.1.2. a) Compound 146 studied by Banerjee et al.\textsuperscript{74} for DNA binding and b) Its X-Ray structure showing the dihedral angle between the two naphthalimides cores.

Further studies have been carried out in the group using TB-Naps as fluorescent sensors in water.\textsuperscript{121} These studies demonstrated the suitability of TB-Naps for \textit{in vitro} studies.

5.1.5 Work described in this chapter

Previous studies within the Gunnlaugsson group developed a one-pot synthesis of TB-Naps from 4-aminonaphthalimide derivatives using formaldehyde in trifluoroacetic acid (TFA).\textsuperscript{226} Since a family of glycosylated naphthalimides containing a 4-amino functionality had previously been synthesised (Chapter 2), the synthesis of glycosylated TB-Naps and their photophysical and biological properties was investigated in this chapter. It was of particular interest to investigate the binding properties of this class of compound towards lectins, as a monovalent system (Naps) could be compared with a divalent system (TB-Naps). Since Con A is a well-studied lectin, it was used as a lectin model to undertake binding studies using compound 44 (\textbf{Man-Nap}) and its Tröger’s base derivative 45 (\textbf{Man-Tb}). Here, the possibility of higher binding affinity for Con A of the divalent \textbf{Man-Tb} versus a monovalent \textbf{Man-Nap}, in accordance with predictions based on the multivalent principle, was studied. Figure 5.1.3 illustrates possible binding modes of \textbf{Man-Nap} and \textbf{Man-Tb} with a tetrameric lectin such as Con A.
5.2 Synthesis

The synthesis of compound Man-Tb was first attempted using compound Man-Nap, the synthesis of which has been discussed in Chapter 2, by reacting it with paraformaldehyde in the presence of TFA for 18 h (Scheme 5.2.1), as has been previously described in the

Scheme 5.2.1. Attempted synthesis of compound the Tröger’s base compound 45.
Unfortunately, this reaction resulted in a complex mixture of products, and purification was complicated by the high polarity conferred to this molecule by the presence of the two α-D-mannose units. Attempts at purification using reverse-phase silica chromatography were unsuccessful and hence this synthetic route was abandoned.

An alternative synthetic route is depicted in Scheme 5.2.2. The synthesis starts using compound 147, which was synthesised by Dr. Sankarasekaran Shanmugaraju, from the Gunnlaugsson group, and its synthesis has been previously described in the literature.\textsuperscript{226}

Reaction of compound 147 with propargylamine in refluxing ethanol gave compound 148 in 94\% yield over 18 h. Compound 148 was subsequently reacted with 3-azido-1-peractylated-α-D-mannose (compound 55c, synthesis described in Chapter 2) under standard CuAAC conditions to furnish compound 149 in 65\% yield over 2 h. Finally,

\begin{center}
\textbf{Scheme 5.2.2.} Successful synthesis of Man-Tb.
\end{center}
deprotection using Zemplén conditions gave the final compound 45 (Man-Tb) in excellent yield of 98%.

The rationale behind using the protected mannose derivative (149) in the CuAAC was to increase the solubility of the substrate in organic solvents and thus facilitate purification by silica gel chromatography. However, its characterisation was nevertheless quite challenging due to issues with aggregation. $^1$H NMR carried out in CDCl$_3$ (Figure 5.2.1), where 149 was highly soluble, was very poorly resolved even when using an 800 MHz spectrometer and 128 scans. This strongly suggested that the compound was aggregating in solution. This aggregation was later confirmed in aqueous solution via fluorescence spectroscopy (section 5.3.1). The characteristic peaks of TB-Naps corresponding to bridging -N-CH$_2$ usually appear as two singlets integrating by 2 and 4 protons, respectively, and can be found around 5 – 5.50 ppm. In this area is also found the anomeric proton (H-1) from the carbohydrate moiety as a doublet. As can be seen in Figure 5.2.1, these 8 protons can be found in different multiplets between 5.40 and 5.18 ppm, demonstrating the formation of compound 149 (Figure 5.2.1). 2D NMR studies were carried out to complete the characterisation of compound 149.

Figure 5.2.1. $^1$H NMR (800 MHz) of compound 149 recorded in CDCl$_3$. 

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Chapter 5. Glycosylated Naphthalimides for Lectin Detection

Compound Man-Tb was characterised by NMR and IR spectroscopy and MS. Due to its strong ability to aggregate and poor solubility, the NMR of compound Man-Tb was recorded in deuterated trifluoroacetic acid (TFA-d). More convenient solvents were tried such as acetone-d₆, CD₃OD and D₂O as well as mixture of them (Figure A.5.1). However the ¹H NMR spectrum was poorly resolved and therefore TFA-d was used (Figure 5.2.2). The ¹H NMR spectra demonstrated the successful de-O-acetylation as their characteristic peaks found ca. 2 ppm were not longer present. Analysis by MS (MALDI) gave an accurate mass of m/z = 1063.3805, which corresponds to C₅₁H₅₅N₁₀O₁₆ ([M+H]⁺).

Figure 5.2.2. ¹H NMR (800 MHz) of Man-Tb recorded in TFA-d.

Having synthesised and fully characterised by a range of techniques compound 45 its spectroscopical properties were analysed by means of absorption, fluorescence and excitation spectra, and quantum yields determination, as well as the monovalent compound 44 in order to compare them. These properties are described in the next section.
5.3 Photophysical Evaluation

5.3.1 Photophysical Characterisation of compounds Man-Nap and Man-Tb

Naphthalimide 44 (Man-Nap) possesses a broad characteristic ICT absorption band due to the 4-amino substitution, with its maximum found at 430 nm (Figure 5.3.1a). The fluorescence spectra recorded in PBS at 25 °C present a band centred at ca. 530 nm (λ_{exc} = 433 nm). Its TB derivative (45, Man-Tb) presents an absorption band at 380 nm, which possesses a shoulder ca. 350 nm. The emission spectra showed a band centred at 510 nm (λ_{exc} = 380 nm). Therefore, both the absorption and emission spectra of the Man-Tb are blue-shifted by ca. 50 nm with respect to the Man-Nap. This blue shift is due to the substitution of the amine at the 4-position, hindering the ICT process. It can be observed that in both compounds the excitation spectra (red lines) matches the absorption spectra (blue lines).

![Figure 5.3.1. a) Absorption, fluorescence emission and excitation spectra of Man-Nap (1 × 10^{-5} M). b) Absorption, fluorescence and excitation spectra of Man-Tb (1 × 10^{-5} M). Experiments recorded in PBS pH 7.2 at 25 °C.](image)

Table 5.1 summarises the relevant photophysical properties of compounds Man-Nap and Man-Tb in PBS. The fluorescence quantum yields were calculated using Fluorescein as the reference, following equation 8.1 described in Chapter 8. The fluorescence quantum yield of Fluorescein is of 0.920 when measured in 0.1 N NaOH.³
Table 5.1. Summary of characteristic bands, ε and ϕ of compounds Man-Nap and Man-Tb, measured in PBS pH 7.2 at 25 °C. Results obtained from three independent studies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ_{max} (nm)</th>
<th>π→π*</th>
<th>ICT (ε (M⁻¹ cm⁻¹) ±10%)</th>
<th>ϕ_F (±SEM %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man-Nap</td>
<td>254, 273</td>
<td>433</td>
<td>(12,000)</td>
<td>11.50 ± 0.09</td>
</tr>
<tr>
<td>Man-Tb</td>
<td>-</td>
<td>380</td>
<td>(8,900)</td>
<td>2.50 ± 0.09</td>
</tr>
</tbody>
</table>

Prior to investigating the binding properties of Man-Nap and Man-Tb towards Con A, concentration studies were carried out in DPBS (Dulbecco’s phosphate-buffered saline) solution. As can be seen in Figure 5.3.2, in both cases, aggregation in DPBS occurred at concentrations higher than 5 × 10⁻⁵ M. However, the fluorescence quenching was more pronounced in the case of compound Man-Nap. Interestingly, a small red shift of the λ_{max} in the absorption and fluorescence emission spectra of Man-Tb was observed, whereas it did not occur in Man-Nap.

Figure 5.3.2. Changes in the absorption spectra of Man-Nap (a) and Man-Tb (c) and fluorescence emission spectra of Man-Nap (b) and Man-Tb (d), respectively, with different concentrations, measured in DPBS at 25 °C. Inserts represent the maximum wavelength plotted versus the concentration. Representative images of three independent experiments.
After characterising the compounds spectroscopically their affinity towards Con A was evaluated, and will be discussed in the next section.

5.3.2 Con A binding studies

5.3.2.1 Con A binding studies at low concentration

The changes in the absorption and fluorescence emission spectra of Man-Nap (1 × 10^{-5} M) upon binding with Con A where recorded in DPBS pH 7.2 (0.1 mM CaCl$_2$ and 0.1 mM MnCl$_2$) at 25 °C and can be seen in Figure 5.3.3. Interestingly, even though precipitation occurs when Man-Nap binds with Con A, due to the complex formed being more insoluble, the UV-vis spectra still reflects the binding with Con A, with not much scattering being observed (Figure 5.3.3a).

**Figure 5.3.3.** Changes in the absorption (a) and fluorescence emission spectra (b) of Man-Nap (1 × 10^{-5} M) upon the addition of Con A recorded in DPBS pH 7.2 (0.1 mM CaCl$_2$ and 0.1 mM MnCl$_2$) at 25 °C. c) changes in the fluorescence emission intensity (λ$_{\text{max}}$ = 545 nm) of Man-Nap vs. Con A equivalents. Figures representative of three independent experiments. d) Changes in the fluorescence emission intensity (λ$_{\text{max}}$ = 545 nm) of Man-Nap vs. Con A equivalents from three independent experiments.
The band observed at $\lambda_{\text{max}} = 280$ belongs to the Con A, as previous concentrations studies with solely Con A demonstrated (see later discussion). Excitation at 433 nm showed that the fluorescence emission intensity increased upon binding with Con A, with a significant increase found between 0.2 and 0.3 equivalents, and a blue shift in the $\lambda_{\text{max}}$ of approximately 20 nm (Figure 5.3.3b).

This notable increase in fluorescence emission intensity can be clearly observed in Figure 5.3.3c, where the emission intensity is plotted versus Con A equivalents. Importantly, this behaviour was completely reproducible, as can be seen in Figure 5.3.3d, which shows the changes in fluorescence emission vs. Con A equivalents of three independent titrations with Con A. It is important to note that above 1 equivalent of Con A, no further increase in the fluorescence intensity of Man-Nap was observed and a plateau was reached, which could suggest a 1:1 binding.

As it was mentioned previously, the band centered at 280 nm in the absorption spectra belongs to the Con A, and it might suggest that the changes seen in the characteristic ICT band from Man-Nap, $\lambda_{\text{max}} = 430$ nm, are a result from the tail of the band at 280 nm. However, as can be seen in Figure 5.3.4, concentrations studies carried out with Con A demonstrated that although there was influence of this band at 430 nm it was not as significant as shown in Figure 5.3.3. Importantly, excitation at 433 nm gave no fluorescence emission (Figure 5.3.4b), therefore, proving that the changes previously seen with Con A correspond solely to changes in the photophysical properties of Man-Nap upon binding with Con A.

![Figure 5.3.4](image_url)

**Figure 5.3.4.** Changes in the absorption (a) and fluorescence emission spectra ($\lambda_{\text{exc}} = 433$ nm) of Con A at different concentrations, recorded in DPBS pH 7.2 (0.1 mM CaCl$_2$ and 0.1 mM MnCl$_2$) at 25 °C.
Unfortunately, attempts to obtain a binding constant were not successful. Recently Bernardi et al.\textsuperscript{227} have reported a glycodendrom-rhenium complex as probe for Con A, and were able to determine the binding constant, by means of $K_d$, using the Hill model, which can quantify the interactions between ligands and binding site and is usually employed to determine cooperativity.\textsuperscript{228} Using this method they obtained a $K_d$ of 7.27 $\mu$M. However, attempts to fit the data obtained with \textbf{Man-Nap} into the Hill plot using GraphPad prism software resulted unsuccessful ($K_d = 0.4 \mu$M ($\pm 0.1$), $R^2 = 0.7268$).

When the studies were carried out using \textbf{Man-Tb}, similar results were obtained (Figure 5.3.5). In this case, the scattering occurring was a lot higher than for the previous compound, therefore, the changes in the absorption spectra did not follow a logical trend. An increase and decrease in the OD were observed alternatively, regardless of the addition of Con A, as a result from the scattering occurring. Interestingly, these changes were observed at lower equivalents of Con A than the ones required for \textbf{Man-Nap} (0.2 – 0.3 equiv). Here, the addition of only 0.1 equivalents of Con A already induced significant changes in the absorption spectra (Figure 5.3.5a).

Nevertheless, as a result of this scattering, these changes were not reproducible at concentrations of Con A above 0.3 equivalents, as can be seen in Figure 5.3.5c, which shows the changes in the absorption ($\lambda_{\text{max}} = 380 \text{ nm}$) vs. Con A equivalents of three independent experiments. The fluorescence emission spectra ($\lambda_{\text{exc}} = 380 \text{ nm}$) did not show significant changes. In Figure 5.3.5b, a sharp band can be seen at $\lambda_{\text{max}} = 420 \text{ nm}$, this band was most likely Raman scattering from the H$_2$O, as a result of having a wide excitation slit (5 nm) to monitor the low fluorescence emission. Similar results have been observed in the literature.\textsuperscript{229} Apart from this band, only small changes can be observed, with a subtle decrease in the fluorescence emission intensity observed at $\lambda_{\text{max}} = 525 \text{ nm}$. Figure 5.3.5d shows the changes in the fluorescence emission ($\lambda_{\text{max}} = 525 \text{ nm}$) vs. Con A equivalents of three independent experiments, and similarly to the changes observed in the UV-vis spectra, no reproducibility was observed. As the changes observed were not reproducible, no attempts to fit the data were made.

As the aforementioned experiments did not give much information about the binding occurring between Con A and the glycosylated naphthalimides and TB-naphthalimides, further studies were performed at higher concentration, and will be discussed in the next section.
Chapter 5. Glycosylated Naphthalimides for Lectin Detection

Figure 5.3.5. Changes in the absorption (a) and fluorescence emission spectra (b) of \( \text{Man-Tb} (1 \times 10^{-5} \text{ M}) \) upon the addition of Con A recorded in DPBS pH 7.2 (0.1 mM CaCl\(_2\) and 0.1 mM MnCl\(_2\)) at 25 °C. Figure representative of three independent experiments. c) Changes in the absorption (\( \lambda_{\text{max}} = 390 \text{ nm} \)) of \( \text{Man-Tb} \) vs. Con A equivalents of three independent studies and d) Changes in the absorption (\( \lambda_{\text{max}} = 525 \text{ nm} \)) of \( \text{Man-Tb} \) vs. Con A equivalents of three independent studies.

5.3.2.2 Con A binding studies at high concentration

The binding affinity of \( \text{Man-Nap} \) and \( \text{Man-Tb} \) towards Con A at high concentration (\( 1 \times 10^{-4} \text{ M} \)) was investigated. It is important to note that at this concentration both compounds were aggregating (see 5.3.1) and therefore, the emission was being quenched by aggregation (self-quenching). As Con A is a large macromolecule, it was expected that the binding of compounds \( \text{Man-Nap} \) and \( \text{Man-Tb} \) to it would prevent their aggregation, leading to a change in the fluorescence emission. Measurements of the ground state by UV-vis spectroscopy were not possible due to scattering problems (Figure A.5.2), as the binding with Con A forms aggregates that precipitate out of the solution, as previously observed.
Figure 5.3.6. Fluorescence emission spectra of a) Man-Nap (1 × 10^{-4} M, \( \lambda_{exc} = 430 \) nm) and b) Man-Tb (1 × 10^{-4} M, \( \lambda_{exc} = 380 \) nm) before (black lines) and after the addition of 0.1 Equivalents of Con A (red lines), respectively, measured in DPBS (0.1 mM CaCl\(_2\) and 0.1 mM MnCl\(_2\)) at 22 °C. Representative images of three independent experiments.

As a qualitative test, 0.1 equivalents of Con A were added to a solution of Man-Nap (1 × 10^{-4} M) and Man-Tb (1 × 10^{-4} M), respectively, in DPBS (0.1 mM CaCl\(_2\) and 0.1 mM MnCl\(_2\)) at 22 °C. As can be observed in Figure 5.3.6, the results varied depending on whether the Man-Nap or Man-Tb derivatives were used. In the case of Man-Nap, the fluorescence was turned on significantly upon the addition of Con A; by contrast, the fluorescence of Man-Tb was turned off (\( \lambda_{em} = 510 \) nm) and a new band appeared at 450 nm. These results are in agreement with the results obtained at lower concentrations.

In this case, where aggregation and self-quenching was occurring, the decrease in the fluorescence signal of Man-Tb was unexpected, but could be explained by the fact that aggregation did not induce as much quenching as for Man-Nap. A new band appeared in the fluorescence spectrum of Man-Tb at 450 nm and increased linearly with the concentration of Con A. This band was not observed at the experiments performed at lower concentrations.

A back titration was carried out with Con A by treating compounds Man-Nap and Man-Tb with 0.1 equivalents of Con A and decreasing its concentration while keeping the concentration of Man-Nap and Man-Tb constant. As the changes observed for Man-Tb were not so noticeable, the deviation from the initial fluorescence (\( F_0 \)) respect to the fluorescence response upon binding Con A (F) was plotted (Figure 5.3.7), for Man-Tb as well as for Man-Nap for direct comparison. In the case of Man-Tb, represents the
Figure 5.3.7. Changes in the fluorescence increase or decrease of a) Man-Nap (1 × 10⁻⁴ M, λ⁺⁺ = 430 nm) and b) Man-Tb (1 × 10⁻⁴ M, λ⁺⁺ = 380 nm) upon addition of different concentrations of Con A. Experiments carried out in DPBS (0.1 mM CaCl₂ and 0.1 mM MnCl₂) at 22 °C. Representative figures of three independent experiments.

increase and decrease observed at 450 and 510 nm, respectively, whereas in the case of Man-Nap only an increase was found at 535 nm.

As can be seen in Figure 5.3.7, the fluorescence intensity of Man-Nap upon treatment with Con A increased linearly up to 9 × 10⁻⁶ M, after which the fluorescence intensity plateaus. Similarly, the lowest concentration of Con A at which the fluorescence emission changed was 1 × 10⁻⁶ M (0.1 equiv).

For compound Man-Tb the fluorescence increased linearly at λ = 410 nm up to 8 × 10⁻⁶ M, reaching a plateau afterwards in a similar manner to Man-Nap. In addition, the lowest concentration of Con A invoking a change was 1 × 10⁻⁶ M as well (0.1 equiv).

Attempts to fit this data into a Hill plot gave a $K_d = 7 \times 10^{-6}$ M (± 0.1) ($R^2 = 0.9892$). However, these values were not reproducible in further titrations (Figure A.5.3), with $K_d$ values obtained from 9 × 10⁻⁶ M to 1 × 10⁻⁶ M, and therefore and accurate binding constant could not be given for this system. It has to be taken into account that there are three different factors taking place in this system: First, Con A binding with Man-Nap inducing a change in the fluorescence emission intensity, as demonstrated with the studies performed at lower concentration. Secondly, de-aggregation of Man-Nap caused by this binding, and therefore also modifying the fluorescence emission intensity, as aggregation of Man-Nap leads to self-quenching (as demonstrated in Figure 5.3.2). And third and last, the Con A binding form a lower water-soluble complex, which makes the solution turn turbid and can have an effect in the emission intensity due to the secondary inner filter.
effect. Therefore, it is not surprising that the fitting in order to get a binding constant was not reproducible, as there are many factors in play. However, the ability of these compounds to bind to Con A at high (and low) concentration was still demonstrated.

Following this, the effect of Con A binding under different conditions was subsequently investigated. Figure 5.3.8 represents the normalised emission intensity of Man-Nap and Man-Tb with Con A after different treatments.

Whereas the addition 0.1 equivalents of Con A led to a ca. 70% increase in fluorescence intensity in the case of Man-Nap and a decrease of 33% for Man-Tb, the addition of an excess of \( \alpha\)-D-mannose (0.2 equiv) to the mixture did not induce any changes. This may suggest that the ligands bind more efficiently to Con A. However, further studies, including computational analysis, would need to be undertaken to confirm this.

In a separate experiment, the ligands were incubated with denatured Con A (heated at 100 °C for 30 min), and no significant change in the emission was observed, demonstrating that the initial changes observed in the fluorescence were due to binding with the \( \alpha\)-D-mannose unit, and not only due to the presence of large macromolecules in solution.

Following this rationale, 0.1 equivalents of BSA were added to the ligands under identical conditions, but in this instance, no significant changes were observed, further demonstrating that the fluorescence change was due to substrate recognition and selective binding with Con A.
In another experiment, 0.1 equivalents of Con A were added to a mixture of BSA with either Man-Nap or Man-Tb, to investigate if the presence of BSA would interfere with binding to Con A. Gratifyingly, the fluorescence changes followed an identical trend as the experiment carried out without BSA, proving that the binding of Con A is as efficient and selective in the presence of other biomacromolecules.

Finally, the binding of Man-Nap to Con A in the presence of another lectin was investigated. When Man-Nap or Man-Tb were incubated with 0.1 equivalents of Peanut Agglutinin (PNA), no changes in fluorescence intensity were observed. However, when Con A (0.1 equiv) was added to the mixture, the same changes in fluorescence intensity were observed to those observed previously, providing further evidence of substrate selectivity in binding Con A.

The previous experiments demonstrated that the compounds are able to bind efficiently with Con A even when other macromolecules were present. After this, a series of negative control studies were carried out, to further demonstrate that the fluorescence changes were only observed when efficient binding was taking place.

Structurally related compounds 63a (Gal-Nap) and 63d (Lac-Nap), synthesised and described in Chapter 2, containing a β-D-galactose and β-D-lactose unit, respectively, were incubated with Con A, as well as compound 149 as it is the acetylated version of Man-Tb.

![Figure 5.3.9](image)

**Figure 5.3.9.** Changes in the normalised intensity of a series glycosylated naphthalimide (1 × 10^{-4} M) upon addition of 0.1 equivalents of Con A in DPBS (0.1 mM CaCl\textsubscript{2} and 0.1 mM MnCl\textsubscript{2}) at 22 °C.
As shown in Figure 5.3.9, none of these exhibited any changes in their fluorescence intensity upon addition of Con A. Thus it is clear that compounds Man-Nap and Man-Tb bind selectively with Con A, and this binding process can be measured via fluorescence spectroscopy, up to low concentrations of the lectin Con A of 1 μM.

Following the demonstration of a successful binding between Man-Nap and Man-Tb to Con A, independently of the presence of other macromolecules, and having proven that the monovalent system (Man-Nap) binds more efficiently, the ability of other glycosylated naphthalimides towards other lectins was investigated. The next section describes the capacity of compounds 63a (Gal-Nap) and 63d (Lac-Nap) to bind to the lectin PNA.

5.3.3 Study of the binding properties of compounds 63a and 63d with PNA.

PNA is a plant lectin protein that binds selectively to the carbohydrate sequence Gal-β-(1-3)-GalNAc, however it also binds to β-D-galactose and β-D-lactose derivatives.230 Similarly to Con A, PNA also possesses a tetrameric quaternary structure.

As the best results for Con A binding were obtained with Man-Nap, it was decided to investigate the affinity of compounds Gal-Nap and Lac-Nap towards PNA. As previously described for Con A, 0.1 equivalents of PNA lectin were added to a solution of 1 × 10^{-4} M of Gal-Nap and Lac-Nap, respectively. The studies were conducted in PBS at 22 °C, as the presence of metal ions is not necessary for binding to occur in this case.

Figure 5.3.10. Fluorescence emission spectra of a) Gal-Nap (1 × 10^{-4} M) and b) Lac-Nap (1 × 10^{-4} M) before (black lines) and after (red lines) the addition of 0.1 equivalents of PNA. Experiment performed in PBS at 22 °C, λ_{exc} = 430 nm. Figure representative of three independent experiments.
Disappointingly, no significant changes in the fluorescence intensity were observed with either of the compounds (Figure 5.3.10). Only a small, but not statistically significant increase in fluorescence intensity was observed for compound Gal-Nap, while for compound Lac-Nap there was a small decrease in emission intensity that was likely a result of dilution effects.

A possible explanation for why no changes were observed in this case could be due to the achievable protein concentration, since if the binding between the glycosylated naphthalimides and PNA was not strong, 0.1 equivalents may not be enough to disrupt the aggregation, leading to the changes in fluorescence emission. Unfortunately, the molecular weight of PNA was too high to use at higher concentrations, since the stock solutions needed were not water-soluble.

Following the spectroscopical characterization, a morphological evaluation was carried out. Although compounds Gal-Nap and Lac-Nap were not capable to binding to PNA or that this binding was not able to induce a luminescence change allowing for its determination, it was proven in section 5.3.2.2 that Con A binding to Man-Nap and Man-Tb was still occurring when the molecules aggregate. This binding can reduce the self-quench caused by the naphthalimide core aggregation, therefore inducing a change in the fluorescence emission intensity. Therefore, studies of the morphology of these aggregates were of great interest. These studies are described in the next section and illustrate the changes of the supramolecular structures upon binding to Con A.

5.4 Morphological changes of Man-Nap and Man-Tb upon Con A binding.
Upon binding with Con A, both compounds Man-Nap and Man-Tb precipitated out of the solution, forming small aggregates. Since it was noted that both compounds Man-Nap and Man-Tb tend to aggregate in solution, the changes in their morphology after binding with Con A using Scanning Electron Microscopy (SEM) were investigated. The images shown in this section have been taken by Jason Delente, from the Gunnlaugsson group.

DPBS and Con A in DPBS (1 × 10^{-5} M) were prepared as controls (Figure 5.4.1 a and b, respectively) to allow for comparison of the morphological changes. Solutions of Man-Nap (1 × 10^{-4} M) and Man-Tb (1 × 10^{-4} M) were prepared in DPBS and the aggregates formed were deposited onto a silicon plate and dried under vacuum prior to SEM imaging.
Although both of the compounds formed aggregates with a spherical-like shape by themselves (Figure 5.4.1, a and b), interaction with Con A led to a very different morphology (Figure 5.4.1, c and d). Whereas the **Man-Nap**/Con A assembly formed an amorphous precipitate (Figure 5.4.1d), the **Man-Tb**/Con A assembly formed regular spheres of *ca.* 2 μm diameter, which were more than ten times larger than the spheres observed for **Man-Tb** alone (Figure 5.4.1f).

These images provided further evidence of the efficient Con A binding disrupting the aggregation of **Man-Nap** and **Man-Tb** in DPBS solution.
After establishing that an efficient binding between Con A and compounds \textbf{Man-Nap} and \textbf{Man-Tb} is occurring and produces morphological changes which modify their emission properties, their suitability for \textit{in vitro} experiments was determined by means of cellular uptake and their anti-proliferative effect. These studies can be found in the next section.

5.5 Biological Evaluation

5.5.1 Uptake studies of Man-Nap and Man-Tb

Uptake studies carried out in HeLa cells with compound \textbf{Man-Nap}, already reported in Chapter 2 (Figure 2.4.1), demonstrated that no cellular uptake was observed even after long incubation times (24 h). In Figure 5.5.1a, it can be seen the green fluorescence signal emitted from the naphthalimide surrounding the cells, demonstrating no cellular uptake occurring.

Uptake studies of compound \textbf{Man-Tb} showed that is being taken up by the cells after incubating for 24 h (Figure 5.5.1b). As can be seen in Figure 5.5.1b, the compound is localised inside the cells forming aggregates. The cells were washed and replaced with fresh media to ensure that the aggregates were inside the cells. Unfortunately, incubation with compound \textbf{Man-Tb} and DRAQ5 (nuclear stain) was not possible as it quenched its fluorescence. This phenomenon was also observed in \textit{in vitro} studies carried out in the Gunnlaugsson group with other TB-Naps. Therefore, \textit{in vitro} studies of compound \textbf{Man-Tb} did not contain nuclear stain.

The protected α-d-mannose units in compound 149 made it more interesting for cell uptake purposes, since it presented higher lipophilicity. However, this compound formed large aggregates in aqueous solution. Incubation of 149 in HeLa cells showed that after short incubation times (1 h) the aggregates became larger and not much cellular uptake had occurred (Figure 5.5.1d). Instead, the aggregates were interacting with the cell membrane. At longer times (24 h) the aggregates observed were smaller and more of them had been taken up by the cells (Figure 5.5.1c). The cells were also washed and cell media replaced before imaging, to ensure that the compound observed in the confocal images was either inside the cells or interacting with their membrane. Z-stack studies proved that the smaller aggregates were inside the cells whereas the larger ones are interacting with the membrane (Figure A.5.4).
Figure 5.5.1. Confocal image of HeLa cells incubated with a) Man-Nap (50 μM) for 24 h, b) Man-Tb (50 μM) for 24 h, b) 149 (50 μM), c) for 24 h and d) 149 (50 μM) for 1 h. Compound Man-Tb was not incubated in HeLa cells with DRAQ5 because it quenched its fluorescence. Representative image of three independent experiments.
The large aggregates found at the 1 h time-point were also emissive at > 600 nm, as can be seen in Figure 5.5.1d, second column, which corresponds to the nuclear stain.

Since both TB-Naps, compounds \textbf{149} and \textbf{Man-Tb}, were localised inside the cells after 24 h incubation, their effect in the cell-proliferation was evaluated \textit{via} Alamar Blue assay and can be found in the next section.

\textbf{5.5.2 Cytotoxicity of Man-Nap and Man-Tb}

The effect on the cell proliferation of compounds \textbf{Man-Tb} and \textbf{149} in HeLa cells was evaluated \textit{via} an Alamar Blue assay in a range of concentrations (100 – 1 µM). The cytotoxicity of \textbf{Man-Nap} was already reported in Chapter 2 and it was found that the compound was not toxic in HeLa cells after 24 h incubation (IC\textsubscript{50} >100 µM), which was not surprising since the cells did not take up the compound. Since compounds \textbf{Man-Tb} and \textbf{149} were found inside the cells at 24 h, their anti-proliferative effect after longer times (72 h) was also assessed. Table 5.2 summarises the IC\textsubscript{50} values obtained after 24 and 72 h incubation, respectively. Even though cell internalization was occurring for compounds \textbf{Man-Tb} and \textbf{149}, no effect on the cell proliferation (\textit{i.e.} IC\textsubscript{50} >100 µM) was observed even after long incubation times of 72 h.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Compound} & \textbf{IC\textsubscript{50} (µM)} & \textbf{t (h)} \\
\hline
\textbf{Man-Tb} & >100 & 24 \\
\textbf{149} & >100 & 72 \\
\hline
\end{tabular}
\caption{IC\textsubscript{50} (µM) values of compound \textbf{Man-Tb} and \textbf{149} in HeLa cells measured at different times.}
\end{table}

The fact that these compounds proved to be non-toxic towards HeLa cells was a gratifying result as it highlighted the potential for these probes to be used as non-toxic probes for the selective detection of lectin \textit{in vitro}.

\textbf{5.5 Conclusions}

A novel Tröger’s base naphthalimide was successfully synthesised and fully characterised spectroscopically. Both of the compounds, \textbf{Man-Nap} and \textbf{Man-Tb}, were shown to successfully bind with Con A, as demonstrated spectroscopically by the significant changes in their fluorescence intensity upon binding. This was demonstrated at low
concentrations of $1 \times 10^{-5}$ M, with a significant increase in the fluorescence emission intensity of Man-Nap being observed. However, this was not the case for the Man-Tb as it showed scattering even at very low concentrations of Con A (0.02 equiv). This scattering is a consequence of the formation of a less soluble complex. Due to the poor resolution observed in the fluorescence emission spectra of Man-Tb, a direct comparison between the two ligands cannot be drawn. However, the fact that more scattering was observer in the case of Man-Tb may suggest a better binding due to the presence of the two $\alpha$-D-mannose. Nonetheless, better results were obtained with Man-Nap, as the binding with Con A invoked a noticeable increase in the fluorescence emission. Importantly, both compounds showed to be able to bind to Con A at low and high concentrations and the Con A binding was measured with both compounds up to 1 $\mu$M concentrations of Con A.

The compounds demonstrated the ability to bind selectively with Con A even in the presence of other macromolecules such as BSA or PNA. Similar compounds were used as negative controls to provide further evidence that the changes in the fluorescence only occur when the compounds bind to Con A.

The binding occurring was supported by SEM studies, where it was observed that notable changes in the morphologies of both compounds were evident upon binding with Con A.

In vitro studies of both compounds in HeLa cells showed that they were non-toxic, even though Man-Tb was internalised by the cells. Compound 149 was also evaluated in vitro due to its higher lipophilicity. Uptake studies showed large aggregates being dissolved and taken up by the cells over time.

In conclusion, these findings offer a successful proof-of-principle study that demonstrates the potential of glycosylated naphthalimide derivatives to be used as molecular probes for lectin detection. Their simple structures could pave the design of more complex probes in the future.
Chapter 6

Supramolecular Self-Assembly of Glycosylated Naphthalimides
6.1 Introduction

Various supramolecular structures have been formed by the glycosylated naphthalimides described in the previous chapters. Taking into account that the spectroscopic and biological properties of the compounds (as free molecules) had already been explored, it was decided to examine their supramolecular structures to investigate if they could be of interest as novel biomaterials. The use of biomaterials for biological applications has been expanded in the recent years.\textsuperscript{231,232} Biomaterials applications range from drug delivery, antibacterial, or the improvement or replacement of natural functions.\textsuperscript{233-235} The main objective in this field is to create materials that respond to artificial stimuli such as pH, temperature, light or magnetic irradiation, \textit{etc}. Recently, examples of biomaterials inducing cancer apoptosis or a drug-delivery mechanism have emerged using hyperthermia.\textsuperscript{236,237} Photothermal therapy (PTT) uses nano-materials that absorb in the near infrared (tissue penetrating) and transform this energy into heat, thus causing apoptosis in the cancerous cells. For instance, Leiyin \textit{et al.}\textsuperscript{236} developed polyethylene glycol (PEG) nanoparticles of compound 150 (Figure 6.1.1), which absorbs in the NIR and transforms this energy into heat. Assays carried out using $\lambda_{\text{exc}} = 808$ nm for only 8 min irradiation showed a temperature increase from 25 °C to 47 °C, leading to a significant size tumour reduction in mice bearing HSC tumour.

![Figure 6.1.1. Compound 150 developed by Leiyin \textit{et al.} forming PEG-nanoparticles that induces apoptosis via PTT.\textsuperscript{236}](image)

However, preventing the accumulation of nanoparticles within the body is still challenging and therefore the development of degradable materials is still needed.\textsuperscript{238,239}
Therefore, many groups have focused their research in the development of biodegradable materials.\textsuperscript{240-243} Chen and co-worker\textsuperscript{240} developed silica nanoparticles bearing Doxorubicin (Dox) coated with collagen and hyaluronic acid, which will improve biocompatibility and cancerous cell delivery, as the CD44 receptor for hyaluronic acid is overexpressed in certain tumours. More importantly, hyaluronidase (HAase) would degrade the nanoparticles thus releasing the drug (Dox) inside the cells and inducing cell death. Similarly, gels have also been used to encapsulate drugs,\textsuperscript{244,245} and DeForest and co-workers,\textsuperscript{243} have recently developed hydrogels that can be selectively released by a combination of stimuli, such as enzymatic, light irradiation or reducing conditions (Scheme 6.1.1). These hydrogels can be engineered depending on the desired combinations of stimuli required to reach the degradation, as certain diseases-states present overexpressed enzymes or reducing environments.\textsuperscript{243}

\begin{center}
\begin{tikzpicture}
\node at (0,0) {R-S-S-R'};
\node at (2,0) {Redcing
\begin{tikzpicture}
\node at (0,0) {R-S-H + HS-R'};
\node at (2,0) {Enzymatic
\begin{tikzpicture}
\node at (0,0) {R-G-P-Q-G-I-W-G-Q-R'};
\node at (2,0) {hv \lambda = 365 \text{nm}};
\node at (0,-1) {\text{Scheme 6.1.1. Different stimuli that trigger hydrogels degradation designed by DeForest group.}^{243}};
\end{tikzpicture}
\end{tikzpicture}
\end{center}

As it was previously discussed, naphthalimides can interact with each other via $\pi-\pi$ interaction of their aromatic cores. This aggregation can lead to the formation of supramolecular structures in solution such as spherical aggregates or gels. The following section describes recent examples of self-assembled naphthalimides and their applications.

### 6.1.1 Supramolecular Structures of Naphthalimides.

Many examples have been reported in recent years regarding the self-assembled structures of naphthalimides.\textsuperscript{128,246-252} Jing \textit{et al.}\textsuperscript{246} developed a naphthalimide-based gel compound \textsuperscript{151} (Figure 6.1.2) that can distinguish between aliphatic and aromatic amines by changes in the gel state and fluorescence quenching; the compound bearing a boric acid ester group at the 4-position of the aromatic ring and an aliphatic chain (C-12) at the imide site. The gels were obtained in polar aromatic solvents such as acetone, ethyl acetate, DMF as well
Chapter 6. Supramolecular Self-Assembly of Glycosylated Naphthalimides

as a mixture of DMSO/H$_2$O (10:1 v/v) (Figure 6.1.2). The concentration necessary to form the gel varies on the solvent, being in the range of 8 – 25 mg/mL. All the gels exhibited fluorescence emission, however a solvatochromic effect was observed, presenting stronger fluorescence emission when formed in EtOAc, which was expected for this class of compounds.\textsuperscript{246}

Figure 6.1.2. Compound 151 and SEM image of gel formed in EtOH at 25 mg/mL.

Substitution of the boric acid ester for $O$-phenyl group yielded compound 152 (Figure 6.1.3), which showed a completely different self-assembly properties and therefore resulted in different morphology.\textsuperscript{247} In this case, a fiber-like material was obtained in organic solvents such as acetonitrile (Figure 6.1.3b). These emissive gels were used for sensing tri-nitrophenols (TNP), as the emission became red-shifted upon interaction with the TNP as there could be an electron-transfer from the electron-rich 152 molecule to the electron-deficient TNP. Interestingly, although no chiral groups are present in the molecule, the fibers observed showed a helical shape. Circular dichroism studies confirmed the presence of a negative band, indicating the chirality.\textsuperscript{247}

Figure 6.1.3. Compound 152 and SEM images of gel formed in acetonitrile.
Similar compounds, having an extended aliphatic chain (C-18) at the imide site were used as sensors for Hg(II) and Fe(III) ions, compounds 153 and 154 in Figure 6.1.4. However, it is worth noting that the small changes in the molecular structure lead to a complete different self-assembly. For instance, changing the 4-position from a 2-pyridil (153) to a 3-pyridil group (154) resulted in different morphologies in acetonitrile. Compound 153 was unable to form a gel, and instead it only crashed out of solution. Upon examination of the precipitate with field emission scanning electron microscopy (FE-SEM), it was observed that microspheres of approximately 30 μm were formed. However, compound 154 was able to form gel in acetone made up of linear fibers, as observed by the FE-SEM (Figure 6.1.4a).

These dramatic changes in the self-assembly are associated with the dihedral angle formed between the pyridine ring and the naphthalimide, being 17.7° for 153 and 92.2° for 154. Small angles enable π-π stacking interactions between the molecules, resulting in stronger non-covalent interactions. For gel formation, these interactions need to be weaker and therefore larger dihedral angles are preferred.

It is easy to understand the aggregation of the previous compounds as they are formed by a naphthalimide core with small aromatic functionalisation and long aliphatic chains that are easy to compact. However, the aggregation of naphthalimides functionalised with more polar units have also been reported. Yi and co-workers have reported nanotubes formed by aggregation of naphthalimides functionalised with δ-glucoronolactone (Figure 6.1.5). These nanotubes were formed in ethanol, and they convert into hydrogels upon sonication in water. The nanotubes proved non-toxic in HeLa cells so their ability to
encapsulate drugs was investigated using tetracycline hydrochloride, as an example, and was demonstrated that 25% of the encapsulated drug was released after 8 h. An increase on the alkynyl chain between naphthalimide and the alkyne functionality in compound 156 (Figure 6.1.5) gave a completely different supramolecular structure with a precipitate being formed in water, resulting in a compact microsphere of 10 μm diameter (Figure 6.1.5a). Sonication and heating of this precipitate in water formed hydrogels (Figure 6.1.5b), as can be seen by the cross-linked fibers formation.

Yi also investigated the effect that water has in the packing mode of self-assembled molecules in organic solution. They observed that a honeycomb structure was obtained from the self-assembly of compound 157, Figure 6.1.6. By varying the water content of the mixture, the sizes of the pores were modified (Figure 6.1.6a-d), increasing the size of the pores with the water content.

These gels were formed by evaporation of initial solutions of CHCl₃ or CH₂Cl₂. It was found that the presence of water weakens the intermolecular interactions, affording larger pores in the gels. Understanding how the presence of water changes the self-assembly process is of great interest, as it allows the formation of multiple structures from a single compound.
6.1.2 Work described in this chapter

This chapter describes the self-assembly of some synthetic glycosylated naphthalimides in order to investigate the possibility to exploit them as biomaterials. The compounds from which self-assembly is described are found in Figure 6.1.7. This research was conducted due to initial self-assembly behaviour observed with compound 63a by previous members of the research group. Interestingly, compounds 63d and 106, which present very similar structure, gave very different morphologies, indicating the possibility of tuning these materials by small structural changes. As it has been mentioned in the previous section, the self-assembly of naphthalimides forming spherical aggregates or gels is well known.\textsuperscript{246-248,251} However, in this chapter attempts have been made to understand the importance of the carbohydrate moiety in the supramolecular structures as the presence of the carbohydrates unit allows for the use of glycosidase enzymes, therefore enabling easy modification of the morphology under physiological conditions.
Compounds 141 and 75, which present a N,N'-dimethylethlenediamine group at the imide side and O-acyl protected carbohydrate moieties, displayed a very different morphology than the previous examples, supporting the hypothesis of the importance of the carbohydrate in the self-assembly. The supramolecular structure of these compounds has been analysed by SEM and confocal fluorescence microscopy.

### 6.2 Supramolecular studies of glycosylated naphthalimides
#### 6.2.1 Supramolecular studies of unprotected glycosylated naphthalimides

Previous work carried out in a collaborative project between the Gunnlaugsson and the Scanlan group using glycosylated naphthalimide structures found that compound 63a formed microspheres spontaneously in CD$_3$OD. This work was performed by Stefan Acherman, as part of a Master’s project, and the size and morphology of these microspheres were examined by Helium-Ion Beam microscopy (HIB) (Figure 6.2.1).

From the HIB images of these microspheres it was observed that they were of approximately 100 – the 150 µm diameter and were formed by fibers that were on average 2-5 µm of length, and were intertwined forming the 3D porous material (Figure 6.2.1c and d). Interestingly, these microspheres presented pores of approximately 10 µm (Figure
6.2.1.b). These microspheres presented a high surface area that was considered to be interesting for gas absorption studies.

![Microsphere images](image.png)

**Figure 6.2.1.** Helium-Ion Beam microscopy images of glycosylated microspheres formed by compound 63a in CD$_3$OD.

The fibres forming these architectures are, most likely, formed by the π-π stacking interactions between the naphthalimide cores, as previously shown for compound 50 in Chapter 2. However, no similar spherical aggregates had been reported with naphthalimides before, suggesting that hydrogen bonding interactions occurring between the hydroxyl groups in the galactose unit play an important role for their structure. It has previously been reported that natural polysaccharides present a well-defined helical structure due to hydrogen-bonding interaction between the carbohydrate units in the polysaccharide structure.\(^{253,254}\) For instance, amylose, a component of starch, is formed by α-D-glucose with 1-4 linkages and displays a helical shape.\(^{255}\) However, since compound 63a possesses only a monosaccharide it was estimated that it would not form a helical structure.

As the microspheres looked highly porous, attempts to measure their capability to be used as gas adsorption, storage or separation systems were performed by nitrogen adsorption/desorption analysis. These experiments were carried out by Kevin Byrne from the Prof. Schmitt group at TCD and consist of first purging the sample with an inert gas (Helium) to remove any possible moisture or residual solvent (methanol) from the surface.
Afterwards, the sample is exposed to a low pressure of gas, Nitrogen in this case. These results obtained were the typical obtained by a porous material (Figure 6.2.2a).

![Figure 6.2.2. Surface measurement of compound 63a. a) adsorption/desorption isotherm and b) BET plot and linear fit.](image)

When the sample was exposed to N\(_2\) a monolayer was formed (horizontal trend, Figure 6.2.2a), this was followed by the formation of a multilayer and finally by a capillary formation, when the gas was filling the pores. This resulted in a noticeable increase of the isotherm (increasing the slope of the graph). When the pores have been completely filled, a plateau is usually reached, which was not observed in this case, and desorption process takes place. The gap between the desorption and adsorption isotherms is an indication of a porous material.

To quantify the surface area of the pores, the data was analysed using the Brunauer, Emmett and Teller (BET) method, which uses the phase of the monolayer formation of the BET, the BET plot is obtained (Figure 6.2.2b) following Equation 8.2, described in Chapter 8. According to this method, the surface area of the pores was found to be 87.215 m\(^2\)/g (R\(^2\) = 0.9887). This was in agreement with a second method use, Dubinn-Radushkevic (DR), which gave a surface area of 84.809 m\(^2\)/g (R\(^2\) = 0.9998). These methods indicated that the surface area of the pores was approximately 85 m\(^2\)/g. Taking into account that both methods gave good correlation coefficient with similar relatively high value surface area, there was a strong evidence that these materials were porous, however further physical examination of the spheres was needed, and thus attempts were made to replicate them.

Besides of the π-π and hydrogen bonding interactions forming these microspheres, it was understood that the environment and conditions in which they were formed was
significantly important for their morphology. As the initial microspheres were formed spontaneously in an NMR tube over time, there was no record of their concentration,

![Figure 6.2.3. HIB microscopy image of microspheres formed by 63a when tried to replicate in CD$_3$OD.](image)

...temperature or time of formation. It is important to note that 63a was not very soluble in deuterated methanol, and therefore the solution had to be heated up to high temperatures and sonicated. Therefore, attempts to replicate them turned out to be quite challenging. Several attempts, initially unsuccessful, were carried out by a different masters student (Lukas Sricker), but microspheres were finally observed (Figure 6.2.3).

The microspheres formed in the replicate experiment were slightly smaller in size (80 – 100 µm), as can be seen in figure 6.2.3a. They were also formed by fibres (Figure 6.2.3b) but in this case the fibres were located more parallel to each other, therefore there was no pores observed in these structures. As can be seen in Figure 6.2.3a, not all the material were forming spheres, which indicated that the process by which these spheres are formed is not arbitrary but it depends on specific conditions such as time, temperature and concentrations. With all this in mind, the controlled formation of these microspheres and their morphology was next investigated.

Compound 63a was re-synthesised (as described in Chapter 2) and, as previously observed, compound 63a formed microspheres spontaneously in CD$_3$OD that can be observed with the naked eye (Figure 6.2.4), similarly to the ones previously found. Other aggregates were observed in H$_2$O or EtOAc, however, none of them presented a regular shape. It is not possible to determine their self-assembly without an X-Ray structure,
however, the X-Ray structure obtained of compound 50 (Chapter 2) reveals that a head-to-tail aggregation was occurring, although the molecules are not completely orthogonal to each other and instead they present a 120°. Very likely, the presence of the galactose unit would modify their self-assembly arrangement due to the larger amount of hydrogen-bonding interactions being able to form.

Figure 6.2.4 shows the formation of spherical aggregates observed inside an NMR tube, which was subsequently investigated using confocal fluorescence microscopy. The microspheres were decanted from the NMR tube and left on a silicon plate overnight to ensure that all CD$_3$OD had been evaporated.

![Figure 6.2.4](image)

As can be noted in Figure 6.2.4a the spheres in solution were interacting with each other but still keeping a spherical shape. However, when the solvent was removed most of them lost partially or completely their spherical shape, suggesting that hydrogen bonding interactions between CD$_3$OD and the hydroxyl groups in the β-D-galactose unit were necessary to maintain their structure. Different drying times (1-7 days) and different temperatures (22 °C and 65 °C) were examined, to understand if the drying process was an important factor for their supramolecular structure. Unfortunately, no improvement in the formation of the spheres was observed and most of them were disrupted after drying, as they were too weak. Figure 6.2.5 shows the self-assembly of compound 63a after drying in CD$_3$OD by a) confocal microscopy and b-d) SEM. SEM images were taken by Jason Delente, from the Gunnlaugsson group.

It can be observed in Figure 6.2.5a that the presence of interweaving fibres is supporting the supramolecular structure. This result agrees with the initial aggregates observed by
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previous students from the group. SEM images (Figure 6.2.5b-d) also demonstrated the presence of fibres, however the spherical shape had been lost.

As glycosylated naphthalimide purification turned quite difficult due to the $\pi-\pi$ stacking interaction of the naphthalimides, previously synthesised compound 63a had been purified by size exclusion column chromatography (Sephadex G-25).

**Figure 6.2.5.** Imaging of the self-assembly of compound 63a in CD$_3$OD by a) confocal fluorescence microscopy ($\lambda_{\text{exc}} = 405$ nm, $\lambda_{\text{em}} = 500 – 550$ nm) and b-d) SEM.

However, this batch of 63a had been purified exclusively by SiO$_2$ column chromatography base-treated with triethyl amine. Sephadex is commonly used for the purification of very polar compounds. Taking into account that Sephadex is a dextran, strong hydrogen bonding interactions would occur with the $\beta$-D-galactose unit. It was hypothesised that glucose units from the Sephadex could have been detached and therefore participate for their self-assembly. Figure 6.2.6 shows the $^1$H NMR of compound 63a previously synthesised and purified by Stefan Acherman (red) and compound 63a after purification using base-treated SiO$_2$ column chromatography (green) and subsequent column with sephadex (blue).

$^1$H NMR showed the disappearance of a broad singlet (bs) at 4.55 ppm and a singlet at 3.57 ppm after Sephadex purification. These peaks were not present on the initial sample.
suggesting that they were impurities. It could be then hypothesised that the presence of these small impurities could have hindered the self-assembly process. However, no other significant changes were found after Sephadex purification.

Unfortunately, further studies using the further purified sample 63a demonstrated that the aggregates found in CD$_3$OD did not form regular spheres and they were still quite weak and their supramolecular structure was disrupted after drying. These spheres were also formed in sample vials and in HPLC methanol (non-deuterated), as showing in Figure 6.2.7.

The experiments carried out with 63a demonstrated that glycosylated naphthalimides aggregate in methanol forming fibers, most likely due to the π-π stacking interactions of the naphthalimide cores and the dipole moments. These fibers arrange forming supramolecular structures with spherical shape. However, controlling the process by which these spheres are formed is quite challenging as would require a thorough examination of concentrations, solvents, time and temperature. Furthermore, these spheres dissemble after
solvent removal, thus suggesting that the hydrogen bonding interactions occurring with molecules of methanol are required to hold the spheres together.

Compound 63d, which possesses an identical naphthalimide core but a β-D-lactose moiety instead of β-D-galactose, also aggregated in CD₃OD, as well as MeOH, forming aggregates of spherical-like shapes. Unfortunately, attempts to control and replicate the formation of these microspheres were also unsuccessful. Interestingly, confocal studies demonstrated that these aggregates were not formed by fibres, as seen for compound 63a. Instead the compound aggregates forming “boomerang-shape” structures (Figure 6.2.8). Confocal studies of the aggregates were taken after leaving the aggregate to dry at rt overnight.

Figure 6.2.8. Compound 63d and confocal fluorescence image (λ_exc = 405 nm, λ_em = 500 – 550 nm) of its aggregate found in CD₃OD after drying overnight.

Examination of the aggregates using SEM also revealed the presence of these “boomerang-shaped” fibres of ca. 2 μm length forming the aggregates. Some of the aggregates were forming spheres of approximately 200 μm diameter, as can be seen in Figure 6.2.9 where the sample had been examined using SEM. SEM images were taken by Amy Lynes, from the Gunnlaugsson group.

The structural differences observed between compound 63a and 63d shows how the carbohydrate units modulate the shape of the aggregates, probably due to the hydrogen-bonding interactions that can be formed between molecules and polar solvents such as methanol.

Another structurally related compound that formed aggregates in CD₃OD was compound 106. Compound 106 formed the largest aggregates of all the glycosylated naphthalimides described. However, its ¹H NMR recorded in CD₃OD was well-resolved in
contrast to compound 45, described in Chapter 5, which strongly aggregated in polar solvents leading to a very poorly resolved $^1$H NMR. As can be seen in Figure 6.2.10 a well-resolved $^1$H NMR of 106 was obtained in CD$_3$OD enabling its characterisation.
Large aggregates of spherical shape formed by 106 in CD$_3$OD were found in the NMR tube after 16 h (Figure 6.2.11). The spheres observed were very large, with sizes varying from 1.5 – 3 mm diameter.

As can be seen in Figure 6.2.11, the aggregates were formed by “growing” from the glass wall. The spheres were left to dry for 24 h and further imaged by confocal microscopy (Figure 6.2.12). Interestingly, after 24 h drying the spheres had become very
hard and kept their shape, in contrast to the ones observed for compound 63a that lost their shape after drying and became very soft.

Figure 6.2.12. Confocal fluorescence microscopy images ($\lambda_{\text{exc}} = 405 \text{ nm}, \lambda_{\text{em}} = 500 – 550 \text{ nm}$) of compound 106 forming spheres in CD$_3$OD.

Confocal images of compound 106 showed the formation of fibres that interact with each other forming large aggregates with spherical shapes (Figure 6.2.12a). As can be seen in Figure 6.2.12b, these aggregates presented a very uniform spherical shape but also presented a flat side from which the spheres were attached to the NMR tube. Figure 6.2.12c is focussed in this area and it can be seen how the fibres are arranged in a linear trend growing from one point.

Interestingly, compound 106 was re-synthesised and the new material obtained was also able to form spheres in CD$_3$OD. However, their sizes were significantly smaller, of ca. 0.5 mm diameter, than the previously observed. Figure 6.2.13 shows the spheres found in the NMR tube containing 106 in CD$_3$OD after overnight.

Figure 6.2.13. Aggregates formed by compound 106 (new batch) in CD$_3$OD.

The solution in the NMR tube containing the spheres was divided in two portions; one was left standing for longer time (up to a week) to investigate the effect of longer incubation times on the size of the spheres. Unfortunately, the increasing time showed no
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effect on these spheres. The second half was sonicated and heated to dissolve the spheres, which turned quite difficult to re-dissolve. After 12 h, new spheres obtained presented similar sizes, confirming the reproducibility of the formation of spheres from compound 106 under these conditions (CD$_3$OD, rt, NMR tubes). Confocal images of these spheres showed very similar results to the previous batch (Figure 6.2.14).

![Confocal fluorescence microscopy images](image1)

**Figure 6.2.14.** Confocal fluorescence microscopy images ($\lambda_{\text{exc}} = 405$ nm, $\lambda_{\text{em}} = 500 – 550$ nm) of compound 106 (new batch) forming spherical aggregates in CD$_3$OD.

As previously observed, confocal images of the new batch illustrate the spherical shape of the aggregates formed from compound 106 (Figure 6.2.14a and b) which showed the fibres arranged parallel to each other and all growing from a particular point (Figure 6.2.14c).

Having demonstrated that compound 106 formed spherical-like aggregates in CD$_3$OD, and that these were replicable, the formation of similar aggregates in aqueous solvents of more biological relevance was investigated. Compound 106 was dissolved in PBS and after 12 h similar spheres as previously described were formed, which were softer in appearance. The spheres were decanted from the NMR tube and let to dry overnight at rt (Figure 6.2.15). Nonetheless, the spheres were not fully dry and attempts to move them disrupted their structure.

![Sphere formed by compound 106](image2)

**Figure 6.2.15.** Sphere formed by compound 106 in PBS after being dried overnight at rt.

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Attempts to remove the solvent by gently heating made the sphere to swollen and redissolve in PBS. Therefore, the confocal fluorescence microscopy imaging of these spheres was performed on the wet materials. Figure 6.2.16 shows the confocal images of the spheres formed by compound 106 in PBS.

As can be seen in Figure 6.2.16, these spheres were also formed by linear fibres and presented a more regular shape, with an average diameter of 90 μm. However, since the removal of solvent was not possible, the applications of these spheres were very limited.

As described in Chapter 5, protein binding could completely change the morphology of these aggregates. In this case morphological changes of compound 106, containing a β-D-galactose unit, were investigated upon enzymatic treatment, which would give compound 158 (Scheme 6.2.1).

![Confocal fluorescence microscopy images](image)

**Figure 6.2.16.** Confocal fluorescence microscopy images ($\lambda_{exc} = 405$ nm, $\lambda_{em} = 500 – 550$ nm) of the spheres formed by 106 in PBS after 16 h.

In order to analyse the changes in the morphology after enzymatic hydrolysis, the spheres were incubated with 1 U of β-galactosidase at 30 °C for 16 h in PBS. A control experiment where the spheres were incubated with PBS at 30 °C without the enzyme showed no changes in their morphology. Since the compounds were forming fibres, it was
hypothesised that the aromatic cores were forming columns via π-π stacking interactions with a head-to-tail arrangement (due to the dipole moments) with the carbohydrate moieties facing outside and therefore being more exposed to the solvent and stabilising the structure by hydrogen-bonding interactions. Hence, if the supramolecular structure is hold in place by these π-π stacking interactions, the enzymatic cleavage should not impart a big effect on it, and only a small layer of the structure should be removed.

**Scheme 6.2.1.** Enzymatic hydrolysis of compound 106 to give compound 158 upon treatment with β-galactosidase enzyme (1 U) in PBS (pH 7.2) at 30 °C for 16 h.

Interestingly, the spheres were completely disrupted after enzymatic treatment forming amorphous aggregates (Figure 6.2.17a). Confocal images of this material revealed that, not only the supramolecular structure forming spheres had disappeared, but also that the compound 106 was not longer forming fibres. Instead it was an amorphous material (Figure 6.2.17b-d).

This result strongly suggests that the carbohydrate moiety also plays an important role in holding the structure in place, and it is not solely depending on the π-π stacking interactions of the naphthalimide core, as it was postulated.
To gain further insight of the morphological changes occurring upon enzymatic hydrolysis, SEM images were recorded before an after enzymatic treatment. As shown in Figure 6.2.18 SEM images of compound 106 before enzymatic treatment showed the presence of fibers are clearly noticeable (Figure 6.2.18a-c). On the contrary, the fibers that were forming the spheres were no longer present after the enzymatic treatment (Figure 6.2.18d-f), and the resulting material was soft and non-spherical, as previously observed from confocal fluorescence microscopy images. Some salt presence from the PBS could also be observed. SEM images were taken by Jason Delente from the Gunnlaugsson group.
Figure 6.2.18. SEM images of compound 106 in PBS before (a-c) and after treatment with 1 U of β-galactosidase for 16 h (d-f).

Figure 6.2.19 provided further evidence of the effect of the carbohydrate moiety modulating the self-assembly of the glycosylated naphthalimides, indicating that upon carbohydrate cleavage the fibers formed by π-π interactions are disrupted, as illustrated in Figure 6.2.19, suggesting a strong participation of the carbohydrate unit.

Figure 6.2.19. Illustration of the fibre disruption occurring after enzymatic treatment of compound 106.
Having described the self-assembly of glycosylated naphthalimides containing unprotected carbohydrates, the next section will describe self-assemblies of two other glycosylated naphthalimides in which the hydroxyl groups in the carbohydrate moiety were protected by O-acetyl (O-Ac) groups (Compounds 75 and 141). This protection would prevent the formation of hydrogen-bonding interactions with the solvents where the hydroxyl groups were acting as hydrogen-bonding donors, and instead, the O-Ac groups could act as hydrogen-bonding acceptors. Section 6.2.2 will describe the effect of the polarity changes on the supramolecular structure.

6.2.2 Supramolecular studies of O-acetylated glycosylated naphthalimides
Compound 75, synthesised and described in Chapter 2, formed a strong gel in water, in concentration approximately of 10 mM. This gel was formed slowly overtime after 7-10 days. The SEM images of this gel showed the formation of an interweaved fibers network. This gel showed that these fibers were closely interwoven and the gaps between them were only of 2 μm (Figure 6.2.20). Therefore, it could be said that this compound is a hydrogel. Hydrogels are structures in which water is encapsulated by cross-linked fibers formed from low molecular weight gelators. This cross-linking can be formed by strong associations or by weak bonds such as Van der Waals, hydrogen bonds or π-π interactions. SEM images of gel formed by compound 75 were taken by Dr. Savyasachi AJ.

The gel formation was in agreement with the fluorescence quench observed above $7 \times 10^{-5}$ M concentrations of compound 75 in PBS, described in Chapter 2, which proved the aggregation of this compound in aqueous media.
Another glycosylated naphthalimide, compound 141 synthesised and described in Chapter 4, formed a gel in MeOH that was found to be emissive (Figure 6.2.21).

Aggregation of compound 141 in aqueous solution (PBS) was previously demonstrated by spectroscopic characterisation (described in Chapter 4), as a fluorescence quench is observed between $4 \times 10^{-5}$ M and $7 \times 10^{-5}$ M concentrations, followed by an increase in the fluorescence signal at higher concentrations.
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Figure 6.2.21. Compound 141 (7.80 mM) forming a gel in MeOH (a) that was emissive under $\lambda_{exc} = 254$ nm (b).

As this gel was formed in MeOH it could be denoted as an organogel. Organogels are thermoreversibles materials in which an organic liquid is trapped within a cross-linked environment. The formation of this type of gel is driven by the self-assembly of low molecular weight gelators that form cross-linked fibers which can immobilise the organic solvent in its 3D structure. These fibers are stabilised by weak interactions such as Van der Waals forces, $\pi-\pi$ interaction or hydrogen bonding. Therefore, it can be easily understood the formation of these gels by glycosylated naphthalimides. This type of gel possesses potential application in a range of fields such as pharmaceuticals, cosmetics or food. For instance Pillay and co-workers have recently developed an injectable thermoresponsive organogel that can act as drug delivery systems upon thermal decomposition of gel and target tumour environments. The current work is focused on the loading of the organogel with 5-fluorouracil to test their anti-proliferative effect in vitro.

It was observed that, although the appearance was the same, the strength of gel increased with the concentration. For instance, the gel shown in Figure 6.2.21 was formed in a solution of 7.80 mM of 141 in MeOH and was distorted and became a solution after gently heating (ca. $> 37 \, ^\circ C$) and movement. The property by which gels become fluids under movement or shaking process is called thixotropy. This is a time-dependant property in which a non-newtonian fluid changes its viscosity over time under shear stress. Some of the natural occurring gels that present this property are some clays, the cytoplasm and the ground substance (substance present in the extracellular space). If the thixotropic fluid is capable to return to its gel state almost instantly after the stress ceases, they are called pseudoplastic fluids, some examples of this type of fluid could be found in paints, cosmetics or batteries.
It was observed that compound 141 formed a gel at higher concentration (13.85 mM) that was more resistant to thermal and movement decomposition, however the external appearance was identical. To gain a better understanding of the nature of the gel, SEM images of the gels formed at different concentrations were recorded and are shown in figure 6.2.22. SEM images were taken by Dr. Savyasachi AJ.

As can be seen in Figure 6.2.22, although the external appearance of the two gels was the same, SEM analysis revealed that the gel formed at higher concentration of 141 was formed by linear fibers that were located parallel to each other (Figure 6.2.22d-f). Whereas the gel formed by lower concentrations did not show any noticeable fibers. This could be the reason why the gel formed at higher concentration was stronger.

Figure 6.2.22. SEM images of the gel formed by 141 in MeOH at 7.80 mM (a-c) and 13.85 mM (d-f).
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6.3 Conclusions and Future Work.
To conclude, it was found that glycosylated naphthalimides in which the hydroxyl groups have been protected as O-acetyl groups formed gels, whereas the de-protected glycosylated naphthalimides formed aggregates, most of them with spherical structures. In most of the cases, these supramolecular structures were formed by fibers that can be localised either parallel to each other or interweaving. Interestingly, all these structures were formed in polar solvents, such as MeOH, CD$_3$OD, H$_2$O and D$_2$O, indicating the importance of the carbohydrate unit on the self-assembly properties by forming hydrogen bonding interactions. This was further proved by treatment of compound 106 with β-galactosidase, to give compound 158, which did not form fibers, as observed by SEM analysis. This demonstrated that not only the π-π interactions from the aromatic core are important but also the hydrogen-bonding interactions are necessary for the fiber formation.

After characterising these supramolecular structures using confocal microscopy and SEM, further tests would need to be done to characterise the gels, such as rheology.

Unfortunately, the spheres formed did not maintain their porous structure after drying. However, further characterisation would be needed as these assemblies present the potential to be used for gas storage or drug delivery systems.

The results obtained are in agreement with the previous self-assemblies reported structures from naphthalimides. However, to date no examples of the self-assembly of glycosylated naphthalimides have been found in the literature.

Future work should focus on the characterisation of these assemblies and understanding and controlling the factors that drive their formation. The use of enzymes to modify or to completely disrupt the morphology of these assemblies could be of great interest. For instance, the morphology formed by the unprotected naphthalimides could be modified by using glycosidase enzymes, as demonstrated for compound 106. This could be used for drug delivery, as the structure could be disrupted completely. For the case of assemblies formed by protected glycosylated naphthalimides, a dual approach using two enzymes could be employed, by using first esterase enzymes to cleave the acetate groups followed by glycosidase enzymes.
Chapter 7

Overall Conclusions
As described in chapters 2-6, glycosylated naphthalimides offer the possibility of being used for multiple medicinal purposes. The conjugation of carbohydrates to naphthalimides provided these molecules with very special properties. On one hand, due to the naphthalimide units, they present interesting photophysical and biological properties that can be exploited for a range of medicinal applications from, fluorescence probes to drug delivery. Although most of the compounds developed in this thesis were not able to get into the cell nuclei and no DNA binding was achieved, cell death was still observed with some of them, with IC$_{50}$ values in the range of 2 μM in some cases. On the other hand, the conjugation to carbohydrates increased their water solubility and changed their behaviour in solution (as demonstrated in Chapter 6) but also, and most importantly, enabled for the use of glycosidase enzymes. It has been demonstrated in this thesis that the use of these enzymes to cleave the carbohydrate moiety and release the naphthalimide core works efficiently, including in vitro. It was also demonstrated in Chapter 4 that endogenous glycosidase enzymes were also able to recognise these compounds as substrates. This enzymatic-dependent release has been exploited for the delivery of a fluorescence probe (Chapter 2), a probe with the capacity to undergo CuAAC (Chapter 3) and a cytotoxic drug (Chapter 4).

To conclude, it can be said that this was a successful PhD project, which covered a wide range of topics. Chapter 2 demonstrated the efficient delivery of naphthalimides via enzymatic-dependent mechanism, and this finding paved the way for the development of the following projects. Unfortunately, no successful in vitro click reaction was achieved in Chapter 3 with the alkyne-containing naphthalimide and the azide-modified carbohydrates. Since this topic has been widely explored by Bertozzi and co-workers, further investigations into the optimal conditions for the click reaction could be done. However, due to time-constrains, in this PhD project it was decided to move onto more interesting topics such as the delivery of a cytotoxic drug via enzymatic delivery.

Although more studies would need to be done, such as in vitro studies with healthy cells and in vivo studies, Chapter 4 successfully demonstrated the delivery of Amonafide into three different cell lines, obtaining similar toxicity results. Perhaps more detailed experiments on the quantification i.e. percentage of Amonafide being released, could be done to gain further understanding on this system. Nevertheless, it was still illustrated that the two derivatives developed here are good candidates as prodrugs of Amonafide.
Chapter 7. Overall Conclusions

On another note, Chapter 5 studied the use of glycosylated naphthalimides as protein binding probes. Although the system’s behaviour turned more complex than expected, due to the aggregation of the compounds occurring, the binding with the lectin Con A was still demonstrated via UV-vis and fluorescence emission, highlighting again the relevance of this class of compounds in a range of medicinal applications.

Furthermore, it was demonstrated in Chapter 6 that the self-assembly, and thus the material formation, of glycosylated naphthalimide can be modified by using glycosidase enzymes. Although Chapter 6 only provides a brief discussion on these properties, it can be envisaged that the use of this class of compound for releasing cytotoxic drugs upon enzymatic exposure.

Taking into account all the aforementioned, it can be said that these glycosylated naphthalimides are a very interesting class of compounds within which applications can be further explored, especially in their material side, as this thesis only provides a short description and the controlled formation of hydrogels and microspheres could be of great interest.
Chapter 8

Experimental
8.1 General Experimental Detail

Unless otherwise stated; all commercial chemicals were obtained from Sigma-Aldrich or Fluka and used without further purification. Deuterated solvents for NMR use were purchased from Apollo. Dry solvents were distilled under Argon and dried over 4 Å molecular sieves prior to use. Solvents for synthesis purposes were used at GPR grade. Analytical TLC was performed using Merck Kieselgel 60 F254 silica gel plates or Polygram Alox N/UV254 aluminium oxide plates. Visualisation was by UV light (254 nm) by molybdenum staining.

NMR spectra were recorded on Bruker DPX–400 Advance spectrometers, operating at 400.13 MHz and 600.1 MHz for \(^1\)H NMR; 100.6 MHz and 150.9 MHz for \(^{13}\)C-NMR. Shifts are referenced to the internal solvent signals. NMR data were processed using Maestrenova software.

HRMS spectra were measured on a Micromass LCT electrospray TOF instrument with a WATERS 2690 autosampler and methanol/acetonitrile as carrier solvent.

LRMS analysis was performed with an Advion compact mass spectrometer (CMS) equipped with a with Z-spray electrospray ionisation (ESI) source.

Melting points were determined using an Electrothermal IA900 digital melting point apparatus.

Infrared spectra were recorded on a Perkin Elmer Spectrum One FT-IR Spectrometer equipped with a Universal ATR sampling accessory.

Carbohydrate positions are named 1 to 6, starting the count in the anomeric position. In the case of the lactose derivatives, the glycoside are named A and B, A being the closest one to the naphthalimide, as shown in Figure 8.1.

![Figure 8.1. Nomenclature of the carbohydrates moiety.](image)

8.2 UV-vis absorption and luminescence spectroscopy

UV-visible absorption spectra and optical density were recorded by means of a Varian CARY 50 spectrophotometer. Solutions were measured in 3 cm (10 mm x 10 mm) cuvettes. The wavelength range was 200-600 nm with a scan rate of 300 nm min\(^{-1}\). The solvents employed were HPLC or spectrophotometric grade. Water used in DNA related
work was triply distilled, autoclaved and filtered (Millipore, HV, 0.45 μm). PBS was obtained from Sigma Aldrich. Baseline correction measurements were used for all spectra. The sDNA was obtained from Sigma Aldrich as its sodium salts and it was stored at −20 °C to prevent bacterial growth. The concentrations of sDNA were accurately determined using quantification by UV-Vis analysis. The DNA concentration per nucleotide was determined spectrophotometrically using the molar extinction coefficient, 6600 M⁻¹cm⁻¹ at 260 nm for sDNA.

Fluorescence measurements were made with a Varian Carey Eclipse Fluorimeter equipped with a 1.0 cm path length quartz cell. The solvents used were of HPLC grade. The concentrations of the compounds under investigation were the same as those used for the UV-visible absorption measurements.

8.2.1. Quantum yield determination
The Φᵢ were determined using reference standards such as Fluorescein and Quinine sulfate in aqueous solutions and, they are stated in the corresponding sections. The studies were carried out using dilute solutions of OD 0.05, so that corrections for self-absorption and of incident and emitted light on the emission intensities were not required. The spectra were recorded using an excitation slit of 20 nm and emission slit of 1.5 nm. Therefore, the reference and the test samples solutions with identical absorbance at the same excitation wavelength can be assumed to be absorbing the same number of photons.

Φᵢ values were calculated by comparing the integrated areas underneath the emission band of the spectra using equation 8.1. Each measurement was repeated twice; calculated quantum yields, following this method, have an error of approximately 10%.

\[
(\Phi_\text{Φ})_x = (\Phi_\text{Φ})_r \cdot A_x / A_r \cdot F_x / F_r \cdot (\eta_x / \eta_r)^2
\]

where, \(x, r, A, F\) and \(\eta\) refer to the test sample, reference standard, absorbance, integrated area and the solvent refraction index, respectively.

8.3 Cell Culture
HeLa, HepG2 and HCT-116 cells were grown in Dulbecco’s Modified Eagle Medium (Glutamax) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 0.2% of plasmocin at 37 °C in a humidified atmosphere of 5% CO₂.
8.4 Alamar blue viability assay
Cells were seeded at a density of $2.5 \times 10^4$ cells/mL in 96-well plates and let to grow for 24 h and subsequently treated with the indicated compounds in a range of concentrations for 24 h, 48 or 72 h. Alamar blue (22 µL) was then added to each well and incubated at 37 °C in the dark for 4 h. Plates were then read on a fluorescence plate reader (SpectraMax Gemini, Molecular Devices) with excitement and emission wavelengths of 544 nm and 590 nm, respectively. Experiments were performed in triplicate on three independent days with activity expressed as percentage cell viability compared to vehicle treated controls. All data points (expressed as means ± SEM) were analysed using GRAPHPAD Prism (Graphpad software Inc., San Diego, CA).

8.5 Confocal microscopy
Cells were seeded at a density of $5 \times 10^4$ cells/dish in glass bottom wells and treated with compounds for the corresponding time in glucose free medium. For enzyme studies, cells were incubated for a further 1.5 h with the corresponding enzyme. Cells were stained with DRAQ5 (red nuclear stain), followed by viewing using Olympus FV1000 confocal microscopy with a 60X oil immersion lens or Leica SP8 STED confocal microscopy with a 40X oil immersion lens. Image analysis was performed using FluoView Version 7.1 Software for the Olympus microscope and Leica Application Suite software. Compounds were excited by a 405 nm argon laser, emission 450-550 nm, DRAQ5 was excited by a 633 nm red helium-neon laser, emission >650 nm. Images are representative of three independent experiments. For some images, compounds were excited with a high, 5-fold increase in laser power, to ensure successful imaging of compounds diluted in cell culture medium, this is noted in the text, where appropriate.

8.6 Two-Photo Microscopy
HeLa cells were seeded at a density of $1 \times 10^5$ in a 6 channel µ-Slide dish, and let to grow for 24 h. The following day the cell media was replaced by phenol red-free media before treatment with the corresponding compounds. For enzyme studies, cells were incubated with the corresponding enzyme. All the images were taken by Dr. Gavin McManus, from the School of Biochemistry at TCD. The compounds were imaged in Olympus BX61WI Upright Microscope with a Coherent Chameleon Laser- Titanium.
Sapphire Laser (Class IV), with tunable excitation ranging from 680 - 1080 nm. The images were analysed using FV-viewer software. The compounds were excited at >750 nm, and the exact excitation wavelength is stated in each case. The fluorescence emission is recorded at 455 – 490 nm (green channel), 500 – 550 nm (yellow channel) and 580 – 638 nm (red channel). The fluorescence recorded at 397 – 420 nm was not recorded as the compounds were not fluorescence in this region.

8.7 Scanning Electron Microscopy
The morphology of the samples were studied using a Carl Zeiss Ultra SEM with an SE2 or in-lens detector in the Advanced Microscopy Laboratory, CRANN, Trinity College Dublin, with the samples deposited on silicon wafers with a thick silicon dioxide layer. Prior to imaging, all samples were coated with a conductive layer of Pd/Au using a Cressington 208 Hr high-resolution sputter coater.

8.8 X-Ray Crystallography
The diffraction data were collected on a Bruker APEX-II Duo dual-source instrument using microfocus Cu-Kα radiation (λ = 1.5405 Å) using ω and φ scans. A single crystal was mounted on Mitegen micromounts in NVH immersion oil, and maintained at a temperature of 100 K using a Cobra cryostream. The diffraction data were reduced and processed using the Bruker APEX suite of programs. Multi-scan absorption corrections were applied using SADABS. The data were solved using the Intrinsic Phasing routine in SHELXT. Although the chiral space group P21212 gave the best statistics and most appropriate structure model, no chirality information is assumed and the structure was refined as a racemic twin with full-matrix least squares procedures using SHELXL-2015 within the OLEX-2 GUI. All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms were placed in calculated positions and refined with a riding model, with isotropic displacement parameters equal to either 1.2 or 1.5 times the isotropic equivalent of their carrier atoms. Due to the severe disorder in the naphthalimide fragments of both unique molecules, DFIX, SADI and RIGU restraints and EADP constraints were necessary to maintain reasonable chemical geometries and Uij tensors, particularly where atoms from both fragments were closely overlapping. Occupancies of the individual conformers were determined with free variable refinement and then fixed to sensible fractional values. Specific collection and refinement strategies
are further outlined in the combined crystallographic information file (cif) under the refine special details heading. CCDC 1866499.

### 8.8.1 Crystal Data for compound 50

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Flack parameter  0.6(8)

8.9 Gas uptake studies
Gas adsorption isotherms were carried out in collaboration with Mr. Kevin Byrne of Prof Wolfgang Schmitt’s group in SNIAMs, School of Chemistry, TCD and were measured using a Quantachrome Autosorb IQ gas sorption analyser. Chemically pure (CP, N4.5) grade He, N\(_2\), H\(_2\) and CO\(_2\) gases were used for the measurements. The calculations are done using equation 8.2.

\[
\frac{1}{W((P/P_0)^{-1}) - 1} = \frac{1}{W_m c} + \frac{c-1}{W_m c} \left(\frac{P}{P_0}\right)
\]

By plotting \(\frac{1}{W((P/P_0)^{-1}) - 1}\) against \(\left(\frac{P}{P_0}\right)\) of these data points the BET plot is resulted. the surface area was calculated by linear fit of the data.

8.10 General Experimental Procedures
8.10.1 General Procedure A; Synthesis of 53(a-d).
To a stirred solution of the corresponding peracetylated sugar (1 equiv) and 3-chloro-1-propanol (2 equiv) in anhydrous CH\(_2\)Cl\(_2\), BF\(_3\).Et\(_2\)O (2.5 equiv) was added dropwise at 0 °C. The reaction mixture was allowed to warm to room temperature (rt). After reaction completion observed by TLC, the mixture was poured into ice water and stirring continued until the ice was melted. After phase separation, the water layer was extracted with CH\(_2\)Cl\(_2\) (3 × 100 mL). The combined organic phases were washed with aqueous NaHCO\(_3\) solution (100 mL), brine (100 mL), dried over MgSO\(_4\) and the solvent evaporated in vacuo. Purification of the crude product by SiO\(_2\) column chromatography gave the product.

8.10.2 General Procedure B; Synthesis of 54(a-d)
The corresponding 2,3,4,6-tetra-O-acetyl-1-O-(3-chloropropyl)-\(\beta\)-D-glycopyranoside (1 equiv) was dissolved in DMF (0.05 M) and NaN\(_3\) (4 equiv) was added in one portion. The mixture was stirred for 18 h at 80 °C, allowed to cool and poured into ice water and extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with water (200 mL) and brine (200 mL), dried over MgSO\(_4\) and concentrated in vacuo. Purification using SiO\(_2\) column chromatography gave the corresponding product.
8.10.3 General Procedure C; Synthesis of 55(a-d), 69 and 45.
The corresponding peracetylated sugars (1 equiv) was dissolved in MeOH/NaOMe (0.4 equiv). After stirring overnight at rt, DOWEX® 50WX8-200 ion exchange resin was added to the mixture until a neutral pH was measured. The reaction mixture was filtered and the filtrate concentrated in vacuo. Column chromatography purification yielded the desired product.

8.10.4 General Procedure D; Synthesis of 63(a-e), 44, 64, 75, 149
The corresponding alkyne derivative (1.1 equiv) and tetrakis(acetonitrile)-copper(I)tetrafluoroborate ([(CH₃CN)₄Cu]BF₄) (0.15 equiv) were added to a solution of the corresponding azide (1 equiv) in DMF (5 mL) in a microwave vial. The reaction mixture was stirred for 1 h at 115 °C in a microwave reactor. The solvent was removed in vacuo and the crude product dissolved in a mixture of MeOH/CH₂Cl₂ (1:2) and filtered through a plug of Celite® to remove the copper catalyst. The filtrate was concentrated in vacuo and purified by SiO₂ column chromatography, previously base treated with Et₃N, using 20-30% MeOH/EtOH (v/v), to afford the corresponding product.

8.11 Experimental for Chapter 2
2,3,4,6-Tetra-O-acetyl-1-O-(3-chloropropyl)-β-D-galactopyranoside (53a)²⁶⁷

Following general procedure A; 1,2,3,4,6-penta-O-acetyl-β-D-galactose (4.00 g, 10.24 mmol), 3-chloro-1-propanol (1.72 mL, 20.48 mmol), BF₃·Et₂O (3.16 mL, 25.60 mmol) in CH₂Cl₂ (40 mL). Reaction time; 72 h. Purification using 1:6 EtOAc/Hexane (v/v) afforded 53a as pale yellow oil (1.40 g, 32%).

Rᵣ = 0.58 (2:3 (v/v), CH₂Cl₂/Hexane).

δH (400 MHz, CDCl₃): 5.38 (d, J₃, ₄ = 3.3 Hz, 1H, H-3), 5.22 – 5.12 (m, 1H, H-2), 5.01 (dd, J₄, ₅ = 10.4 Hz, J₄, ₃ = 3.3 Hz, 1H, H-4), 4.46 (d, J₁, ₂ = 7.9 Hz, 1H, H-1), 4.23 – 4.05 (m, 2H, H-6, H-6’), 4.03 – 3.94 (m, 1H, H-7), 3.91 (app. t, 1H, H-5), 3.74 – 3.63 (m, 1H, H-7’), 3.60 (dd, J = 6.8, 5.5 Hz, 2H, H-9, H-9’), 2.14 (s, 3H, OCOCH₃), 2.08 (m, 2H, H-8, H-8’), 2.06, 2.04, 1.97 (s, 9H, OCOCH₃).

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2,3,4,6-Tetra-O-acetyl-1-O-(3-chloropropyl)-β-D-glucopyranoside (53b) 268

Following procedure A; 1,2,3,4,6-penta-O-acetyl-β-D-glucose (4.00 g, 10.24 mmol), 3-chloro-1-propanol (1.72 mL, 20.48 mmol), BF₃·Et₂O (6.29 mL, 50.00 mmol, 5 equiv) in CH₂Cl₂ (50 mL). Reaction time: 14 h. Purification using 1:6 EtOAc/Hexane (v/v) afforded 53b as pale yellow oil (1.29 g, 30%).

Rf. = 0.62 (2:3 (v/v), EtOAc/Hexane)

δH (600 MHz, CDCl₃): 5.07 (app. t, 1H, H-3), 4.92 (app. t, 1H, H-4), 4.82 (dd, J = 9.8 Hz, J₂, J = 8.0 Hz, 1H, H-2), 4.40 (d, J₁, J = 8.0 Hz, 1H, H-1), 4.13 (dd, J = 12.3 Hz, J = 4.9 Hz, 1H, H-6), 4.02 – 3.95 (m, 2H, H-6’, H-5), 3.90 – 3.77 (m, 2H, H-7, H-7’), 3.64 – 3.52 (m, 2H, H-9, H-9’), 3.51 – 3.41 (m, 2H, H-9, H-9’), 1.93, 1.91, 1.88, 1.85 (4 s, 12H, OCOCH₃).


2,3,4,6-Tetra-O-acetyl-1-O-(3-chloropropyl)-α-D-mannopyranoside (53c) 267

Following procedure A; 1,2,3,4,6-penta-O-acetyl-α-D-mannose (6.00 g, 15.30 mmol), 3-chloro-1-propanol (6.40 mL, 76.70 mmol, 5 equiv), BF₃Et₂O (13.16 mL, 107.10 mmol, 7 equiv) in dry CH₂Cl₂ (50 mL). Reaction time: 14 h. Purification using 1:3 EtOAc/Hexane (v/v) afforded 53c as pale yellow oil (2.90 g, 45%).

Rf. = 0.68 (3:2 (v/v), EtOAc/Hexane)

δH (400 MHz, CDCl₃): 5.31 – 5.27 (m, 2H, H-3, H-4), 5.26 – 5.24 (m, 1H, H-2), 4.82 (d, J₁, J = 1.7 Hz, 1H, H-1), 4.28 (dd, J = 12.2, 5.4 Hz, 1H, H-6), 4.16 – 4.09 (m, 2H, H-6’ and H-5), 4.02 – 3.97 (m, 1H, H-7), 3.94 – 3.88 (m, 2H, H-8, H-8’), 3.69 – 3.64 (m, 1H, H-7’), 3.63 – 3.55 (m, 2H, H-9 and H-9’), 2.16, 2.10, 2.05, 1.99 (s, 12H, OCOCH₃).


3-chloropropyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (53d) 269

Following procedure A. 2,3,4,6-tetra-O-acetyl-β-D-galacto-pyranosyl)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (4.00 g, 5.90 mmol) and 3-
chloro-1-propanol (1.25 mL, 11.80 mmol) dissolved in CH₂Cl₂ (50 mL), BF₃·Et₂O (1.80 mL, 14.75 mmol) was added at 0 °C. Reaction time: 3 days. Purification using 3:2 EtOAc/Hexane (v/v) a colourless oil (1.25 g, 30%).

Rf = 0.6 (3:2 (v/v), EtOAc/Hex).

δH (400 MHz, CDCl3): 5.34 (d, J = 3.3 Hz, 1H, H-4b), 5.19 (app. t, 1H, H-3a), 5.10 (app. t, 1H, H-2b), 4.95–4.88 (m, 3H, H-6a, H-5b, H-6′b), 3.92–3.99 (m, 1H, H-6a), 3.87 (app. t, 1H, H-5a), 3.79 (app. t, 1H, H-4a), 3.64–3.72 (m, 1H, H-7), 3.56–3.64 (m, 1H, H-9), 2.15, 2.12, 2.06, 2.05, 2.04, 1.96 (s, 21H, OCOCH₃), 1.26 (app. t, 2H, H-8, H-8′).


2,3,4,6-Tetra-O-acetyl-1-O-(3-azidopropyl)-β-D-galactopyranoside (54a) 270

Following procedure B; 53a (1.37 g, 3.23 mmol) and NaN₃ (800 mg, 12.27 mmol) and DMF (50 mL). Purification using 1:6 EtOAc/Hexane (v/v) gave the product as yellow oil (1.15 g, 83%).

Rf = 0.40 (2:3 (v/v), EtOAc/Hexane)

δH (400 MHz, CDCl3): 5.38 (d, J = 3.3 Hz, 1H, H-4), 5.19 (dd, J₂, 3 = 10.5 Hz, J₂, 1 = 8.0 Hz, 1H, H-2), 5.01 (dd, J₃, 2 = 10.5 Hz, J₃, 4 = 3.3 Hz, 1H, H-3), 4.46 (d, J₁, 2 = 8.0 Hz, 1H, H-1), 4.08–4.21 (m, 2H, H-6, H-6′), 4.00–3.88 (m, 2H, H-7, H-5), 3.63–3.56 (m, 1H, H-7′), 3.41–3.32 (m, 2H, H-9, H-9′), 2.14, 2.06, 2.04, 1.98, (s, 12H, OCOCH₃), 1.95–1.76 (m, 2H, H-8, H-8′).


2,3,4,6-Tetra-O-acetyl-1-O-(3-azidopropyl)-β-D-glucopyranoside (54b) 268

Following general procedure B; 53b (1.29 g, 3.04 mmol) and NaN₃ (790 mg, 12.2 mmol) and DMF (50 mL). Solvent evaporation gave the product as a colourless oil (1.35 g, 99%).

Rf = 0.31 (2:3 (v/v), EtOAc/Hexane)

δH (400 MHz, CDCl3): 5.11 (app. t, 1H, H-3), 4.97 (app. t, 1H, H-4), 4.88 (dd, J = 9.6 Hz, J₂, 1 = 8.0 Hz, 1H, H-2), 4.43 (d, J₁, 2 = 8.0 Hz, 1H, H-1), 4.16 (dd, J = 12.3, 4.8 Hz, 1H, H-
6), 4.04 (dd, $J = 12.3$ Hz, $J = 2.5$ Hz, 1H, H-6’), 3.88 – 3.82 (m, 1H, H-9), 3.66 – 3.60 (m, 1H, H-9’), 3.57-3.48 (m, 1H, H-5), 3.30 – 3.24 (m, 2H, H-8, H-8’), 1.98, 1.95, 1.92, 1.90 (s, 12H, OCOCH$_3$), 1.78-1.70 (m, 2H, H-7, H-7’).

HRMS (m/z-ESI): Found: 454.1444, ([M+Na]$^+$). C$_{17}$H$_{25}$N$_3$O$_{10}$Na, Required: 454.1438.

2,3,4,6-tetra-O-acetyl-1-O-(3-azidopropyl)-$\alpha$-D-mannopyranoside (54c) $^{267}$

Following general procedure B, 53c (1.22 g, 2.87 mmol) and NaN$_3$ (150 mg, 11.48 mmol) and DMF (60 mL). Purification using 1:3 EtOAc/Hexane (v/v) yielded 25 as a pale yellow oil (430 g, 40%).

R$_f$. = 0.48 (2:3 (v/v), EtOAc/Hexane)

$\delta$$_H$ (600 MHz, CDCl$_3$): 5.37 – 5.26 (m, 2H, H-3, H-5), 5.25 – 5.21 (m, 1H, H-2), 4.81 (d, $J_{1, 2} = 2.5$ Hz, 1H, H-1), 4.27 (dd, $J = 8.2$ Hz, $J = 18.4$ Hz, 1H, H-7), 4.14 – 4.07 (m, 1H, H-7’), 4.00 – 3.92 (m, 1H, H-4), 3.85 – 3.77 (m, 1H, H-6), 3.56 – 3.48 (m, 1H, H-6’), 3.43 (app. t, 2H, H-9, H-9’), 1.98 – 1.83 (m, 2H, H-8, H-8’).

HRMS (m/z - ESI$^+$): Found: 454.1143, ([M+Na]$^+$). C$_{17}$H$_{25}$N$_3$O$_{10}$Na, Required: 454.1438.

3-Azidopropyl-4-O-(2,3,4,6-tetra-O-acetyl-$\beta$-D-galactopyranosyl)-2,3,6-tri-O-acetyl-$\beta$-D-glucopyranoside (54d) $^{271}$

Following general procedure B, 53d (290 mg, 0.40 mmol) and NaN$_3$ (104 mg, 1.60 mmol) in DMF (20 mL). Purification using 2:1 EtOAc/Hexane (v/v) afforded 24 as a pale yellow oil (270 g, 95%).

R$_f$. = 0.55 (3:2 (v/v), EtOAc/Hex)

$\delta$$_H$ (600 MHz, CDCl$_3$): 5.34 (d, $J = 3.3$ Hz, 1H, H-4b), 5.19 (app. t, 1H, H-3a), 5.10 (app. t, 1H, H-3b), 4.95 (dd, $J = 8.0$ Hz, $J = 10.4$ Hz, 1H, H-2b), 4.88 (app. t, 1H, H-2a), 4.44 - 4.52 (m, 3H, H-1a, H-1b, H-6b), 4.05 – 4.15 (m, 3H, H-6a, H-5b, H-6’b), 3.84 – 3.82 (m, 2H, H-6’a, H-7), 3.79 (app. t, 1H, H-4a), 3.55 – 3.62 (m, 2H, H-5a, H-7’), 3.30 – 3.39 (m, 2H, H-9, H-9’), 2.15, 2.12, 2.06, 2.06, 2.04, 2.04 1.96 (s, 21H, OCOCH$_3$), 1.76 – 1.82 (m, 2H, H-8, H-8’).

HRMS (m/z-ESI$^+$): Found: 742.2278, ([M+Na]$^+$). C$_{29}$H$_{41}$N$_3$O$_{18}$Na, Required: 742.2283.)
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1-O-(3-azidopropyl)-β-D-galactopyranoside (55a) 272

Following general procedure C; 54a (1.15 g, 2.66 mmol) and NaOMe (72 mg, 1.33 mmol) and MeOH (60 mL). Purification using 1:9 MeOH/EtOAc (v/v) gave 55a as a colourless oil (625 mg, 90%).

\[ R_f = 0.30 \text{ (1:4 (v/v), MeOH/EtOAc)} \]

\[ \delta_H (600 \text{ MHz, CD}_3\text{CD}): 4.24 (d, J = 7.6 \text{ Hz, 1H, H-1}), 4.03 – 3.97 (m, 1H, H-6), 3.85 (d, J = 2.7 Hz, 1H, H-5), 3.82 – 3.73 (m, 2H, H-7, H-7’), 3.71 – 3.66 (m, 2H, H-6’, H-4), 3.57 – 3.52 (m, 2H, H-2, H-9), 3.51 – 3.47 (m, 3H, H-3, H-9’). \]


1-O-(3-azidopropyl)-β-D-glucopyranoside (55b) 268

Following general procedure C, 54b (1.36 g, 3 mmol), NaOMe (72 mg, 1.33 mmol) and MeOH (50 mL). The product was recrystallised from hexane yielding a colourless oil (524 mg, 67%).

\[ R_f = 0.30 \text{ (1:4 (v/v), MeOH/EtOAc)}. \]

\[ \delta_H (400 \text{ MHz, CD}_3\text{OD}): 4.48 (d, J = 8.7 \text{ Hz, 1H}), 4.30 (d, J = 7.5 \text{ Hz, 1H}), 3.86 (dd, J = 12.3 \text{ Hz, J = 2.4 Hz, 1H}), 3.80 (dd, J = 12.3 \text{ Hz, J = 4.0 Hz, 1H}), 3.77 – 3.73 (m, 1H), 3.73 – 3.69 (m, 1H), 3.64 (dd, J = 11.4 \text{ Hz, J = 4.0 Hz, 1H}), 3.57 – 3.39 (m, 6H). \]


1-O-(3-azidopropyl)-α-β-mannopyranoside (55c) 267

Following general procedure C; 54c (423 mg, 0.98 mmol), NaOMe (22 mg, 0.40 mmol) and MeOH (40 mL). Purification using 1:9 MeOH/EtOAc (v/v) yielded to a colourless oil (220 mg, 85%).

\[ R_f = 0.48 \text{ (3:2 (v/v), Hex/EtOAc)}. \]

\[ \delta_H (400 \text{ MHz, D}_2\text{O}): 4.87 (d, J = 2.3 \text{ Hz, 1H, H-1}), 3.98 – 3.94 (m, 1H, H-2), 3.93 – 3.72 (m, 4H, H-6, H-6’, H-7, H-7’), 3.70 – 3.57 (m, 3H, H-3, H-4, H-5), 3.50 – 3.41 (m, 2H, H-9, H-9’), 1.99 – 1.84 (m, 2H, H-8, H-8’). \]

3-Azidopropyl(O-β-D-galactopyranosyl)-(1-4)-O-β-d-glucopyranoside (55d)$^{273}$

Following general procedure C; compound 54d (0.27 g, 0.37 mmol), NaOMe (30 mg, 0.15 mmol) and MeOH (50 mL). Solvent removal affords compound 9 (158 mg, quantitative) as a white foam.

R$_f$ = 0.10 (1:3 (v/v), Hex/EtOAc).

δ$_H$ (600 MHz, CD$_3$OD): 4.36 (d, $J$ = 7.7 Hz, 1H, H-1b), 4.28 (d, $J$ = 7.8 Hz, 1H, H-1a), 3.98 – 3.92 (m, 1H, H-6a), 3.92 – 3.87 (m, 1H, H-6b), 3.86 – 3.75 (m, 3H, H-6’b, H-7, H-7’), 3.69 (dd, $J$ = 4.6 Hz, $J$ = 11.3 Hz, 1H, H-4b), 3.76 – 3.62 (m, H-6’a), 3.60 – 3.38 (m, 7H, H-3a, H-4a, H-2b, H-3b, H-5b, H-9, H-9’), 3.24 (app. t, 1H, H-2a), 1.89 – 1.84 (m, 2H, H-8, H-8’).


1-Bromo-(2,3,4,6-tetra-O-1-bromo-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-2,3,6-tri-O-acetyl-α-D-glucopyranoside (56)$^{114}$

Hydrobromic acid (2.04 mL, 35.4 mmol, 6.00 equiv) is added at 0 °C to peracetylated lactose (4.00 g, 5.90 mmol, 1.00 equiv) in 60 mL of CH$_2$Cl$_2$. After 1 h of stirring at 0 °C, the mixture was let to stirred overnight at rt. The reaction mixture was filtered off and quenched with NaHCO$_3$ until orange colour disappeared. The aqueous phase was extracted with EtOAc (100 mL) and the combined organic layers dried under MgSO$_4$. Solvent removed in vacuo led to 56, obtained as a colourless oil (1.94 g, 48%).

R$_f$ = 0.54 (1:1 (v/v), EtOAc/Hex)

δ$_H$ (400 MHz, CD$_3$Cl): 6.53 (d, $J_{1b, 2b}$ = 4.0 Hz, 1H, H-1b), 6.25 (d, $J$ = 3.5 Hz, 1H, H-1a), 5.60 – 5.50 (m, 1H, H-3b), 5.46 (dd, $J$ = 10.2 Hz, $J$ = 9.3 Hz, 1H), 5.38 – 5.34 (m, 1H) 5.16 – 5.10 (m, 2H), 5.04 - 4.95 (m, 2H), 4.76 (dd, $J$ = 10.0 Hz, $J_{2b, 1b}$ = 4.0 Hz, 1H, H-2b), 4.55 – 4.45 (m, 2H, H-6a, H-6’a), 4.24 – 4.04 (m, 2H), 2.17, 2.15, 2.13, 2.09, 2.06, 2.06, 1.97, (s, 21H, OCOCH$_3$).

HRMS (m/z-ESI$^+$): Found: 721.0950, ([M+Na]$^+$). C$_{26}$H$_{35}$O$_{17}$Na$^{79}$Br, Required: 721.0955.
1-Azido-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (57) \(^{114}\)

A mixture containing 56 (1.90 g, 2.70 mmol, 1.00 equiv), sodium azide (85 mg, 1.36 mmol, 5.00 equiv), tetrabutylammonium hydrogensulphate (923.17 mg, 4.08 mmol, 1.50 equiv) and 40 mL of NaHCO\(_3\)/CH\(_2\)Cl\(_2\) (1:1 (v/v)), was stirred overnight at rt. The organic layer was diluted with CH\(_2\)Cl\(_2\), washed with water and brine, dried over Mg\(_2\)SO\(_4\), filtered off and concentrated. Column chromatography of the residue on silica gel (35% EtOAc/Hexane) gave the product as a white foam (890 mg, 49%).

R\(_f\) = 0.73 (3:2 (v/v), EtOAc/Hex)

δ\(^H\) (600 MHz, CDCl\(_3\)): 5.26 (dd, \(J_{4b, 3b} = 3.5\) Hz, \(J = 1.1\) Hz, 1H, H-4b), 5.12 (app. t, 1H, H-3a), 5.01 (dd, \(J_{2b, 3b} = 10.4\) Hz, \(J = 7.8\) Hz, 1H, H-2b), 4.88 (dd, \(J_{3b, 2b} = 10.4\) Hz, \(J_{3b, 4b} = 3.5\) Hz, 1H, H-3b), 4.76 (dd, \(J = 9.5\) Hz, \(J_{2a, 1a} = 8.8\) Hz, 1H, H-2a), 4.58 (d, \(J_{1a, 2a} = 8.8\) Hz, 1H, H-1a), 4.47 – 4.39 (m, 2H, H-6a, H-1b), 4.07 – 3.97 (m, 3H, H-6’a and H-6b), 3.86 - 3.81 (m, 1H, H-6’b), 3.75 (app. t, 1H, H-4a), 3.68 – 3.62 (m, 1H, H-5a), 2.06, 2.04, 1.98, 1.97, 1.96, 1.95 (s, 21 H, OCOCH\(_3\)).

HRMS (m/z - ESI\(^+\)): Found: 684.1870, ([M+Na]\(^+\), C\(_{26}\)H\(_{35}\)N\(_3\)O\(_{17}\)Na, Required: 684.1864).

1-Azido-(β-O-D-galactopyranosyl)-(1-4)-O-β-D-glucopyranoside (58) \(^{114}\)

Following general procedure C; compound 57 (890 mg, 1.30 mmol) was dissolved in 25% NaOMe/MeOH solution (60 mL). Compound 58 (386 mg, 86%) was obtained without further purification as a white foam.

R\(_f\) = 0.10 (1:3 (v/v), Hex/EtOAc).

δ\(^H\) (400 MHz, CD\(_3\)OD): 4.48 (d, \(J = 8.7\) Hz, 1H, H-1a), 4.30 (d, \(J = 7.5\) Hz, 1H, H-1b), 3.86 (dd, \(J = 12.3\) Hz, \(J = 2.4\) Hz, 1H), 3.80 (dd, \(J = 12.3\) Hz, \(J = 4.0\) Hz, 1H), 3.77 – 3.73 (m, 1H), 3.73 – 3.68 (m, 1H), 3.64 (dd, \(J = 11.4\) Hz, \(J = 4.0\) Hz, 1H), 3.57 – 3.50 (m, 3H), 3.50 – 3.39 (m, 4H).

HRMS (m/z-ESI\(^+\)): Found: 390.1132, ([M+Na]\(^+\), C\(_{12}\)H\(_{22}\)N\(_3\)O\(_{10}\)Na, Required: 390.1135).

N-Propargyl-4-nitro-1,8-naphthalimide (61) \(^{149}\)

2-Propyn-1-amine (0.30 mL, 4.60 mmol, 1.10 equiv) was added to a sparingly soluble solution of 4-nitro-1,8-naphthalic anhydride (1.00 g, 4.19 mmol, 1.00 equiv) in ethanol (25
mL). After 6 h refluxing under argon, the reaction was cooled to room temperature, filtered and washed with cool ethanol (10 mL). The residue was dried in vacuo to afford the product 61 as a brown powder (1.01 g, 87%).

\[ R_f = 0.45 \text{ (2:3 (v/v), EtOAc:Hex).} \]

\[ \delta \text{H} (400 MHz, CDCl}_3\): 8.86 (d, \[ J_{6,8} = 7.8 \text{ Hz}, 1\text{H}, H-6\]), 8.79 (d, \[ J_{8,6} = 7.8 \text{ Hz}, 1\text{H}, H-8\]), 8.74 (d, \[ J_{5,6} = 8.0 \text{ Hz}, 1\text{H}, H-5\]), 8.41 (d, \[ J_{6,5} = 8.0 \text{ Hz}, 1\text{H}, H-6\]), 8.01 (app. t, 1H, H-7), 4.97 (d, \[ J_{3,1} = 2.4 \text{ Hz}, 2\text{H}, H-3\]), 2.22 (t, \[ J_{1,3} = 2.4 \text{ Hz} 1\text{H}, H-1\]).

HRMS (m/z - ESI\(^+\)): Found: 303.03792 ([M+Na\(^+\]). C\(_{15}\)H\(_8\)N\(_2\)NaO\(_4\) Required: 303.0376).

N-Propargyl-4-amino-1,8-naphthalimide (62)\(^{149}\)

A solution of SnCl\(_2\) (4.77 g, 25.6 mmol, 7.00 equiv) in HCl (10 mL) was carefully added to a stirred suspension of 61 (1.00 g, 3.60 mmol, 1.00 equiv) in EtOH (40 mL). The reaction mixture was stirred for 2 h at rt and quenched with an aqueous solution containing 10% NaHCO\(_3\) (150 mL). The mixture was filtered and the collected solids given into CH\(_2\)Cl\(_2\) (500 mL). Filtration followed by washing the residue with CH\(_2\)Cl\(_2\) (200 mL) and drying off the residue in vacuo afforded the product 62 as orange powder (895 mg, 86%).

\[ R_f = 0.33 \text{ (50\% EtOAc/Hex) } \]

\[ \delta \text{H} (400 MHz, DMSO): 8.64 (d, \[ J_{8,6} = 7.3 \text{ Hz}, 1\text{H}, H-8\]), 8.45 (d, \[ J_{6,8} = 7.3 \text{ Hz}, 1\text{H}, H-6\]), 8.21 (d, \[ J_{4,5} = 8.4 \text{ Hz}, 1\text{H}, H-4\]), 7.67 (app. t, 1H, H-7), 7.53 (br. s, 2H, NH\(_2\)), 6.85 (d, \[ J_{5,4} = 8.4 \text{ Hz}, 1\text{H}, H-5\]), 4.73 (d, \[ J_{3,1} = 2.4 \text{ Hz}, 2\text{H}, H-3\]), 3.06 (t, \[ J_{1,3} = 2.4 \text{ Hz}, 1\text{H}, H-1\]).

HRMS (m/z - ESI\(^+\)): Found: 249.0661 ([M+Na\(^+\]). C\(_{15}\)H\(_{11}\)N\(_2\)O\(_2\) Required: 249.0664).

N-((1-(3-(β-D-galactopyranosyl)-(1-4)-O-β-D-glucopyranosyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl)-4-amino-1,8-naphthalimide (63a)

Following general procedure D; 55a (312 mg, 1.20 mmol), 62 (327 mg, 1.30 mmol), [(CH\(_3\)CN)\(_4\)Cu]BF\(_4\) (66.00 mg, 0.18 mmol) and DMF (15 mL). Compound 63a was obtained as an orange powder (345 mg, 58%).

\[ R_f = 0.13 \text{ (1:4 (v/v), MeOH/EtOAc) } \]

\[ [\alpha]\text{D}_{20} = 19 \text{ deg cm}^2 \text{ g}^{-1} \text{ dm}^{-1} \text{ (0.01, MeOH) } \]
δ<sub>H</sub> (400 MHz, CD<sub>3</sub>OD): 7.99 (s, 1H, H-10), 7.78 (d, J<sub>16,14</sub> = 7.8 Hz, 1H, H-16), 7.71 (d, J<sub>14,16</sub> = 7.8 Hz, 1H, H-14), 7.55 (d, J<sub>12,13</sub> = 8.4 Hz, 1H, H-12), 7.03 (app. t, 1H, H-15), 6.33 (d, J<sub>13,12</sub> = 8.4 Hz, 1H, H-13), 5.16 – 5.06 (m, 2H, H-11, H-11'), 4.57 (t, J<sub>9,8</sub> = 6.7 Hz, 2H, H-9, H-9'), 4.05 (d, J<sub>1,2</sub> = 8.4 Hz, 1H, H-1), 3.92 – 3.84 (m, 1H, H-8), 3.73 (d, J<sub>4,3</sub> = 3.5 Hz, 1H, H-7), 3.70 – 3.53 (m, 3H, H-2, H-8, H-8'), 3.30 – 3.25 (m, 1H, H-5), 3.20 (dd, J<sub>3,2</sub> = 9.9 Hz, J<sub>3,4</sub> = 3.5 Hz, 1H, H-3), 2.24 - 2.24 (m, 2H, H-7, H-7').

δ<sub>C</sub> (100 MHz, CD<sub>3</sub>OD): 165.8 (CO), 164.8 (CO), 153.4 (q, Ar-C), 144.6 (q, Ar-C), 135.2 (C-12), 132.5 (C-16), 129.9 (C-15), 129.6, 126.1 (C-10), 124.9 (C-14), 120.8, 119.4, 109.8, 107.9 (C-13), 104.0 (C-1), 75.9 (C-5), 73.5 (C-3), 71.6 (C-2), 69.4 (C-6), 67.2 (C-4), 61.7 (C-7), 47.9 (C-9), 35.6 (C-11), 30.4 (C-8).

ν<sub>max</sub> (ATR)/cm<sup>-1</sup>: 1045 (C-N), 1409 (N=N), 1559 (ar. C-C), 1656 (C=O), 3363 (OH/NH<sub>2</sub>).

HRMS (m/z - ESI<sup>+</sup>): Found: 536.1753, ([M+Na]<sup>+</sup>). C<sub>24</sub>H<sub>27</sub>N<sub>5</sub>O<sub>8</sub>, Required: 536.1757).

**N-((1-(3-(β-D-glucopyranosyl)propyl)-1H-1,2,3-triazol-4-yl)methyl)-4-amino-1,8-naphthalimide (63b)**

Following general procedure D; 55b (60.00 mg, 0.23 mmol), 63 (62.50 mg, 0.25 mmol), [(CH<sub>3</sub>CN)<sub>4</sub>Cu]BF<sub>4</sub> (13 mg, 0.03 mmol) and DMF (5 mL). An orange powder was obtained (108 mg, 91%).

R<sub>f</sub> = 0.14 (1:4 (v/v), MeOH/EtOAc)

[α]<sub>D</sub><sup>20</sup> = 46 deg cm<sup>3</sup> g<sup>-1</sup> dm<sup>-1</sup> (0.01, MeOH)

δ<sub>H</sub> (600 MHz, D<sub>2</sub>O): 7.99 (s, 1H, H-10), 7.52 (d, J<sub>16,14</sub> = 7.1 Hz, 1H, H-16), 7.44 (d, J<sub>14,16</sub> = 7.1 Hz, 1H, H-14), 7.32 (d, J<sub>12,13</sub> = 8.2 Hz, 1H, H-12), 6.77 (app. t, 1H, H-15), 6.10 (d, J<sub>13,12</sub> = 8.2 Hz, 1H, H-13), 5.00 (s, 2H, H-11), 4.58 (t, J<sub>9,8</sub> = 7.26 Hz, 2H, H-9, H-9'), 4.32 (d, J<sub>1,2</sub> = 8.4 Hz, 1H, H-1), 3.92 – 3.84 (m, 1H, H-8), 3.73 (d, J<sub>4,3</sub> = 3.5 Hz, 1H, H-7), 3.70 – 3.53 (m, 3H, H-2, H-8, H-8'), 3.30 – 3.25 (m, 1H, H-5), 3.20 (dd, J<sub>3,2</sub> = 9.9 Hz, J<sub>3,4</sub> = 3.5 Hz, 1H, H-3), 2.24 - 2.24 (m, 2H, H-7, H-7').
Chapter 8. Experimental

N-((1-(3-(a-d-manosepyranosyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl)-4-amino-1,8-naphthalimide (44)

Following general procedure D, 55c (50 mg, 0.18 mmol), 62 (51 mg, 0.20 mmol), [(CH3CN)4Cu]BF4 (10 mg, 0.03 mmol) and DMF (5 mL). Product 44 afforded as an orange powder (75 mg, 78%).

\[ \delta_{H} (600 \text{ MHz, CD}_{3}OD): \]
8.49 (dd, \( J_{16,15} = 7.3 \text{ Hz}, J_{16,15} = 1.2 \text{ Hz}, 1H, H-16 \)), 8.45 (dd, \( J_{14,15} = 8.4 \text{ Hz}, J_{14,16} = 1.2 \text{ Hz}, 1H, H-14 \)), 8.24 (d, \( J_{12,13} = 8.4 \text{ Hz}, 1H, H-12 \)), 7.84 (s, 1H, H-10), 7.58 (dd, \( J_{15,14} = 8.4 \text{ Hz}, J_{15,16} = 7.3 \text{ Hz}, 1H, H-15 \)), 6.82 (d, \( J_{13,12} = 8.4 \text{ Hz}, 1H, H-13 \)), 5.36 (s, 2H, H-11, H-11’), 4.62 (d, \( J_{1,2} = 2.0 \text{ Hz}, 1H, H-1 \)), 4.40 (td, \( J = 6.9 \text{ Hz}, J = 3.0 \text{ Hz}, 2H, H-9, H-9’ \)), 3.76 – 3.66 (m, 3H, H-6, H-8, H-3), 3.64 – 3.57 (m, 2H, H-6’, H-2), 3.52 (app. t, 1H, H-4), 3.45 – 3.39 (m, 2H, H-8’, H-5), 2.14 – 2.05 (m, 2H, H-7, H-7’).

\[ \delta_{C} (150 \text{ MHz, CD}_{3}OD): \]
133.2 (C-16), 131.2 (C-12), 128.8 (C-13), 124.7 (C-10), 124.0 (C-15), 108.4 (C-14), 99.9 (C-1), 70.3, 69.6, 66.7, 64.7 (C-6), 60.7 (C-8), 48.7, 47.9 (C-9), 34.6 (C-11), 28.8 (C-7).

\[ \nu_{\text{max}} (\text{ATR})/\text{cm}^{-1}: \]
1534 (ar. C-C), 1312 (C-12), 1288 (C-13), 1247 (C-10), 1240 (C-15), 1084 (C-14), 999 (C-1), 703, 696, 667, 647 (C-6), 607 (C-8), 487, 479 (C-9), 346 (C-11), 288 (C-7).

\[N-((1-(3-(\beta-D-galactopyranosyl)-(1-4)-O-\beta-D-glucopyranosyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl)-4-amino-1,8-naphthalimide (63d)\]

Following general procedure D; \[55d\] (110 mg, 0.15 mmol), \[62\] (42 mg, 0.17 mmol), [(CH\textsubscript{3}CN)\textsubscript{4}Cu]BF\textsubscript{4} (8.40 mg, 0.02 mmol) and DMF (5 mL). Orange powder precipitated (68 mg, 67%).
\[
R_f = 0.12 \ (1:1 \ (v/v), \text{MeOH/EtOAc})
\]
\[\alpha\]D\textsubscript{20} = 36 deg cm\textsuperscript{-1} g\textsuperscript{-1} dm\textsuperscript{-1} (0.01, MeOH)

\[
\delta_H \ (600 \text{ MHz, CD}_3\text{OD}): \ 8.56 \ (dd, J_{16, 15} = 7.2 \text{ Hz, } J_{16, 14} = 1.1 \text{ Hz, } 1H, \ H-16), \ 8.52 \ (dd, J_{14, 15} = 8.4 \text{ Hz, } J_{14, 16} = 1.1 \text{ Hz, } 1H, \ H-14), \ 8.31 \ (d, J_{12, 13} = 8.4 \text{ Hz, } 1H, \ H-12), \ 7.99 \ (s, 1H, \ H-10), \ 7.66 \ (dd, J_{15, 14} = 8.4 \text{ Hz, } J_{15, 16} = 7.2 \text{ Hz, } 1H, \ H-15), \ 6.90 \ (d, J_{13, 12} = 8.4 \text{ Hz, } 1H, \ H-13), \ 5.43 \ (s, 2H, \ H-11), \ 4.52 \ (t, J_{9, 8} = 6.6 \text{ Hz, } 2H, \ H-9, \ H-9'), \ 4.36 \ (d, J_{1a, 2a} = 7.6 \text{ Hz, } 1H, \ H-1a), \ 4.16 \ (d, J_{1b, 2b} = 7.7 \text{ Hz, } 1H, \ H-1b), \ 3.91 - 3.78 \ (m, 5H), \ 3.73 \ (dd, J = 11.4, 4.7 \text{ Hz, } 1H), \ 3.64 - 3.54 \ (m, 2H), \ 3.55 - 3.48 \ (m, 2H), \ 3.22 \ (dd, J = 9.3 \text{ Hz, } J = 7.7 \text{ Hz, } 1H), \ 3.11 \ (q, J = 7.3 \text{ Hz, } 2H), \ 2.17 - 2.11 \ (m, 2H, \ H-7, \ H-7').
\]

\[
\delta_C \ (150 \text{ MHz, CD}_3\text{OD}): \ 134.4 \ (C-16), \ 131.3 \ (C-Ar), \ 129.0 \ (C-Ar), \ 128.7 \ (C-13), \ 124.1 \ (C-10), \ 123.8 \ (C-Ar), \ 103.9 \ (C-1a), \ 102.7 \ (C-1b), \ 79.0, \ 77.9, \ 75.5, \ 73.3, \ 71.0, \ 68.7, \ 65.3, \ 60.3, \ 46.9 \ (C-9), \ 29.1 \ (C-7).
\]

\[\nu_{\text{max}} \ (\text{ATR/cm}^{-1}): \ 1014 \ (C-N), \ 1407 \ (N=N), \ 1518 \ (ar. C-C), \ 1660 \ (C=O), \ 3257 \ (OH/NH_2). \]

HRMS (m/z - ESI\textsuperscript{+}): Found: 698.2290, ([M+Na]\textsuperscript{+}. C\textsubscript{30}H\textsubscript{37}N\textsubscript{5}O\textsubscript{13}Na, Required: 698.2286).

\[N-((1-(\beta-D-galactopyranosyl)-(1-4)-O-\beta-D-glucopyranosyloxy)-1H-1,2,3-triazol-4-yl)methyl)-4-amino-1,8-naphthalimide (63e)\]

Following general procedure D, \[58\] (55 mg, 0.15 mmol), \[63\] (42 mg, 0.17 mmol), [(CH\textsubscript{3}CN)\textsubscript{4}Cu]BF\textsubscript{4} (8 mg, 0.02 mmol) and DMF (5 mL). Compound \[63e\] obtained as an orange powder (80 mg, 87%).
\[
R_f = 0.07 \ (1:1 \ (v/v), \text{MeOH/EtOAc, base treated with Et}_3\text{N}).
\]
\( \delta_H \) (400 MHz, D\(_2\)O): 8.27 (s, 1H, H-7), 7.60 (d, \( J_{13,11} = 7.1 \) Hz, 1H, H-13), 7.42 (d, \( J_{11,13} = 7.1 \) Hz, 1H, H-11), 7.37 (d, \( J_{9,10} = 8.4 \) Hz, 1H, H-9), 6.79 (br. s, 1H, H-12), 6.07 (d, \( J_{10,9} = 8.4 \) Hz, 1H, H-10), 5.84 (d, \( J_{1a,2a} = 9.2 \) Hz, 1H, H-1a), 5.08 (s, 2H, H-3), 4.53 (d, \( J_{1b,2b} = 7.8 \) Hz, 1H, H-1b), 4.12 (app. t, 1H), 3.99 – 3.85 (m, 4H), 3.84 – 3.74 (m, 2H), 3.73 – 3.66 (m, 1H), 3.60 (dd, \( J = 9.9 \) Hz, \( J = 7.7 \) Hz, 1H), 3.32 – 3.25 (m, 1H), 3.23 – 3.16 (m, 1H), 1.34 - 1.25 (m, 1H).

\( \delta_C \) (100 MHz, D\(_2\)O): 164.4 (CO), 163.5 (CO), 151.1 (q, Ar-C), 133.9 (C-9), 131.4 (C-13), 126.9 (C-11), 124.4 (C-7), 122.3 (C-12), 118.0 (q, Ar-C), 109.3 (C-10), 108.5 (q, Ar-C), 102.4 (C-1), 86.9 (C-1'), 77.2, 76.8, 74.9, 72.0, 71.5, 68.1, 67.4, 59.3, 48.3, 36.4, 30.8.

\( \nu_{\text{max}} \) (ATR)/cm\(^{-1}\): 1033 (C-N), 1422 (N=N), 1575 (ar. C-C), 2854, 2924.

HRMS (m/z - ESI\(^+\)): Found: 618.2043, ([M+\( \text{H} \)]\(^+\). C\(_{27}\)H\(_{32}\)N\(_5\)O\(_{12}\), Required: 618.2047).

**N-(3'-propanol)-1H-1,2,3-triazol-4-yl)methyl)-4-amino-1,8-naphthalimide (50)**

Compound 63a (55 mg, 0.11 mmol, 1.00 equiv) was dissolved in 5 mL of NaOAc buffer (10 mM, pH = 5) and a solution of galactosidase enzyme (2 mL, 1.4 g/L, 0.10 equiv) in NaOAc buffer (10 mM, pH = 5) was added. The reaction was stirred at 30 °C overnight. Solvent was evaporated in vacuo and product purified using SiO\(_2\) chromatography column (1:24 MeOH/EtOAc, v/v). Compound 50 was obtained as an orange powder (29 mg, 99%).

**Rf. = 0.6 (1:9 (v/v), MeOH/EtOAc)**

\( \delta_H \) (400 MHz, CD\(_3\)OD): 8.56 (dd, \( J_{10,9} = 7.3 \) Hz, \( J_{10,8} = 1.1 \) Hz, 1H, H-10), 8.53 (dd, \( J_{8,9} = 8.2 \) Hz, \( J_{8,10} = 1.1 \) Hz, 1H, H-8), 8.32 (d, \( J_{6,7} = 8.4 \) Hz, 1H, H-6), 7.91 (s, 1H, H-4), 7.66 (app. t, 1H, H-9), 6.90 (d, \( J_{7,6} = 8.4 \) Hz, 1H, H-7), 5.43 (s, 2H, H-5, H-5'), 4.46 (t, \( J_{3,2} = 7.0 \) Hz, 2H, H-3), 3.54 (t, \( J_{1,2} = 6.1 \) Hz, 2H, H-1), 2.12 – 2.03 (m, 2H, H-2).

\( \delta_C \) (100 MHz, CD\(_3\)OD): 166.0 (CO), 165.4 (CO), 154.8 (q, Ar-C), 145.5 (q, Ar-C), 135.8 (C-6), 132.8 (C-10), 130.43 (C-8), 125.3 (C-9), 124.9 (C-4), 123.4 (q, Ar-C), 121.2 (q, Ar-C), 109.7 (C-7), 59.3 (C-1), 49.0 (C-3), 35.9 (C-5), 33.9 (C-2).

\( \nu_{\text{max}} \) (ATR)/cm\(^{-1}\): 1033 (C-N), 1260 (N=N), 1660 (C=O), 3248 (OH/NH\(_2\)).

HRMS (m/z - ESI\(^+\)): Found: 352.1404, ([M+\( \text{H} \)]\(^+\). C\(_{18}\)H\(_{18}\)N\(_5\)O\(_3\), Required: 352.1404).
2,3,4,6-Tetra-O-acetyl-1-O-(propargyl)-β-D-galactopyranoside (68) 274

1,2,3,4,6-penta-O-acetyl-β-D-galactose (3.00 g, 7.70 mmol, 1 equiv) and propargyl alcohol (1.84 mL, 30.80 mmol, 4 equiv) and BF₃·Et₂O (4.72 mL, 38.50 mmol, 5.00 equiv) were stirred overnight in anhydrous CH₂Cl₂ (40 mL). The crude product was purified by SiO₂ column chromatography SiO₂ using 1:5 EtOAc/Hexane (v/v), affording 68 as pale colourless oil (1.76 g, 60%).

Rᶠ = 0.75 (1:1 (v/v), EtOAc/Hexane)

δH (400 MHz, CDCl₃): 5.39 (dd, J₄,₃ = 3.5 Hz, J₄,₅ = 1.1 Hz, 1H, H-4), 5.21 (dd, J₂,₃ = 10.4 Hz, J₂,₁ = 7.9 Hz, 1H, H-2), 5.05 (dd, J₃,₂ = 10.4 Hz, J₃,₄ = 3.5 Hz, 1H, H-3), 4.73 (d, J₁,₂ = 7.9 Hz, 1H, H-1), 4.38 (d, J₇,₉ = 2.4 Hz, 2H, H-7), 4.22 – 4.07 (m, 2H, H-6, H-6’), 4.02 – 3.87 (m, 1H, H-5), 2.46 (t, J₉,₇ = 2.4 Hz, 1H, H-9), 2.14, 2.07, 2.05, 1.98 (s, 12H, OCOCH₃).


Propargyl-β-D-galactopyranoside (69) 274

Following general procedure C; 68 (1.30 g, 3.36 mmol) and NaOMe (72 mg, 1.33 mmol) were stirred in MeOH (60 mL). Purification using 1:6 EtOH/Hexane (v/v) gave the product as a colourless oil (674 mg, 92%).

Rᶠ = 0.68 (50% MeOH/EtOAc)

δH (600 MHz, D₂O): 4.59 (d, J₁,₂ = 7.9 Hz, 1H, H-1), 4.55 – 4.44 (m, 2H, H-7), 3.94 (dd, J₄,₃ = 3.5 Hz, J₄,₅ = 1.0 Hz, 1H, H-4), 3.84 – 3.70 (m, 3H, H-6, H-6’, H-5), 3.68 (dd, J₃,₂ = 9.9 Hz, J₃,₄ =3.5 Hz, 1H, H-3), 3.54 (dd, J₂,₃ = 9.9 Hz, J₂,₁ = 7.9 Hz, 1H, H-2), 2.93 (t, J₉,₇ = 2.4 Hz, 1H, H-9).


N-(2-(dimethylamino)ethyl)-4-bromo-1,8-naphthalimide (72) 275

N,N-Dimethylethylenediamine (0.14 mL, 2.16 mmol, 1.20 equiv) was quickly added to a cloudy solution of 4-bromo-1,8-naphthalic anhydride (500 mg, 1.80 mmol, 1.00 equiv) in ethanol (20 mL). After 6 h refluxing under argon, the reaction was allowed to cool to room temperature, filtered off and washed with cool ethanol (10 mL). The residue was dried in vacuo to afford the product as a brown powder (471 mg, 76%).
\( R_f = 0.10 \) (EtOAc).

\( \delta_H \) (400 MHz, CDCl3): 8.66 (dd, \( J_{5, 4} = 7.3 \) Hz, \( J_{5, 3} = 1.1 \) Hz, 1H, H-5),
8.58 (dd, \( J_{3, 4} = 8.5 \) Hz, \( J_{3, 5} = 1.1 \) Hz, 1H, H-3), 8.41 (d, \( J_{1, 2} = 7.9 \) Hz, 1H, H-1), 8.04 (d, \( J_{2, 1} = 7.9 \) Hz, 1H, H-2), 7.85 (dd, \( J_{3, 4} = 8.5 \) Hz, \( J_{3, 5} = 7.3 \) Hz, 1H, H-3), 4.41 (t, \( J = 6.8 \) Hz, 2H, CH2), 2.91 (br. s, 2H, CH2), 2.54 (s, 6H, H-8).

HRMS (m/z - ESI\(^+\)): Found: 347.0392 ([M+H]+. C\textsubscript{16}H\textsubscript{16}N\textsubscript{2}O\textsubscript{2}79 Br

Required: 347.0395).

\textit{N-(2-(dimethylamino)ethyl)-4-azide-1,8-naphthalamide (73)}

Compound 72 (190 g, 0.55 mmol, 1.00 equiv), NaN\(_3\) (143 mg, 2.2 mmol, 4.00 equiv) were stirred in DMF (25 mL) at 80 °C overnight. Solvent was removed \textit{in vacuo} and the crude was dissolved in hot methanol, subsequent hot filtration removed NaBr salt. Solvent removal and further purification using SiO\(_2\) chromatography column gave the product as brown oil (1.70 g, neat).

\( R_f \). 1.6 (1:5 (v/v), MeOH/EtOAc).

\( \delta_H \) (400 MHz, CD\(_3\)OD): 8.58 (dd, \( J_{3, 4} = 7.3 \) Hz, \( J_{3, 5} = 1.1 \) Hz, 1H, H-3), 8.54 (d, \( J_{2, 1} = 8.0 \) Hz, 1H, H-2), 8.48 (dd, \( J_{3, 4} = 8.4 \) Hz, \( J_{5, 3} = 1.1 \) Hz, 1H, H-5), 7.80 (dd, \( J_{4, 5} = 8.4 \) Hz, \( J_{4, 3} = 7.3 \) Hz, 1H, H-4), 7.64 (d, \( J_{1, 2} = 8.0 \) Hz, 1H, H-1), 4.33 (t, \( J = 7.0 \) Hz, 2H, H-6), 2.72 (t, \( J = 7.0 \) Hz, 2H, H-7), 2.38 (s, 6H, H-8).

\( \delta_C \) (100 MHz, CD\(_3\)OD): 166.3 (CO), 165.6 (CO), 154.6 (q, Ar-C), 135.6 (C-1), 132.6 (C-5), 131.4 (q, Ar-C), 130.5 (C-3), 125.2 (C-4), 123.0 (q, Ar-C), 120.9 (q, Ar-C), 109.6 (C-2), 57.7 (C-7), 45.5 (C-8), 38.1 (C-6).

HRMS (m/z - ESI\(^+\)): Found: 310.1299, ([M+H]+. C\textsubscript{16}H\textsubscript{16}NO\textsubscript{2}, Required: 310.1304).

\textit{N-(2-(dimethylamino)ethyl)-4-(5-((\beta-D-glucopyranosyl)methyl)-1H-1,2,3-triazol-5-yl)1,8-naphthalamide (64)}

Following general procedure D; 69 (70 mg, 0.22 mmol) 73 (101 mg, 0.26 mmol), [(CH\(_3\)CN)\(_4\)Cu]BF\(_4\) (10.00 mg, 0.02 mmol) and DMF (15 mL). Compound 12 obtained as an orange powder (34 mg, 29%).

\( R_f \). 0.22 (MeOH).

[\( \alpha \)]\(_{D}^{20}\) = 21 deg cm\(^{-3}\) g\(^{-1}\) dm\(^{-1}\) (0.01, MeOH)

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δH (400 MHz, CD3OD): 8.74 (d, J10, 9 = 7.8 Hz, 1H, H-10), 8.71 (dd, J11, 12 = 7.3 Hz, J11, 13 = 1.1 Hz, 1H, H-11), 8.64 (s, J1, H-8), 8.26 (dd, J13, 12 = 8.6 Hz, J13, 11 = 1.1 Hz, 1H, H-13), 8.03 (d, J9, 10 = 7.8 Hz, 1H, H-9), 7.95 (dd, J12, 13 = 8.6 Hz, J13, 11 = 1.1 Hz, 1H, H-12), 7.50 (dd, J7, 7' = 12.7 Hz, 1H, H-7), 5.17 (d, J7, 7' = 12.7 Hz, 1H, H-7'), 4.48 (d, J1, 2 = 7.8 Hz, 1H, H-1), 4.39 (t, J14, 15 = 6.8 Hz, 2H, H-14), 3.87 (dd, J3, 4 = 3.3 Hz, J4, 5 = 1.0 Hz, 1H, H-4), 3.86 – 3.74 (m, 2H, H-6, H-6'), 3.66 – 3.59 (m, 2H, H-2, H-5), 3.53 (dd, J3, 2 = 9.7 Hz, J3, 4 = 3.3 Hz, 1H, H-3), 2.77 (t, J15, 14 = 6.8 Hz, 2H, H-15), 2.40 (s, 6H, H-16).

δC (100 MHz, CD3OD): 146.9 (CO), 139.5 (CO), 132.9 (C-11), 131.7 (C-10), 130.5 (C-13), 129.8 (C-12), 128.0 (C-8), 125.3 (C-9), 104.6 (C-1), 76.9 (C-2 or C-5), 74.9 (C-3), 72.5 (C-2 or C-5), 70.4 (C-4), 63.2 (C-7), 62.6 (C-6), 57.7 (C-15), 45.8 (C-16), 38.9 (C-14).

νmax (ATR)/cm⁻¹: 1047 (C=N), 1435 (N=N), 1589 (ar. C=C), 1655 (C=O), 2496, 3368 (OH/NH2).


N-(2'(dimethylamino)ethyl)-4-(4'-hydroxymethyl)-1''',2''',3'''-triazol-1'''-yl)-1,8-napthalimide (74)

Compound 64 (45 mg, 0.08 mmol, 1.00 equiv) was dissolved in 5 mL of NaOAc buffer (10 mM, pH = 5) and galactosidase enzyme (1 mg, 8 U/mg, 0.01 equiv) was added. The reaction was stirred at 30 °C overnight. Solvent was evaporated in vacuo and product purified by SiO2 chromatography column using 1:6 (v/v), MeOH/EtOAc. Compound 74 was obtained as an orange powder (27 mg, 87%).

Rf = 0.33 (MeOH)

δH (400 MHz, CD3OD): 8.76 (d, J5, 4 = 7.8 Hz, 1H, H-5), 8.73 (dd, J6, 7 = 7.3 Hz, J6, 8 = 1.0 Hz, 1H, H-6), 8.51 (s, 1H, H-9), 8.26 (dd, J8, 7 = 8.6 Hz, J8, 6 = 1.0 Hz, 1H, H-8), 8.04 (d, J4, 5 = 7.8 Hz, 1H, H-4), 7.95 (dd, J7, 8 = 8.6 Hz, J7, 6 = 7.3 Hz, 1H, H-7), 4.89 (br. s, 2H, H-10, H-10'), 4.45 (t, J3, 2 = 6.6 Hz, 2H, H-3), 3.00 (t, J2, 3 = 6.6 Hz, 2H, H-2), 2.58 (s, 6H).
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\[ \delta_c (150 \text{ MHz, CD}_3 \text{OD}): 165.5 \text{ (CO)}, 164.9 \text{ (CO)}, 149.9 \text{ (q, Ar-C)}, 139.8 \text{ (q, Ar-C)}, 133.2 \text{ (C-6)}, 131.9 \text{ (C-5)}, 130.7 \text{ (C-8)}, 129.8 \text{ (C-7)}, 128.0 \text{ (q, Ar-C)}, 126.7 \text{ (C-9)}, 125.3 \text{ (C-4)}, 124.2 \text{ (q, Ar-C)}, 57.6 \text{ (C-10)}, 56.5 \text{ (C-2)}, 45.0 \text{ (C-1)}, 37.9 \text{ (C-3)}. \]

\[ \nu_{\text{max}} \text{ (ATR)/cm}^{-1}: 949.9, 1010 \text{ (C-N)}, 1638 \text{ (C=O)}, 3360 \text{ (OH/NH}_2). \]

HRMS \((m/z - \text{ESI}^+): \text{Found: 366.1563, (}[\text{M+H}]^+. \text{C}_{19}\text{H}_{20}\text{N}_5\text{O}_3, \text{Required: 366.1566).} \)

\(N-(2\text{-}(\text{dimethylamino})\text{ethyl})-4-(5-(2,3,4,6-\text{Tetra-}\text{O-}\text{acetyl-}\beta\text{-D-}\text{glucopyranosylmethyl})-1\text{H}-1,2,3-\text{triazol-5-yl})-1,8\text{-naphthalimide (75)}\)

Following general procedure D; Compound 69 (86 mg, 0.224 mmol, 1.1 equiv), compound 73 (63 mg, 0.204 mmol, 1 equiv) and Cu(MeCN)\(_2\text{BF}_4\) (9.6 mg, 0.030 mmol, 0.15 equiv) were dissolved in 5 mL of DMF. The reaction was carried out in a microwave reactor for 3 h at 40 °C. After this, work up of the reaction and puridication by SiO\(_2\) column (1:5 (v/v), MeOH/EtOAc) gave the desired product as a yellow wax (21 mg, 15%). Formation of Amonafide was also observed (24%). After column purification the product was dissolved in 2 mL of MeOH and stirred in activated charcoal to give the titled compound as a yellow wax (20 mg, 15%).

\(R_f = 0.05 \text{ (1:4 (v/v), MeOH/EtOAc).} \)

\([\alpha]D^{30} = 2 \text{ deg cm}^3 \text{ g}^{-1} \text{ dm}^{-1} \text{ (0.1, MeOH)} \)

\(\delta_h \text{ (400 MHz, CDCl}_3): 8.68 \text{ (d, J}_{10,9} = 7.8 \text{ Hz, 1H, H-10)}, 8.65 \text{ (dd, J}_{11,12} = 7.3, J_{11,13} = 1.1 \text{ Hz, 1H, H-11)}, 8.54 \text{ (s, 1H, H-8)}, 8.17 \text{ (dd, J}_{13,12} = 8.6 \text{ Hz, J}_{13,11} = 1.1 \text{ Hz, 1H, H-13)}, 7.99 \text{ (d, J}_{9,10} = 7.8 \text{ Hz, 1H, H-9)}, 7.90 \text{ (dd, J}_{12,13} = 8.6 \text{ Hz, J}_{12,11} = 7.3 \text{ Hz, 1H, H-12)}, 5.42 \text{ (d, J = 2.6 Hz, 1H)}, 5.18 - 5.12 \text{ (m, 2H)}, 5.09 \text{ (d, J = 12.6 Hz, 1H)}, 4.97 \text{ (d, J = 12.6 Hz, 1H)}, 4.93 - 4.89 \text{ (m, 1H)}, 4.37 \text{ (t, J}_{1j,15} = 6.7 \text{ Hz, 2H, H-14)}, 4.17 \text{ (app t, 3H, H-7)}, 2.83 \text{ (t, J}_{15,14} = 6.7 \text{ Hz, 2H, H-15)}, 2.45 \text{ (s, 6H, H-16)}, 2.14, 2.01, 2.00, 1.94 \text{ (4s, 12H, OCOCH}_3). \)

\(\delta_c \text{ (100 MHz, CDCl}_3): 144.7, 138.0, 131.6 \text{ (C-11)}, 130.3 \text{ (C-10)}, 128.9 \text{ (C-13)}, 128.4 \text{ (C-12)}, 126.6 \text{ (C-8)}, 123.9 \text{ (C-9)}, 122.8, 100.2 \text{ (C-1)}, 70.9 \text{ (C-3)}, 70.6 \text{ (C-5)}, 68.9 \text{ (C-2)}, 67.5 \text{ (C-4)}, 61.8 \text{ (C-6)}, 61.2 \text{ (C-7)}, 56.3 \text{ (C-15)}, 44.2 \text{ (C-16)}, 37.2 \text{ (C-14)}. \)

\(\nu_{\text{max}} \text{ (ATR)/cm}^{-1}: 557, 785, 1042, 1231, 1370, 1590 \text{ (ar. C-C)}, 1662 \text{ (C=O)}, 1703, 1745, 3394. \)

HRMS \((m/z - \text{ESI}^+): \text{Found: 696.2514, ([M+Na]}. \text{C}_{33}\text{H}_{38}\text{N}_5\text{O}_{12}, \text{Required: 696.2511).} \)
8.11 Experimental for Chapter 3

2-Deoxy-2-(p-methoxybenzylideneamino)-β-D-glucopyranose (93) ¹⁸⁶

D-Glucosamine hydrochloride (92) (3.00 g, 14 mmol, 1 equiv) was dissolved in a stirred solution of 1 M sodium hydroxide (15 mL, 15 mmol, 1.1 equiv). p-Anisaldehyde (1.9 mL, 16.0 mmol, 1.1 equiv) was added and after 10 min a white precipitate began to form. The reaction mixture was cooled in an ice bath for 2 h and monitored by TLC (8:2 (v/v), EtOAc/Hex). After 2 h the white precipitate was isolated by vacuum filtration and washed thoroughly with cold water (100 mL) followed by a 1:1 mixture of Et₂O/EtOH (4 × 25 mL). The white solid was then dried further under vacuum to afford the desired product (93) as a white crystalline solid (1.52 g, 37%).

M.p: 157-160 °C (lit: 161-163 °C) ¹⁸⁶

δH (400 MHz, CD₃OD): 8.21 (s, 1H, N=CHAr), 7.75 – 7.66 (m, 2H, Ar-H), 7.01 – 6.90 (m, 2H, Ar-H), 4.85 (d, J₁,₂ = 8.0 Hz, 1H, H-1), 3.91 – 3.87 (m, 1H, H-6), 3.82 (s, 3H, -OCH₃), 3.71 – 3.61 (m, 2H, H-3, H-4) 3.42 – 3.33 (m, 2H, H-5, H-6'), 2.95 – 2.91 (app.t, 1H, H-2).


1, 3, 4, 6-Tetra-O-acetyl-2-deoxy-2-(p-methoxybenzylideneamino)-β-D-glucopyranose (94) ¹⁸⁶

Compound 93 (1.52 g, 5 mmol, 1 equiv) was added to a cooled (0 °C) mixture of pyridine (8 mL, 100 mmol, 20 equiv) and acetic anhydride (7 mL, 74 mmol, 14.8 equiv). The reaction mixture was stirred in an ice-bath for 1 h and then let to warm up to rt and stirred for further 5 h. After such time, TLC (6:4 EtOAc/Hex) of the reaction mixture showed product formation. The colourless solution was poured into a beaker of cold water (100 mL) and a white precipitate was observed. EtOAc was added (50 mL) and a work up was carried out using CuSO₄ solution (5 mL) and water (30 mL). The aqueous layer was removed and the organic layer was washed again with water (2 × 30 mL) and brine (50 mL). The organic layer was then dried with MgSO₄ and concentrated in vacuo to afford a crude yellow solid.
Purification by column chromatography (2:8 (v/v), EtOAc/Hex) yielded the desired product (94) as a white solid (1.64 g, 69%).

M.p: 178-181 °C (lit. 180-182 °C)

δH (400 MHz, CDCl3): 8.15 (s, 1H, N=CHAr), 7.68 (d, J = 9.0 Hz, 2H, Ar-H), 6.92 (d, J = 9.0 Hz, 2H, Ar-H), 5.96 (d, J1,2 = 8.5 Hz, 1H, H-1), 5.44 (app t, 1H, H-3), 5.14 (app t, 1H, H-4), 4.38 (dd, J6',6 = 12.5 Hz, J6',5 = 4.5 Hz, 1H, H-6'), 4.15 – 4.11 (m, 1H, H-6), 3.99 – 3.95 (m, 1H, H-5), 3.85 (s, 3H, -OCH3), 3.49 – 3.44 (app t, 1H, H-2), 2.10, 2.04, 2.02, 1.88 (4s, 12H, COCH3).


1, 3, 4, 6-Tetra-O-acetyl-β-D-glucosamine hydrochloride (95)

To a solution of 94 (1.50 g, 3.2 mmol, 1.00 equiv) in warm acetone (30 °C, 20 mL) was added 5 M HCl (0.8 mL, 4.0 mmol, 1.3 equiv) dropwise. The reaction mixture was then cooled in an ice bath and a white precipitate was formed. Et2O (20 mL) was added and let to stir in the ice bath for 3 h. After such time the white precipitate was isolated by vacuum filtration, washed with cold Et2O (100 mL) and dried in vacuo, affording the desired product (95) as a white solid (1.14 g, 92%).

M.p: 212-213 °C (lit. 217-222 °C)

δH (400 MHz, CD3OD): 5.86 (d, J1,2 = 9.0 Hz, 1H, H-1), 5.34 (dd, J3,2 = 10.5 Hz, J3,4 = 9.0 Hz, 1H, H-3), 5.07 (dd, J4,5 = 10.0 Hz, J4,3 = 9.0 Hz, 1H, H-4), 4.29 (dd, J6,6' = 12.5 Hz, J6,5 = 4.5 Hz, 1H, H-6), 4.10 (dd, J6',6 = 12.5 Hz, J6',5 = 2.5 Hz, 1H, H-6'), 4.01 (ddd, J5,4 = 10.0 Hz, J5,6 = 4.5 Hz, J5,6' = 2.5 Hz, 1H, H-5), 3.61 (dd, J2,3 = 10.5 Hz, J2,1 = 9.0 Hz, 1H, H-2), 2.18, 2.08, 2.02, 2.01 (4s, 12H, COCH3).


1, 3, 4, 6-Tetra-O-acetyl-2-(chloroacetamido)-2-deoxy-β-D-glucopyranose (96)

A stirred suspension of 95 (325 mg, 0.9 mmol, 1.00 equiv) and chloroacetic anhydride (580 mg, 3.4 mmol, 3.80 equiv) in dry CH2Cl2 (20 mL) was cooled in an ice bath before adding TEA (0.5 mL, 3.40 mmol, 3.80 equiv). The resulting mixture was then allowed to warm up to rt and let to stir for 18 h. After such time the reaction mixture was diluted with CH2Cl2 (30 mL) and a work up was carried out by
washing with 1 M HCl (2 × 50 mL), saturated NaHCO₃ (2 × 50 mL) and brine (50 mL). The organic layer was dried with MgSO₄ and concentrated in vacuo to afford a black crude solid. The product was purified by SiO₂ column chromatography (3:7 (v/v), EtOAc/Hex), yielding the desired product (96) as a white solid (267 mg, 74%).

**M.p:** 160-164 °C (lit. 169-169.5 °C).²⁷⁶

δ\(\text{H}(400 MHz, CDCl₃): 6.69 (d, } J = 9.5 Hz, 1H, NH), 5.82 (d, } J_{1,2} = 8.5 Hz, 1H, H-1), 5.30 (dd, } J_{3,2} = 10.5 Hz, J_{3,4} = 9.5 Hz, 1H, H-3), 5.13 (app t, 1H, H-4), 4.3 – 4.2 (m, 2H, H-2, H-6), 4.13 (dd, } J_{6',6} = 12.5 Hz, J_{6',5} = 2.5 Hz, 1H, H-6’), 3.98 (d, } J = 0.9 Hz, 2H, CH₂Cl), 3.86 (m, 1H, H-5), 2.11, 2.09, 2.05, 2.04 (4s, 12H, COCH₃).

**HRMS (m/z - ESI⁺):** Found: 446.0843 ([M + Na]⁺, C₁₆H₂₂ClN₁₀, Required: 446.0830).

1, 3, 4, 6-Tetra-O-acetyl-2-(azidoacetyl)amino)-2-deoxy-β-D-glucopyranose (84)²⁷⁷

A solution of 96 (250 mg, 0.6 mmol, 1 equiv) and sodium azide (153 mg, 2.4 mmol, 4 equiv) in DMF (15 mL) was stirred for 18 h at rt and monitored by TLC (6:4 EtOAc/Hex). After such time, the yellow reaction mixture was diluted with EtOAc (50 mL). The organic phase was washed with water (2 × 50 mL) and brine (50 mL), dried with MgSO₄ and concentrated under vacum. The crude white solid was purified by column chromatography (3:7 (v/v), EtOAc/Hex) to afford the desired product (84) as a white crystalline solid (207 mg, 81%).

**M.p:** 187-190 °C (lit. 201-203 °C).²⁷⁷

δ\(\text{H}(400 MHz, CDCl₃): 6.58 (d, } J_{NH,2} = 9.5 Hz, 1H, NH), 5.79 (d, } J_{1,2} = 8.5 Hz, 1H, H-1), 5.32 – 5.23 (m, 1H, H-3), 5.13 (app t, 1H, H-4), 4.34 – 4.19 (m, 2H, H-2, H-6), 4.12 (dd, } J_{6',6} = 12.5 Hz, J_{6',5} = 2.0 Hz, 1H, H-6’), 3.91 (s, 2H, CH₂N₃), 3.88 – 3.82 (m, 1H, H-5), 2.11, 2.09, 2.04 (3s, 6H, COCH₃).

δ\(\text{C}(100 MHz, CDCl₃): 171.5, 171.1, 169.8, 169.7, 167.6 (C=O), 92.6 (C-1), 73.3 (C-5), 72.6 (C-3), 68.2 (C-4), 62.1 (C-6), 53.6 (C-2), 53.0 (CH₂N₃), 21.3, 21.2, 21.1, 21.0 (COCH₃).

**HRMS (m/z - ESI⁺):** Found: 453.1236 ([M + Na]⁺, C₁₆H₂₂N₄O₁₀, Required: 453.1234).
2-Deoxy-2-((2-hydroxynaphthylmethylidene)amino]-α-D-mannopyranose (100)\(^{187}\)

To a mixture of D-mannosamine hydrochloride (500 mg, 2.3 mmol, 1.00 equiv) in H\(_2\)O (0.5 mL) was added sodium acetate (248 mg, 3.00 mmol, 1.3 equiv), 2-Hydroxynaphthaldehyde (740 mg, 4.3 mmol, 1.9 equiv) and methanol (38 mL). An orange precipitate formed after 20 min. The reaction mixture was let to stir at rt overnight. The mixture was cooled in an ice-water bath for 30 min and the orange precipitate was filtered using a cintered funnel. The crude material was washed with cold water (3 × 20 mL), CH\(_2\)Cl\(_2\) (2 × 20 mL) and Et\(_2\)O (2 × 20 mL). The material was dried under vacuum to afford the desired product (100) as an orange solid (692 mg, 89%).

**M.p:** 195-200 °C (lit. 191.5-193.5 °C).\(^{187}\)

\(\delta\)H (400 MHz, D\(_2\)O): 8.96 (s, 1H, CH=N), 8.03 – 7.96 (m, 1H, Ar-H), 7.75 – 7.67 (m, 1H, Ar-H), 7.65 – 7.58 (m, 1H, Ar-H), 7.45 – 7.37 (m, 1H, Ar-H), 7.22 – 7.15 (m, 1H, Ar-H), 6.74 – 6.65 (m, 1H, Ar-H), 5.14 (d, \(J_{1,2} = 1.0\) Hz, 1H, H-1), 3.95 – 3.08 (m, 6H, H-2, H-3, H-4, H-5, H-6).

**HRMS (m/z - ESI\(^+\)):** Found: 334.1302 ([M + H]\(^+\), C\(_{17}\)H\(_{19}\)N\(_4\)O\(_6\) Required: 334.1291).

2-(2-Acetoxynaphthylmethylidene)amino)-1,3,4,6-tetra-\(O\)-acetyl-2-deoxy-\(\alpha\)-D-mannopyranose (101)\(^{187}\)

Compound 100 (520 mg, 1.56 mmol, 1.00 equiv) was dissolved in excess of pyridine (12 mL) and the mixture was cooled in an ice-water bath. Acetic anhydride (11.9 mL) and a DMAP (4 mg, 0.03 mmol, 0.02 equiv) were added and the reaction mixture was allowed to warm to rt. The orange solution gradually turned yellow and then colourless after 2 h. The reaction mixture was left to stir overnight. After 18 h the reaction mixture was concentrated under vacuum and the crude residue was deissolved in CH\(_2\)Cl\(_2\) (50 mL) and washed with dilute sodium bicarbonate (1% (v/v), 3 × 40 mL). The combined organic layer were then dried with MgSO\(_4\), filtered and concentrated to afford the crude product as a yellow viscous liquid. Purification by column chromatography (1:9 EtOAc/Hex) yielded the desired product (100) as yellow crystalline solid (549 mg, 65%).

**M.p:** 153-157 °C (lit. 157.5-160 °C).\(^{187}\)
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δH (400 MHz, CDCl3): 9.00 (d, J = 8.5 Hz, 1H, Ar-H), 8.76 (s, 1H, CH=N), 8.01 – 7.11 (m, 5H, Ar-H), 6.12 (d, J1,2 = 1.5 Hz, 1H, H-1), 5.74 (app t, 1H, H-4), 5.47 (dd, J3,4 = 10.0 Hz, J3,2 = 4.0 Hz, 1H, H-3), 4.43 – 3.93 (m, 4H, H-2, H-5, H-6), 2.39, 2.21, 2.07, 2.06, 1.99 (5s, 15H, COCH3).

**HRMS (m/z - ESI+):** Found: 544.1821 ([M + H]+, C27H29NO11, Required: 544.1819).

1,3,4,6-Tetra-O-acetyl-2-amino-2-deoxy-α-D-mannopyranose oxalate (102)\(^{187}\)

A stirred solution of 101 (525 mg, 0.97 mmol, 1.00 equiv) in acetone (20 mL) was cooled in an ice-water bath and oxalic acid dihydrate (668 mg, 5.3 mmol, 5.5 equiv) was added followed by extra acetone (8 mL). After 30 min, precipitation began to occur and the yellow reaction mixture was allowed to warm to rt. The reaction mixture was stirred at rt for 1.5 h and then cooled again in an ice-water bath for 15 min. The white precipitate was filtered through a cintered funnel and washed with Et2O (3 × 30 mL). After drying under vacuum the desired product (102) was obtained as a white solid (250 mg, 59%).

**M.p:** 147-150 ºC (lit. 154-155.5 ºC)\(^{187}\)

δH (400 MHz, CD3OD): 6.27 (d, J1,2 = 1.5 Hz, 1H, H-1), 5.57 (dd, J3,4 = 9.5 Hz, J3,2 = 5.0 Hz, 1H, H-3), 5.36 (app. t, 1H, H-4), 4.38 (dd, J6,6' = 12.5 Hz, J6,5 = 5.1 Hz, 1H, H-6), 4.34 – 4.27 (m, 1H, H-5), 4.13 (dd, J6,6' = 12.5 Hz, J6,5 = 2.0 Hz, 1H), 3.99 (dd, J2,3 = 5.0 Hz, J2,1 = 1.5 Hz, 1H, H-2), 2.22, 2.14, 2.10, 2.10 (4s, 12H, COCH3).

**HRMS (m/z - ESI+):** Found: 348.1299 ([M + H]+, C14H22NO9, Required: 348.1298).

1, 3, 4, 6-Tetra-O-acetyl-2-(chloroacetamido)-2-deoxy-α-D-mannopyranose (103)\(^{278}\)

A stirred mixture of 102 (220 mg, 0.5 mmol, 1.00 equiv) in anhydrous CH2Cl2 (20 mL) was cooled in an ice bath. Chloroacetic anhydride (342 mg, 2.0 mmol, 4 equiv) was added to the solution followed by Et3N (0.3 mL, 2 mmol, 4 equiv) and the reaction mixture was allowed to come to rt and stir overnight. After 18 h, the reaction mixture was diluted in CH2Cl2 (30 mL) and washed with 1 M HCl (2 × 50 mL), saturated NaHCO3 (2 × 50 mL) and brine (50 mL). The organic phase was then dried using MgSO4 and concentrated in vacuo to afford the crude product (102) as a colourless oil (160 mg, 76%).

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\[ \delta_H (400 \text{ MHz, CDCl}_3): 6.85 (d, J_{NH,2} = 9.0 \text{ Hz, } 1\text{H, NH}), 6.06 (d, J_{1,2} = 2.0 \text{ Hz, } 1\text{H, H-1}), 5.34 (dd, J_{3,4} = 10.0 \text{ Hz, } J_{3,2} = 4.1 \text{ Hz, } 1\text{H, H-3}), 5.26 (\text{app t, } 1\text{H, H-4}), 4.65 – 4.58 (m, 1\text{H, H-5}), 4.21 (dd, J_{6,6'} = 12.5 \text{ Hz, } J_{6,5} = 4.0 \text{ Hz, } 1\text{H, H-6}), 4.13 – 4.01 (m, 4\text{H, H-6', H-2, CH}_2), 2.18, 2.09, 2.05, 2.00 (4s, 12\text{H, COCH}_3). \]  

**HRMS (m/z - ESI\(^+\)):** Found: 446.0841 ([M + H]\(^+\), C\(_{16}\)H\(_{22}\)ClNO\(_{10}\), Required: 348.1298).

**1, 3, 4, 6-Tetra-O-acetyl-2-((azidoacetyl)amino)-2-deoxy-\(\alpha\)-D-mannopyranose (81)**

To a solution of 102 (148 mg, 0.35 mmol, 1.00 equiv) in DMF (5 mL) was added sodium azide (91 mg, 1.4 mmol, 4 equiv). The yellow reaction mixture was then stirred at rt overnight. After 24 h, the reaction mixture was diluted in EtOAc (50 mL) and the organic layers were washed with water (50 mL) and brine (50 mL), dried using MgSO\(_4\) and concentrated in vacuo to afford the crude product as a colourless oil. Purification by column chromatography (3:7 EtOAc/Hex) yielded the desired product (81) as a colourless oil (90 mg, 60%).

\[ \delta_H (600 \text{ MHz, CDCl}_3): 6.57 (d, J_{NH,2} = 9.0 \text{ Hz, } 1\text{H, NH}), 6.04 (d, J_{1,2} = 2.0 \text{ Hz, } 1\text{H, H-1}), 5.33 (dd, J_{3,4} = 10.0 \text{ Hz, } J_{3,2} = 4.5 \text{ Hz, } 1\text{H, H-3}), 5.21 (\text{app t, } 1\text{H, H-4}), 4.64 - 4.58 (m, 1\text{H, H-5}), 4.23 (dd, J_{6,6'} = 12.5 \text{ Hz, } J_{6,5} = 4.2 \text{ Hz, } 1\text{H, H-6}), 4.12 – 4.02 (m, 4\text{H, H-6', H-2, CH}_2), 2.18, 2.10, 2.05, 1.99 (4s, 12\text{H, COCH}_3). \]  

**HRMS (m/z - ESI\(^+\)):** Found: 453.1237 ([M+Na]\(^+\), C\(_{16}\)H\(_{22}\)N\(_4\)O\(_{10}\), Required: 453.1234).

**N-Propargyl-4-bromo-1,8-naphthalimide (105)**

2-Propyn-1-amine (0.14 mL, 2.16 mmol, 1.20 equiv) was added to a solution of 4-bromo-1,8-naphthalic anhydride (0.50 g, 1.80 mmol, 1.00 equiv) in ethanol (20 mL). After 3 h refluxing, the reaction was dried in vacuo to afford the product 105 as a brown powder (0.56 g, 99%).

\[ \delta_H (400 \text{ MHz, CDCl}_3): 8.70 (dd, J_{8,7} = 7.3 \text{ Hz, } J_{8,6} = 1.1 \text{ Hz, } 1\text{H, H-8}), 8.60 (dd, J_{6,7} = 8.5 \text{ Hz, } J_{6,8} = 1.1 \text{ Hz, } 1\text{H, H-6}), 8.46 (d, J_{4,5} = 7.9 \text{ Hz, } 1\text{H, H-4}), 8.06 (d, J_{5,4} = 7.9 \text{ Hz, } 1\text{H, H-5}), 7.87 (dd, J = 8.5 \text{ Hz, } J = 7.3 \text{ Hz, } 1\text{H, H-7}), 4.95 (d, J_{2,1} = 2.5 \text{ Hz, } 2\text{H, H-3}), 2.20 (t, J_{1,2} = 2.5 \text{ Hz, } 1\text{H, H-1}). \]
HRMS (m/z - ESI\(^{+}\)): Found: 313.98254 ([M+H]\(^{+}\), C\(_{13}\)H\(_9\)BrNO\(_2\), Required: 313.98111).

\(N\)-((1-(3-\(\beta\)-D-galactopyranosyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl)-4-bromo-1,8-naphthalimide (106)

Following general procedure D: 55a (0.28 g, 1.06 mmol, 1.3 equiv), compound 105 (0.256 g, 0.818 mmol, 1.0 equiv), \([(\text{CH}_3\text{CN})_4\text{Cu}]\text{BF}_4\) (38.00 mg, 0.12 mmol, 0.15 mmol) and DMF (15 mL). Product 106 afforded as an orange powder (0.46 g, 98%).

\(R_f\) = 0.20 (1:1 (v/v), EtOAc/Hex)

[\(\alpha\)]\(_D\)\(^{20}\) = 20 \(^{\circ}\) cm\(^{3}\) g\(^{-1}\) dm\(^{-1}\) (0.1, MeOH)

\(\delta\)\(_H\) (400 MHz, DMSO): 8.59 (app t, 2H, H-14, H-16), 8.36 (d, \(J_{12,13} = 7.9\) Hz, H-1, H-12), 8.24 (d, \(J_{13,12} = 7.9\) Hz, H-1, H-13), 8.05 – 7.98 (m, 2H, H-15, DMF), 7.93 (s, 1H, H-10), 5.28 (s, 2H, H-11), 4.85 (d, \(J = 4.6\) Hz, 1H, OH), 4.61 (d, \(J = 5.6\) Hz, 1H, OH), 4.50 (app t, 1H, OH), 4.38 (app t, 2H, H-9), 4.30 (d, \(J = 4.6\) Hz, 1H, OH), 4.03 – 3.96 (m, 2H, H-1, OH), 3.70 (dt, \(J = 11.1\) Hz, \(J = 5.8\) Hz, 1H, H-6), 3.57 (t, \(J = 4.1\) Hz, 1H), 3.52 – 3.38 (m, 2H, H-7), 3.19 – 3.11 (m, 3H), 2.04 – 1.93 (m, 2H, H-8).

\(\delta\)\(_C\) (100 MHz, DMSO): 133.3 (q, C-16), 132.4 (q, C-15), 131.6 (q, C-13), 131.5 (q, C-12), 129.0 (q, C-14), 104.5 (C-1), 73.5, 70.9, 68.5, 65.4 (C-6), 61.07 (C-7), 47.9 (C-8), 35.7 (C-11), 30.3 (C-8).

\(\nu\)\(_{\text{max}}\) (ATR)/cm\(^{-1}\): 3354 (OH), 2923, 1702, 1661 (C=O), 1587 (ar. C-C), 1368, 1344, 1235, 1055, 1033, 953, 783, 577

HRMS (m/z - ESI\(^{+}\)): Found: 599.073311, ([M+Na]\(^{+}\). C\(_{24}\)H\(_{25}\)BrN\(_4\)NaO\(_8\), Required: 599.074797).

\(N\)-((1-(3-(\(\beta\)-D-galactopyranosyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl)-4-trimethylsilylacetylene-1,8-naphthalimide (107)

To a solution of compound 106 (120 mg, 0.21 mmol, 1.00 equiv) in anhydrous THF (3 mL) CuI (2 mg, 0.02 mmol, 0.05 equiv), Pd(PPh\(_3\))\(_4\) (12 mg, 0.01 mmol, 0.05 equiv), trimethylsilylacetylene (32.6 \(\mu\)L, 0.23 mmol, 1.1 equiv) and NEt\(_3\) (0.2 mL, 1.45 mmol, 7 equiv) were added under an Argon atmosphere and the mixture was warmed up to 40 °C. After 24 h, the
reaction mixture was concentrated under pressure and the crude material was filtered through a plug of SiO₂ using 1:24 (v/v) MeOH/EtOAc. Solvent removal afforded the desired compound 107 as a yellowish wax (113 mg, 92%).

\[ \text{Rf} = 0.15 \ (1:5 \ (v/v), \ \text{MeOH/EtOAc}). \]

\[ \delta \ (400 \ \text{MHz, CD₃OD}): 8.34 – 8.26 \ (m, \ 2H, \ H-14, \ H-16), \ 8.17 \ (d, \ J_{12,13} = 7.6 \ \text{Hz}, \ 1H, \ H-12), \ 8.06 \ (s, \ 1H, \ H-10), \ 7.69 – 7.62 \ (m, \ 2H, \ H-15, \ H-13), \ 5.32 \ (s, \ 2H, \ H-11), \ 4.53 \ (t, \ J_{9,8} = 6.8 \ \text{Hz}, \ 3H, \ H-9), \ 4.18 \ (dd, \ J_{1,2} = 7.7, \ 1.6 \ \text{Hz}, \ 1H, \ H-1), \ 3.86 – 3.83 \ (m, \ 1H, \ H-4), \ 3.78 – 3.68 \ (m, \ 3H, \ H-3, \ H-6), \ 3.55 – 3.40 \ (m, \ 4H, \ H-2, \ H-7, \ H-5), \ 2.19 – 2.10 \ (m, \ 3H, \ H-8) \]

\[ \text{HRMS (m/z - ESI⁺): Found: 617.2036,} \ \text{([M+Na]⁺. C}_{29}\text{H}_{34}\text{N}_{4}\text{O}_{8}\text{Si, Required: 617.2038}). \]

\[ N-((1-(3-(β-D-galactopyranosyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl)-4-acetylene-1,8-naphthalimide (90) \]

\[ \text{Compound 107 (104 mg, 0.175 mmol, 1.00 equiv) was dissolved in a mixture of MeOH/THF (1:1, 10 mL) and excess of K₂CO₃ (180 mg, 1.30 mmol, 7.00 equiv) was added. The reaction was let to stir at rt. After 4 h, the mixture was vaced down and columned using SiO₂ column chromatography (2:8 EtOAc, Hex) to give the desired compound 90 as clear yellow wax (35 mg, 38%).} \]

\[ \text{Rf} = 0.72 \ (1:9 \ (v/v), \ \text{EtOAc/Hex}). \]

\[ [\alpha]_{D}^{30} = 2 \ \text{deg cm}^3 \ \text{g}^{-1} \ \text{dm}^{-1} \ (0.05, \ \text{MeOH}) \]

\[ \delta \ (400 \ \text{MHz, CD₃OD}): 8.60 \ (dd, \ J_{16,15} = 8.4 \ \text{Hz}, \ J_{16,14} = 1.2 \ \text{Hz}, \ 1H, \ H-16), \ 8.53 \ (dd, \ J_{14,15} = 7.3 \ \text{Hz}, \ J_{14,16} = 1.2 \ \text{Hz}, \ 1H, \ H-14), \ 8.43 \ (d, \ J_{13,12} = 7.6 \ \text{Hz}, \ 1H, \ H-13), \ 8.06 \ (s, \ 1H, \ H-10), \ 7.90 \ (d, \ J_{12,13} = 7.6 \ \text{Hz}, \ 1H, \ H-12), \ 7.83 \ (dd, \ J_{15,16} = 8.4 \ \text{Hz}, \ J_{15,14} = 7.3 \ \text{Hz}, \ 1H, \ H-15), \ 5.40 \ (s, \ 2H, \ H-11), \ 4.56 – 4.49 \ (m, \ 2H, \ H-9), \ 4.38 \ (s, \ 1H, \ H-17), \ 4.15 \ (d, \ J_{1,2} = 7.7 \ \text{Hz}, \ 1H, \ H-1), \ 3.90 – 3.82 \ (m, \ 1H, \ H-6), \ 3.80 \ (d, \ J_{4,5} = 3.4 \ \text{Hz}, \ J_{4,3} = 1.0 \ \text{Hz}, \ 1H, \ H-4), \ 3.76 – 3.65 \ (m, \ 2H, \ H-3, \ H-7), \ 3.53 – 3.42 \ (m, \ 3H, \ H-2, \ H-6’), \ 3.39 \ (dd, \ J_{5,6} = 9.7 \ \text{Hz}, \ J_{5,4} = 3.4 \ \text{Hz}, \ 1H, \ H-5), \ 2.18 - 2.10 \ (m, \ 2H, \ H-8). \]

\[ \delta \ (100 \ \text{MHz, CD₃OD}): 163.5, \ 143.3, \ 132.0 \ (C-16), \ 131.8 \ (C-14), \ 131.3 \ (C-12), \ 129.9 \ (C-13), \ 127.6 \ (C-15), \ 126.6, \ 124.3 \ (C-10), \ 122.7, \ 103.6 \ (C-1), \ 75.3, \ 73.5, \ 71.0, \ 68.8, \ 65.3 \ (C-6), \ 61.0 \ (C-7), \ 47.8 \ (C-9), \ 34.8 \ (C-11), \ 30.0 \ (C-8). \]
\( v_{\text{max}} \) (ATR)/cm\(^{-1}\): 3360 (OH), 2925, 1698, 1656 (C=O), 1589 (ar. C-C), 1383, 1235, 1070, 786.

**HRMS** (m/z - ESI\(^+\)): Found: 545.16322, ([M+Na]\(^+\). C\(_{26}\)H\(_{26}\)N\(_4\)NaO\(_8\), Required: 545.1642).

8.13 Experimental for Chapter 4

2-(2-dimethylamino-ethyl)-5-nitro-benzo[de]isoquinoline-1,3-dione (27) \(^{280}\)

To a solution of 3-nitro-1,8-naphthalic anhydride (0.40 g, 1.64 mmol, 1.00 equiv) in 20 mL of EtOH \( N,N \)-Dimethylthelyenediamine (0.22 mL, 1.97 mmol, 1.20 equiv) was added. After refluxing for 4 h, the mixture was let to cool down to rt and diluted in CH\(_2\)Cl\(_2\) (20 mL), washed with brine (2 x 15 mL) and water (15 mL) and the combined organic layers were dried under MgSO\(_4\), filtered off and solvent removed *in vacuo*. The product was afforded as a brownish powder (497 mg, 97%).

\( R_t = 0.10 \) (3:2 (v/v), EtOAc/Hex).

\( \delta \)H (400 MHz, CDCl\(_3\)): 9.31 (d, \( J_{4,5} = 2.2 \) Hz, 1H, H-4), 9.13 (d, \( J_{5,4} = 2.2 \) Hz, 1H, H-5), 8.78 (dd, \( J_{6,7} = 7.3 \) Hz, \( J_{6,8} = 1.2 \) Hz, 1H, H-6), 8.42 (dd, \( J_{8,7} = 8.3 \) Hz, \( J_{8,6} = 1.2 \) Hz, 1H, H-8), 7.93 (dd, \( J_{7,8} = 8.3 \) Hz, \( J_{7,6} = 7.3 \) Hz, 1H, H-7), 4.43 (t, \( J_{3,2} = 6.5 \) Hz, 2H, H-3), 2.91 (bs, 2H, H-2), 2.51 (s, 6H, H-1).

**HRMS** (m/z - ESI\(^+\)): Found: 314.1139, ([M+H]\(^+\). C\(_{16}\)H\(_{16}\)N\(_3\)O\(_4\), Required: 314.1142).

\( N-[1\text{-Dimethylamino-ethyl}]\text{-3-amino-1,8-naphthalimide} \) (26) \(^{281}\)

Compound 27 (100 mg, 0.32 mmol, 1.00 equiv) was dissolved in MeOH/EtOH (1:1, v/v) (10 mL) and Pd/C (10 wt. %) (6.80 mg, 0.06 mmol, 0.20 equiv) was added. After stirring under H\(_2\) atmosphere for 18 h, the mixture was filtered through a plug of Celite and solvent removed *in vacuo*. Column chromatography on silica gel (7:3 EtOAc/Hexane (v/v) lead to compound 26 as a yellow powder (70 mg, 77%).

\( \text{M.p:} \) 160 – 162 ºC (lit. 162 - 164 ºC)\(^{282}\)

\( R_t = 0.36 \) (50% EtOAc/MeOH)

\( \delta \)H (400 MHz, CD\(_3\)OD): 8.23 (d, \( J_{8,7} = 7.3 \) Hz, 1H, H-8), 8.07 (d, \( J_{5,4} = 2.3 \) Hz, 1H, H-5), 8.01 (d, \( J_{6,7} = 8.3 \) Hz, 1H, H-6), 7.63 (dd, \( J_{7,8} = 8.3 \) Hz, \( J_{7,6} = 7.3 \) Hz, 1H, H-7), 7.39 (d, \( J_{4,5} = 2.3 \) Hz, 1H, H-4), 4.32 (t, \( J_{3,2} = 6.6 \) Hz, 2H, H-3), 2.70 (t, \( J_{2,3} = 6.6 \) Hz, 2H, H-2), 2.38 (s, 6H, H-1).
HRMS (m/z - ESI\(^+\)): Found: 284.1394, ([M+H] \(^+\). C\(_{16}\)H\(_{18}\)N\(_3\)O\(_2\), Required: 284.1399).

2,3,4,6-Tetra-O-acetyl-\(\beta\)-d-galactopyranose (123) \(^{283}\)

Compound 124 (750 mg, 1.83 mmol, 1.00 equiv) was dissolved in a mixture of acetones/H\(_2\)O (9:1) (10 mL) with Ag\(_2\)CO\(_3\) (755 mg, 2.74 mmol, 1.50 equiv) at 0 °C and let warm up to rt. The reaction was taken off after 16 h, filtered through celite, and solvent removed \textit{in vacuo}. The mixture was re-dissolved in CH\(_2\)Cl\(_2\) and washed with H\(_2\)O (3 × 10 mL) and brine (10 mL). The organic layers were combined and the solvent was removed, the product was purified by SiO\(_2\) (7:3, EtOAc/Hex (v/v)) to give compound 123 as a white wax (420 mg, 66%).

\(RF = 0.6\) (3:2 (v/v), EtOAc/Hex).

\(\delta\)\(_H\) (400 MHz, CDCl\(_3\)): 5.55 (d, \(J_{1,2} = 3.6\) Hz, 1H, H-1), 5.51 (d, \(J_{2,3} = 3.2\) Hz, 1H, H-2), 5.44 (dd, \(J_{3,4} = 10.9\) Hz, \(J_{3,2} = 3.2\) Hz, 1H, H-3), 5.20 (dd, \(J_{4,3} = 10.9\) Hz, \(J_{4,5} = 3.6\) Hz, 1H, H-4), 4.50 (app t. 1H, H-5), 4.21 – 4.15 (m, 2H, H-6), 2.18, 2.13, 2.11, 2.08 (4s, 12H, OCOCH\(_3\)).

HRMS (m/z - ESI\(^+\)): Found: 371.0955, ([M+Na] \(^+\). C\(_{14}\)H\(_{20}\)O\(_{10}\)Na, Required: 371.0954).

2,3,4,6-Tetra-O-acetyl-\(\alpha\)-d-galactopyranosyl bromide (124) \(^{284}\)

To a solution of 2,3,4,6-tetraacetate-d-galactopyranose (5.00 g, 5.12 mmol, 1.00 equiv) in anhydrous CH\(_2\)Cl\(_2\) (30 mL), HBr in acetic acid solution (1.75 mL, 30.70 mmol, 6.00 equiv) was added at 0 °C. After 30 min, the reaction was let to warm up and left it overnight at rt. The mixture was poured into ice and extracted with CH\(_2\)Cl\(_2\). The organic phase was washed with brine and dried over MgSO\(_4\), filtered off and concentrated. Column chromatography of the residue on SiO\(_2\) (3:1 EtOAc/Hexane, v/v) gave the product as a white foam (1.51 g, 72%).

\(RF = 0.88\) (1:1 (v/v), EtOAc/Hex)

\(\delta\)\(_H\) (400 MHz, CDCl\(_3\)): 6.68 (d, \(J_{1,2} = 4.0\) Hz, 1H, H-1), 5.51 – 5.49 (m, 1H, H-4), 5.39 (dd, \(J_{3,2} = 10.6\) Hz, \(J_{3,4} = 3.3\) Hz, 1H, H-3), 5.03 (dd, \(J_{2,3} = 10.6\) Hz, \(J_{2,1} = 4.0\) Hz, 1H, H-2), 4.47 (app t, 1H, H-5), 4.17 (dd, \(J_{6,6'} = 11.4\) Hz, \(J = 6.3\) Hz, 1H, H-6a), 4.10 (dd, \(J_{6',6} = 11.4\) Hz, \(J = 6.3\) Hz, 1H, H-6b), 2.14, 2.10, 2.05, 2.00 (4s, 12H, OCOCH\(_3\)).

HRMS (m/z - ESI\(^+\)): Found: 433.0114, ([M+Na] \(^+\). C\(_{14}\)H\(_{19}\)O\(_6\)BrNa, Required: 433.0110).
3-Nitro-4-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-benzaldehyde (126) 199

Compounds 124 (0.50 g, 1.22 mmol, 1.00 equiv), 4-hydroxy-3-nitro-benzaldehyde (367.66 mg, 2.20 mmol, 1.8 equiv) and BnEt₃NCl (278.50 mg, 1.50 mmol, 0.80 equiv) were dissolved in CHCl₃ (5 mL) and NaOH solution (1.25 M, 1.75 mL, 2.20 mmol, 1.8 equiv). After stirring at 70 °C for 3 h, the mixture was diluted in H₂O/CHCl₃ (2:1 (v/v), 15 mL), the organic layer was separated and successively washed with NaOH solution (1.25 M, 2 × 5 mL) and brine, dried over MgSO₄ and the solvent evaporated in vacuo. The product was obtained as a white foam (417 mg, 70 %).

Rₖ = 0.32 (2:3 (v/v), EtOAc/Hex)

δH (400 MHz, CDCl₃): 9.98 (s, 1H, Ar-CHO), 8.30 (d, J₉,₈ = 2.0 Hz, 1H, H-9), 8.07 (dd, J₈,₇ = 8.6 Hz, 1H, H-8), 7.48 (d, J₇,₆ = 8.6 Hz, 1H, H-7), 5.62 – 5.55 (m, 1H, H-2), 5.49 (dd, J₄,₃ = 3.5 Hz, J = 1.1 Hz, 1H, H-4), 5.21 (d, J₁,₂ = 7.9 Hz, 1H, H-1), 5.13 (dd, J = 10.4 Hz, J₃,₄ = 3.5 Hz, 1H, H-3), 4.29 – 4.23 (m, 1H, H-6), 4.22 – 4.09 (m, 2H, H₅, H₆'), 2.19, 2.13, 2.08, 2.02 (4s, 12H, OCOCH₃).


2,3,4,6-Tetra-O-acetyl-(2-nitro-4-(hydroxymethyl)phenyl)-β-D-galactopyranoside (127). 105,199

Compounds 126 (420 mg, 0.82 mmol, 1.00 equiv) was dissolved in degassed CH₂Cl₂ (5.5 mL) and iPrOH (3 mL), silica gel (950 mg) and ion exchanger IR-120 H⁺ form (10 mg) were added and the solution was cooled to 0 °C. After 30 min, NaBH₄ (120.00 mg, 3.20 mmol, 4.0 equiv) was added portionwise and stirring continued at 0 °C for 3 h. The reaction was quenched by addition of ice-cold sat. NH₄Cl solution (10 mL) and extracted with CH₂Cl₂ (2 × 15 mL). The combined organic layers were washed with water (10 mL) and brine (10 mL) and dried under MgSO₄. Solvent removal afforded 126 as a white foam (340 mg, 83%).

Rₖ = 0.25 (2:3 (v/v), EtOAc/Hex)

δH (400 MHz, CDCl₃): 7.80 (d, J₉,₈ = 2.0 Hz, 1H, H-9), 7.51 (dd, J₈,₇ = 8.5 Hz, J₈,₉ = 2.0 Hz, 1H, H-8), 7.35 (d, J₇,₆ = 8.5 Hz, 1H, H-7), 5.57 – 5.50 (m, 1H, H-2), 5.46 (dd, J₃,₄ = 3.4 Hz, J₃,₅ = 1.2 Hz, 1H, H-4), 5.10 (dd, J₃,₂ = 10.5 Hz, J₃,₄ = 3.4 Hz, 1H, H-3), 5.05 (d, J₁,₂ = 8.0 Hz, 1H, H-1), 4.75 – 4.71 (m, 2H, H-10), 4.25 (dd, J₆,₇ = 11.3 Hz, J = 7.0 Hz, 236
1H, H-6), 4.16 (dd, $J_{6',6} = 11.3$ Hz, $J = 6.0$ Hz, 1H, H-6'), 4.10 – 4.02 (m, 1H, H-5), 2.19, 2.13, 2.07, 2.01 (4s, 12H, OCOCH$_3$).

**HRMS** ($m/z$ – ESI$^+$): Found: 522.1230, ([M+Na]$^+$). C$_{21}$H$_{25}$NO$_{13}$Na, Required: 522.1224.

1-Bromo-2,3,4-tri-O-acetyl-α-D-glucopyranuronic acid, methyl ester (129) $^{285}$

1,2,3,4-Tetra-O-β-D-glucopyranuronic acid, methyl ester (2.0 g, 5.32 mmol, 1.00 equiv) was cooled to 0 ºC and HBr (33% in AcOH, 8 mL) was added to this and let to stir for 30 min. After this time, the reaction was let to stir overnight at 4 ºC (in the fridge). The reaction mixture was diluted with CH$_2$Cl$_2$, quenched with iced NaHCO$_3$, washed with water, brine and combined organic layer dried over MgSO$_4$. After solvent removal under reduced pressure the product was obtained as a white wax (1.46 g, 70%). The MS could not be obtained due to its fast decomposition.

**R$_f$** = 0.38 (1:1 (v/v), EtOAc/Hex)

$\delta$H (400 MHz, CDCl$_3$): 6.64 (d, $J_{1,2} = 4.0$ Hz, 1H, H-1), 5.61 (app t, 1H, H-3), 5.31 – 5.21 (m, 1H, H-4), 4.85 (dd, $J = 10.0$ Hz, $J_{2,1} = 4.0$ Hz, 1H, H-2), 4.58 (d, $J = 10.3$ Hz, 1H, H-5), 3.76 (s, 3H, COOCH$_3$), 2.10, 2.05 (3s, 9H, OCOOCH$_3$).

Methyl-1-(4-formyl-2-nitrophenyl)-2,3,4-tri-O-acetyl-β-D-glucopyronuronate (130) $^{286}$

Compound 129 (1.40 g, 3.53 mmols, 1.00 equiv), 4-hydroxy-3-nitrobenzaldehyde (1.06 g, 6.36 mmol, 1.8 equiv) and BnEtNCl (0.52 g, 2.82 mmol, 0.8 equiv) were dissolved in CH$_3$Cl$_3$ (15 mL) and NaOH (5 mL, 1.25 M) was added. The mixture was let to stir overnight (16 h) at rt. The mixture was diluted in CH$_2$Cl$_2$ washed with NaOH (2 × 15 mL, 2 M) and brine (15 mL). The combined organic layers were dried under MgSO$_4$, filtered off and solvent removed in vacuo to afford a white wax (653 mg, 38%).

**R$_f$** = 0.44 (3:2 (v/v), EtOAc/Hex)

$\delta$H (400 MHz, CDCl$_3$): 9.98 (s, 1H, CHO), 8.32 (d, $J = 2.0$ Hz, 1H, H-Ar), 8.10 (d, $J = 2.1$ Hz, 1H, H-Ar), 8.08 (d, $J = 2.1$ Hz, 1H, H-Ar), 5.46 – 5.40 (m, 2H, H-1, H-3), 5.35 – 5.21 (m, 2H, H-2, H-4), 4.32 (d, $J = 8.4$ Hz, 1H, H-5), 3.71 (s, 3H, COOCH$_3$), 2.10, 2.05 (2s, 9H, OCOOCH$_3$).

**HRMS** ($m/z$ – ESI$^+$): Found: 482.090431, ([M-H]$^-$). C$_{20}$H$_{20}$NO$_{13}$, Required: 482.094013.)
Methyl-1-(4-hydroxymethyl-2-nitrophenyl)-2,3,4-tri-O-acetyl-β-D-glucopyranuronate (131)

Compound 130 (659 mg, 1.34 mmol, 1.00 equiv) was dissolved in degassed CH₂Cl₂ (6 mL) and iPrOH (3 mL), silica gel (950 mg) was added and the solution was cooled to 0 °C. After 30 min, NaBH₄ (0.101 g, 2.69 mmol, 2.0 equiv) was added portionwise and stirring continued at 0 °C for 1 h. The reaction was filtered through celite and quenched by addition of ice-cold sat. NH₄Cl solution (10 mL) and extracted with CH₂Cl₂ (2 × 15 mL). The combined organic layers were washed with water (10 mL) and brine (10 mL) and dried under MgSO₄. Solvent removal followed by SiO₂ column chromatography (1:1 (v/v), EtOAc/Hex) afforded 131 as a white solid (202 mg, 32%).

Mp: 170 – 172 ºC (lit. 167 – 168 ºC)

Rf = 0.20 (3:2 EtOAc/Hex).

δH (400 MHz, CDCl₃): 7.82 (d, J = 2.1 Hz, 1H, H-Ar), 7.56 – 7.51 (m, 1H, H-Ar), 7.37 (d, J = 8.5 Hz, 1H, H-Ar), 5.40 – 5.26 (m, 3H, H-2, H-3, H-4), 5.19 (d, J = 6.7 Hz, 1H, H-1), 4.73 (d, J = 5.8 Hz, 2H, CH₂), 4.20 (d, J = 8.8 Hz, 1H, H-5), 3.75 (s, 3H, COOCH₃), 2.06, 2.05, 2.04 (3s, 9H, OCOOCH).


2,3,4,6-Tetra-O-acetyl-[2-nitro-4-(4-nitrophenoxycarbonyloxymethyl)phenyl]-β-D-galactopyranoside (135)

To a solution of 127 (390 mg, 0.68 mmol, 1.00 equiv) in CH₂Cl₂ (15 mL), p-nitrophenylchloroformate (302.34 mg, 1.50 mmol, 2.20 equiv) and pyridine (0.15 mL, 1.70 mmol, 2.50 equiv) were added. After 1 day stirring at rt, the solution was diluted in water and extracted with CH₂Cl₂ (2 × 20 mL). The combined organic layers were washed with water, dried over MgSO₄ and concentrated under reduced pressure. Column chromatography on silica gel (2:3 EtOAc/Hexane v/v) gave the product as a colourless oil (356 mg, 79%).

Rf = 0.17 (3:2 (v/v), EtOAc/Hex).

δH (400 MHz, CDCl₃): 8.29 – 8.24 (m, 2H, H-12), 7.88 (d, J₉,₈ = 2.2 Hz, 1H, H-9), 7.59 (dd, J₈,₇ = 8.6 Hz, J₈,₉ = 2.2 Hz, 1H, H-8), 7.40 – 7.34 (m, 3H, H-11, H-7), 5.53 (dd, J =
10.5 Hz, $J = 7.9$ Hz, 1H, H-2), 5.45 (dd, $J = 3.5$ Hz, $J = 1.2$ Hz, 1H, H-4), 5.27 (s, 2H, H-10), 5.12 – 5.06 (m, 2H, H-1, H-3), 4.24 (dd, $J = 11.3$, 6.9 Hz, 1H, H-6), 4.17 – 4.07 (m, 2H, H-5, H-6’), 2.17, 2.11, 2.05, 2.00 (4s, 12H, OCOCH$_3$).

**HRMS (m/z - ESI+):** Found: 687.1282, ([M+Na]$^+$). C$_{28}$H$_{28}$N$_2$O$_{17}$Na, Required: 687.1286.

2,3,4,6-Tetra-O-acetyl-[2-nitro-4-(N,N-diehtylenediamine)phenyl]-β-D-galacto-pyranoside (137c)

![Structural formula of 2,3,4,6-Tetra-O-acetyl-[2-nitro-4-(N,N-diehtylenediamine)phenyl]-β-D-galacto-pyranoside (137c)](image)

Compound 135 (20 mg, 0.03 mmol, 1.00 equiv) and HOBt (5 mg, 0.03 mmol, 1.00 equiv) were dissolved in DMF (5 mL) and diethylamine (3 uL, 0.06 mmol, 2 equiv) was added to the mixture, which was warme up to 50 °C. The reaction was let to stir for 3 h, after which consumption of compound 135 was observed and the reaction was taken off. The reaction was extracted with CH$_2$Cl$_2$ (2 × 15 mL). The combined organic layers were washed with water (10 mL) and brine (10 mL) and dried under MgSO$_4$. Purification by SiO$_2$ column cromatography (1:5 (v/v) EtOAc/Hex) afforded compound 137c as a colourless oil (9 mg, 50%)

R$_f$ = 0.3 (60% EtOAc/Hex)

δH (400 MHz, CDCl$_3$): 7.79 (s, 1H, H-9), 7.53 – 7.46 (m, 1H, H-7), 7.33 (dd, $J = 8.6$ Hz, $J = 5.8$ Hz, 1H, H-8), 5.52 (dd, $J = 10.5$, 8.0 Hz, 1H, H-3), 5.45 (d, $J_{1-2} = 3.3$ Hz, 1H, H-1), 5.28 (s, 2H, H10), 5.12 – 5.02 (m, 3H), 4.24 (dd, $J = 11.3$ Hz, $J = 7.0$ Hz, 1H, H-6), 4.15 (dd, $J = 11.3$, 6.4 Hz, 1H, H-6’), 3.47 (s, 4H, H-11), 2.17, 2.11, 2.05, 2.00 (4s, 12H, OCOCH$_3$).

**HRMS (m/z - ESI+):** Found: 621.1902, ([M+Na]$^+$). C$_{26}$H$_{34}$N$_2$O$_{14}$Na, Required: 621.1908.
Chapter 8. Experimental

(2R,3S,4S,5R,6S)-2-(acetoxyethyl)-6-((4-(((2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-5-yl)carbamoyloxy)methyl)-2-nitrophenoxyl-tetrahydro-2H-pyran-3,4,5-triyl triacete (136)

Amonafide (100 mg, 0.35 mmol, 1.00 equiv) and DMAP (128 mg, 1.05 mmol, 3 equiv) were dissolved in anhydrous CH$_2$Cl$_2$ (25 mL) under a nitrogen atmosphere, at rt. The reaction mixture was cooled to 0 °C for 10 min before the slow addition of phosgene (1.4 mL, 2.10 mmol, 6.00 equiv). The reaction was stirred for 4 h at rt. N$_2$ was bubbled through the reaction mixture for 1 h to remove the phosgene. Compound 127 (192 mg, 0.385 mmol, 1.10 equiv) was dissolved in anhydrous CH$_2$Cl$_2$ (15 mL) under a nitrogen atmosphere, and added to the reaction mixture at 0 °C. The reaction was stirred for further 18 h at rt followed by washed with 0.1 M HCl (20 mL) and brine (20 mL). The organic layer was dried over Na$_2$SO$_4$, filtered and the solvent removed under reduced pressure. The crude was purified by SiO$_2$ column chromatography (EtOAc/Pet. Ether (2:1, v/v)) to yield the product as a while wax (91 mg, 32%).

$[\alpha]_{D}^{20} = 1$ deg cm$^3$ g$^{-1}$ dm$^{-1}$ (0.05, MeOH)

$\delta$H (400 MHz, CD$_3$OD): 8.73 (d, $J_{15-13} = 2.2$ Hz, 1H, H-15), 8.52 – 8.47 (m, 2H, H-9, H-11), 8.29 (d, $J = 8.0$ Hz, 1H, H-12), 8.08 (s, 1H, NH), 7.96 (d, $J_{13-15} = 2.2$ Hz, 1H, H-13), 7.81 (app. t, 1H, H-14), 7.75 (dd, $J_{8.7} = 8.6$ Hz, $J_{8.9} = 2.2$ Hz, 1H, H-8), 7.50 (d, $J_{7.8} = 8.6$ Hz, 1H, H-7), 5.50 – 5.47 (m, 1H), 5.43 – 5.38 (m, 2H, H-1), 5.31 (s, 2H, H-10), 5.27 (dd, $J = 9.3$ Hz, $J = 4.7$ Hz, 1H), 4.56 (t, $J_{16-17} = 5.9$ Hz, 2H, H-16), 4.37 (t, $J = 6.5$ Hz, 1H), 4.25 – 4.21 (m, 2H, H-6), 3.57 (t, $J_{17-16} = 5.9$ Hz, 2H, H-17), 3.06 (s, 6H, H-18), 2.20, 2.10, 2.06, 1.99 (4s, 12H, OAc).

$\delta$c (100 MHz, CD$_3$OD): 171.9, 171.4, 166.0, 165.8, 161.2, 155.3, 142.5, 139.5, 135.2 (C-12), 134.5 (C-8), 130.8 (C-9 or C-11), 128.7 (C-14), 125.9 (C-13), 125.5 (C-15), 123.2, 121.9 (C-9 or C-11), 119.8 (C-7), 101.0 (C-1), 72.6, 72.0, 69.6, 68.6, 66.28 (C-10), 62.6 (C-6), 57.7 (C-17), 44.3 (C-18), 36.6 (C-16), 20.6, 20.6, 20.4 (OAc).

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\( \nu_{\text{max}} \) (ATR)/cm\(^{-1}\): 2972 (arC-H), 1748 (C=O), 1666 (NO\(_2\)), 1629, 1601, 1536, 1511 (NH), 1468, 1431, 1370, 1231 (NCOO), 1178, 1131, 1071, 1044, 970, 896, 833, 799, 785, 748, 721, 675, 653, 598, 552.

HRMS (m/z – ESI\(^{-}\)): Found: 807.242529, ([M-H] \( \cdot \) C\(_{38}\)H\(_{39}\)N\(_4\)O\(_{16}\), Required: 807.236655).

1,3-bis-2-[2-(dimethylamino)ethyl]-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-5-yl]-urea (140) \(^{108}\)

Compound 140 was obtained as a side product when compound 136 was synthesised. The product was obtained as an orange solid (25 mg, 15%).

\( \delta^H \) (400 MHz, CD\(_3\)OD): 8.60 (d, \( J_{4.8} = 2.2 \) Hz, 2H, H-4), 8.45 (d, \( J_{8.4} = 2.2 \) Hz, 2H, H-8), 8.41 (dd, \( J_{5.6} = 7.3 \) Hz, \( J_{5.7} = 1.1 \) Hz, 2H, H-6), 8.21 (dd, \( J_{6.7} = 8.4 \) Hz, \( J_{7.5} = 1.1 \) Hz, 2H, H-7), 7.75 (dd, \( J_{6.5} = 8.4 \) Hz, \( J_{5.7} = 1.1 \) Hz, 2H, H-6), 4.40 (t, \( J_{3.2} = 6.5 \) Hz, 4H, H-3), 3.80 (s, 4H, H-2), 2.66 (s, 12H, H-1).

LRMS (m/z - ESI\(^{+}\)): Found: 689.2, ([M+3x(CH\(_3\)OH)+H]\(^{+}\). C\(_{34}\)H\(_{36}\)N\(_6\)O\(_6\), Required: 689.3).

\((2S,3S,4S,5R,6S)-6-(4-(((2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-5-yl)carbamoyl)oxy)methyl)-2-nitrophenoxy)-3,4,5-trihydroxy-tetrahydro-2H-pyran-2-carboxylic acid (141)

Amonafide (65 mg, 0.190 mmol, 1.00 equiv) and DMAP (69 mg, 0.56 mmol, 2.10 equiv) were dissolved in anhydrous CH\(_2\)Cl\(_2\) (20 mL) under a nitrogen atmosphere, at rt. The reaction mixture was cooled to 0 °C for 10 min before the addition of phosgene (1.7 mL, 1.69 mmol, 8.00 equiv). The reaction was stirred for 4 h at rt and N\(_2\) was bubbled through the reaction mixture for 1 h until phosgene was removed. Compound 131 (100 mg, 0.206 mmol, 1.1 equiv) was dissolved in anhydrous CH\(_2\)Cl\(_2\) (15 mL) under a nitrogen atmosphere, and added to the reaction mixture at 0 °C. The reaction was stirred for 18 h at rt and washed...
with 0.1 M HCl (20 mL) and brine (20 mL). The organic layer was dried over Na₂SO₄, filtered and the solvent removed *in vacuo*. The product was furnished as white wax (80 mg, 49%).

\[
\alpha \beta = 0.5 \text{ deg cm}^{-3} \text{ g}^{-1} \text{ dm}^{-1} (0.05, \text{MeOH})
\]

δH (400 MHz, MeOD): 8.76 (d, J₁₁-₁₂ = 2.2 Hz, 1H, H-11), 8.55 - 8.51 (m, 2H, H-9, H-15), 8.32 (dd, J₁₃-₁₄ = 8.4 Hz, J₁₃-₁₅ = 1.1 Hz, 1H, H-13), 7.99 (d, J₁₂-₁₁ = 2.2 Hz, 1H, H-12), 7.83 (app. t, 1H, H-14), 7.76 (dd, J₈-₇ = 8.6 Hz, J₈-₉ = 2.2 Hz, 1H, H-8), 7.53 (d, J₇-₈ = 8.6 Hz, 1H, H-7), 5.56 (d, J₁-₂ = 7.6 Hz, 1H, H-1), 5.46 (app. t, 1H), 5.32 (s, 2H, H-10), 5.31 - 5.23 (m, 2H), 4.60 - 4.54 (m, 3H, H-16), 3.76 (s, 3H, COOMe), 3.48 (app. t, 2H, H-17), 2.99 (s, 6H, H-18), 2.11, 2.07, 2.05 (3s, 9H, OAc).

δC (100 MHz, CD₃OD): 171.2, 170.9, 168.8, 149.9, 135.3 (C-12), 134.6 (C-13), 130.8 (C-9 or C-11), 128.7 (C-14), 125.6 (C-7), 124.1 (C-15), 123.2, 121.9 (C-9 or C-11), 119.9 (C-8), 100.4 (C-1), 73.2, 72.8, 71.8, 70.4, 66.3 (C-10), 57.7 (C-17), 53.4 (COOMe), 44.3 (C-18), 36.6 (C-16), 20.5, 20.5, 20.4.

νmax (ATR)/cm⁻¹: 2983 (arC-H), 1688 (C=O), 1380, 1208 (NCOO), 1075, 623.


3-nitro-4-(((2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl(2-(2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo-[de]isoquinolin-5-yl)carbamate (119)

Following general procedure D; Compound 136 (35 mg, 0.04 mmol, 1.00 equiv). Compound was obtained as a yellowish solid without further purification (27 mg, 98%).

Rr = 0.15 (MeOH).

M.p: 155 – 158 ºC

\[
\alpha \beta = 1 \text{ deg cm}^{-3} \text{ g}^{-1} \text{ dm}^{-1} (0.05, \text{MeOH})
\]

δH (600 MHz, DMSO): 10.50 (d, J = 16.3 Hz, 1H, NH), 8.61 (d, J₁₁-₁₂ = 2.2 Hz, 1H, H-11), 8.57 (d, J₁₅-₁₄ = 8.5 Hz, 1H, H-15), 8.41 - 8.36 (m, 2H, H-13, 12), 8.03 (dd, J = 11.4 Hz, J₉-₈ = 2.2 Hz, 1H, H-9), 7.83 (app t, 1H, H-14), 7.80 - 7.77 (m, 1H, H-7), 7.50 (d, J₈-₇
= 8.8 Hz, 1H, H-8), 6.54 (s, 1H, OH), 5.46 (d, J = 5.0 Hz, 1H), 5.32 – 5.20 (m, 2H, H-1, H-10), 4.24 (s, 2H), 3.37, 3.31, 3.27, 2.45 (6H, H-18).

δc (150 MHz, DMSO): 163.6, 163.3, 157.7, 157.5, 153.3, 149.0, 139.9, 138.0, 134.1, 133.6, 132.2, 130.2, 128.9, 127.7, 124.6, 123.8, 123.3, 122.8, 121.9, 119.6, 118.4, 117.1, 116.4, 99.9 (C-1), 76.0, 72.8, 71.3, 69.8, 64.8, 44.5 (C-18), 29.0.

νmax (ATR)/cm⁻¹: 568, 1239, 1347 (ar. C-C), 1656 (C=O), 2853, 2923, 3414 (OH).


(2S,3S,4S,5R,6S)-6-(4-(((2-(2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-5-yl)carbamoyl)oxy)methyl)-2-nitrophenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (120) Compound 141 (40 mg, 0.05 mmol, 1.00 equiv), was dissolved in NaOH (4 mL, 0.1 M) and let to stir at rt for 2 h or until TLC completion. The reaction was then quenched with DOWEX resin, filtered and solvent removed in vacuo. The compound was purified by C18 HPLC (33 mg, 99%).

M.p: 179 – 182 °C (decomposition)

[α]D³⁰ = 40 deg cm³ g⁻¹ dm⁻¹ (0.05, MeOH)

δH (600 MHz, DMSO): 10.50 (d, J = 16.3 Hz, 1H, NH), 8.61 – 8.55 (m, 1H), 8.36 (app. t, H-2), 8.01 (d, J = 2.2 Hz, 1H), 7.81 (app t, 1H, H-8), 7.77 – 7.75 (m, 1H), 7.50 (d, J = 8.8 Hz, 1H, H-7), 7.38 (bs, 1H), 5.24 (s, 2H, H-10), 5.10 (d,1H, H-1) 4.24 (s, 2H), 4.15 (app. t, 2H, H-16), 3.44 (d, J = Hz, 1H), 3.25 – 3.20 (m, 2H), 2.20 (s, 6H, H-18).

δc (150 MHz, DMSO): 134.7, 134.1, 129.2, 128.0 (C-14), 124.9, 123.7, 119.7, 117.7 (C-8), 101.0 (C-1), 77.35, 73.84, 73.4, 72.3, 65.3 (C-10), 38.0 (C-16), 46.1 (C-18)

νmax (ATR)/cm⁻¹: 1064, 1246, 1342 (ar. C-C), 1627 (C=O), 2922, 3398 (OH/NH₂).

4-(Hydroxymethyl)-2-nitrophenol (116) \(^{288}\)

Sodium borohydride (68 mg, 1.79 mmol, 3.00 equiv) were added to 4-hydroxy-3-nitrobenzaldehyde (100 mg, 0.60 mmol, 1 equiv) in MeOH (6 mL) at 0 °C. The mixture was stirred overnight. Solvent was removed \textit{in vacuo} and the crude material was redisolved in EtOAc and filtered through a plug of silica. Solvent removal yielded compound 116 (88 mg, 89%) as a yellow powder.

\textbf{Mp:} 90 - 92 °C, (lit. 96 – 97 °C)\(^{289}\)

\textbf{Rf} = 0.46 (50% EtOAc/Hex).

\(\delta H (\text{400 MHz, DMSO})\): 7.78 (dd, \(J_{1-2} = 2.2 \text{ Hz}, J = 1.0 \text{ Hz}, 1\text{H, H-1}\)), 7.43 (dd, \(J_{2-3} = 8.6 \text{ Hz}, J_{2-1} = 2.2 \text{ Hz}, 1\text{H, H-2}\)), 7.04 (d, \(J_{3-2} = 8.6 \text{ Hz}, 1\text{H, H-3}\)), 5.24 (s, 1H, OH)), 4.40 (s, 2H, H-4).

\textbf{HRMS} (\(m/z\) – ESI): Found: 168.02726, ([M-H]\(^{+}\). C\(_7\)H\(_6\)NO\(_4\), Required: 168.0302).

8.14 Experimental for Chapter 5

Bis-[N-(1-propargyl)-9,18-methano-1,8-naphthalimide- [b,f ][1,5]diazocine (148)

Compound 147 was synthesised reacting troger-base naphthalic anhydride (100 mg, 0.21 mmol, 1 equiv) with propargylamine (35 \(\mu\)L, 0.54 mmol, 2.50 equiv) in EtOH (10 mL) at 80 °C for 16 h. After this time, the reaction mixture was filtered through celite leaving an orange solid (108 mg, 94%).

\textbf{M.p:} 292 - 294 °C (decomposition)

\textbf{Rf} = 0.04 (25% MeOH/EtOAc).

\(\delta H (\text{600 MHz, DMSO-d}_6)\): 8.77 (d, \(J_{6-5} = 8.3 \text{ Hz, 2H, H-6}\)), 8.53 (d, \(J_{4-5} = 7.3 \text{ Hz, 2H, H-4}\)), 8.17 (s, 2H, H-3), 8.00 (app. t, 2H, H-5), 5.75 (s, 4H, H-2), 5.19 (dd, \(J_{7-7'} = 17.2 \text{ Hz, 7H, H-7}\)), 4.77 – 4.68 (m, 4H, H-8), 3.07 (t, \(J_{1-2} = 2.5 \text{ Hz, 2H, H-1}\)).

\(\delta C (\text{151 MHz, DMSO-d}_6)\): 162.5, 131.6 (C-3), 131.0 (C-4), 130.1 (C-6), 127.8 (C-5), 127.3, 122.6, 117.8, 79.81, 73.3, 57.2 (C-7), 29.4 (C-8).

\(\nu_{\text{max}}\) (ATR)/\text{cm}^{-1}: 1033, 1661 (C=O), 1340 (ar. C-C), 2935 (CCH)

\textbf{HRMS} (\(m/z\) - MALDI\(^{+}\)): Found: 537.1578, ([M+Na]\(^{+}\). C\(_{33}\)H\(_{54}\)N\(_{10}\)O\(_{16}\)Na, Required: 537.15557).
Bis-[N-(1-(3-(2',3',4',6'-tetra-O-acetylation-α-D-mannopyranosyloxo)propyl)-1H-
1''',2''',3'''-triazol-4'''-yl)methyl]-9,18-methano-1,8-naphthalimide-[b,f][1,5]diazocine
(149)

Following general procedure D; Compound 148 (28 mg, 0.05 mmol, 1.00 equiv), 55c (45 mg, 0.1 mmol, 2.00 equiv), [Cu(MeCN)₄](BF₄) (10 mg, 0.03 mmol, 0.30 equiv) and DMF (5 mL). Reaction carried out for 2 h.

The product was obtained as an orange wax (40 mg, 66%).

Rₘ = 0.5 (1:5 (v/v), MeOH/EtOAc).

δH (799.7 MHz, CDCl₃): 8.69 (bs, 2H, H-15), 8.62 (d, J = 11.4 Hz, 2H, H-13), 8.13 (s, 2H, H-12), 8.03 (s, 2H, H-10), 7.86 (s, 2H, H-14), 7.61 (s, 2H), 5.42 (bs, 3H), 5.27 – 5.24 (m, 4H, H-11), 5.21 (bs, 2H), 5.14 (app t, 3H, H-16), 4.76 (s, 2H, H-1), 4.66 – 4.64 (m, 2H), 4.61 – 4.56 (m, 3H), 4.43 – 4.39 (m, 2H), 4.38 – 4.34 (m, 2H), 4.35 – 4.20 (m, 3H), 4.05 (d, J = 12.6 Hz, 2H), 3.96 – 3.92 (s, 2H), 3.71 – 3.67 (s, 2H), 3.40 – 3.35 (s, 2H), 2.14, 2.06, 2.05, 1.99 (s, 24H, OCOCH₃), (2 × CH₂ buried under OCOCH₃).

δc (201.1 MHz, CDCl₃): 153.6, 133.5 (C-13), 129.8 (C-14), 100.4 (C-1), 71.9, 71.6 (C-11), 71.3, 69.6, 68.5 (C-11’), 67.3, 67.2, 65.0, 59.7 (C-16), 59.6, 39.2, 37.8, 34.1, 33.7, 33.6, 23.6, 23.5, 23.4, 23.3, 22.4 (7C, OCOCH₃).

νmax (ATR)/cm⁻¹: 789, 1051 (C-N), 1229 (C-N), 1373, 1597 (ar. C-C), 1659 (C=O), 1747, 2923.

Bis-[N-(1-(3’-(α-D-manosepyranosyloxy)propyl)-1H-1”’,2”’,3”’-triazol-4”-yl)methyl]-9,18-methano-1,8-naphthalimide-[b,f ][1,5]diazocine (45)

Following general procedure C; 149 (40 mg, 0.03 mmol, 1.00 equiv), MeOH (5 mL). The reaction was left on for 4 h. The product was obtained as an orange powder (28 mg, 98%).

M.p: 120.5 – 122.0 (decomposition).

Rf = 0 (1:5, v/v, % MeOH/EtOAc).

δH (799.99 MHz, TFA-d): 8.85 (bs, 2H, H-15), 8.78 (bs, 2H, H-13), 8.64 (bs, 2H, H-12), 8.40 (bs, 2H), 8.16 (bs, 2H, H-10), 5.72 (s, 2H, H-17), 5.67 (s, 4H, H-16), 5.62 (s, 1H, H-11), 5.19 – 5.16 (m, 2H, H-1), 5.06 (s, 3H), 5.02 (s, 2H), 4.77 (s, 4H), 4.37 – 4.20 (m, 6H), 4.11 (s, 2H), 4.00 (s, 2H), 3.95 (bs, 2H), 3.90 (bs, 2H), 2.34 (bs, 4H, H-8).

δC (200.1 MHz, TFA-d): 146.1 (C-13), 143.2 (C-10), 142.3 (C-14), 140.4 (C-15), 68.6, 44.6, 80.7, 63.6, 75.0, 76.3, 81.8, 42.4, 76.3, 41.15, 42.07, 40.1 (C-8).

νmax (ATR)/cm⁻¹: 980, 1100, 1490 (ar. C=C), 1750 (C=O), 3410 (OH/NH2).

Chapter 9

References
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5. Spiro, R. G. Glycobiology 2002, 12, 43R-56R.
23. Daly, R.; Vaz, G.; Davies, A. M.; Senge, M. O.; Scanlan, E. M. Chemistry 2012, 18, 14671-14679.
References

34. WC, B. Accademic Press 1954, 2, 756 - 844.
Chapter 9. References

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264. De Rosa, M.; Msb S.r.i., Italy; 2015.


289. Fluorochem, (October, 2018), 018723 - 4-Hydroxy-3-nitrobenzyl alcohol. [http://www.fluorochem.co.uk/Products/Product?code=018723], retrieved October 2018.
Appendix
Appendix

NMR spectra of novel compounds

**Compound 63a**

![NMR spectra of compound 63a](image)

**Figure A.1.** $^1$H NMR and $^{13}$C-NMR of compound 63a.
Figure A.2. $^1$H NMR and HSQC-NMR of compound 63b.
Appendix

Compound 63d

Figure A.3. $^1$H NMR and $^{13}$C NMR of compound 63b.
Appendix

Compound 44

Figure A.4. $^1$H NMR and $^{13}$C NMR of compound 44.
Figure A.5. $^1$H NMR and $^{13}$C NMR of compound 50.
Compound 63e

Figure A.6. $^1$H NMR and $^{13}$C NMR of compound 63e.
Appendix

Compound 64

Figure A.7. $^1$H NMR and $^{13}$C NMR of compound 64.
Figure A.8. $^1$H NMR and $^{13}$C NMR of compound 74.
Appendix

Compound 106

Figure A.9. $^1$H NMR and HSQC NMR of compound 106.
Figure A.10. $^1$H NMR and $^{13}$C NMR of compound 90.
Appendix

Compound 75

Figure A.11. $^1$H NMR and $^{13}$C NMR of compound 75.

270
Compound 148

Figure A.12. $^1$H NMR and $^{13}$C NMR of compound 148.
Appendix

Compound 149

Figure A.13. $^1$H NMR and HSQC NMR of compound 149.

272
Appendix

Figure A.14. $^1$H NMR and HSQC NMR of compound 75.
Appendix

Compound 136

Figure A.15. $^1$H NMR and $^{13}$C NMR of compound 136.
Figure A.16. $^1H$ NMR and $^{13}C$ NMR of compound 141.
Figure A.17. $^1$H NMR and $^{13}$C NMR of compound 119.
Compound 120

Figure A.18. $^1$H NMR and $^{13}$C NMR of compound 120.
Appendix

Appendix Chapter 2

Figure A.2.1. MS (ES') of compound 63a incubated with β-galactosidase (1 U) for 1 h at 30 °C, finding the mass for the released naphthalimide core, compound 74.

![MS (ES') Spectrum](image)

Figure A.2.1. Changes in the ε of compound 50 (a) and 74 (b), respectively, vs. DNA equivalents. Measurements were recorded at 37 °C.

![Graph A](image)

![Graph B](image)
Figure A.3.1. Z-stacks confocal images of HeLa cells incubated with compound 90 (50 μM) for 1 h in the presence of β-galactosidase (1 U), showing that the compound is distributed throughout the cell.
Appendix

Appendix Chapter 4

Figure A.4.1. $^1$H NMR (600 MHz) of compound 102 in D$_2$O.

Figure A.4.2. Absorption, fluorescence emission ($\lambda_{exc} = 347$ nm) and excitation spectra of Amonafide (1 x $10^{-3}$ M) recorded in PBS at 25 °C.
Figure A.4.3. Changes in the absorption (a and c) and fluorescence emission (b and d) spectra of compounds 136 ($1 \times 10^{-5}$ M, a and b) and 141 ($1 \times 10^{-5}$ M, c and d) plotted as $\lambda_{\text{max}}$ vs. pH, carried out in PBS at 25 °C in a back titration. Figure representative of two independent experiments.
Figure A.4.4. Fluorescence emission spectra inside HCT-116 (a and c) and HepG2 (b and d) cells incubated with 119 (50 μM) (a and b) and 120 (50 μM) (c and d), respectively, before and after the addition of the enzyme. Fluorescence spectra of Amonafide (50 μM, black line) is also shown for comparison. Images representative of three independent experiments.

Figure A.4.5. MS (ESI+) analysis of compound 119 (1 × 10^{-5} M) incubated with 1 U of β-galactosidase at 30 °C for 1 h. m/z = 284.1382 obtained for C_{16}H_{18}N_{3}O_{2}, corresponding to [Amonafide + H]^+. 
Figure A.4.6. MS (ESI+) analysis of compound 120 (1 × 10^{-5} M) incubated with 1 U of β-galactosidase at 30 °C for 1 h. m/z = 284.140043 obtained for C_{16}H_{18}N_{3}O_{5}, corresponding to [Amonafide + H]^+.
Figure 4.4.7. Confocal microscopy of HepG2 cells incubated with 119 (50 μM) for a) 24 h, b) 1 h and c) 1 h and the addition of β-galactosidase (1 U). Images representative of three independent experiments.
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**Figure 4.4.8.** Confocal microscopy of HCT-116 cells incubated with 119 (50 μM) for a) 24 h, b) 1 h and c) 1 h and the addition of β-galactosidase (1 U). Images representative of three independent experiments.
Figure 4.4.9. Confocal microscopy of HepG2 cells incubated with 120 (50 μM) for a) 24 h, b) 1 h and c) 1 h and the addition of β-galactosidase (1 U). Images representative of three independent experiments.
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**Figure A.4.10.** Confocal microscopy of HCT-116 cells incubated with 120 (50 μM) for a) 24 h, b) 1 h and c) 1 h and the addition of β-galactosidase (1 U). Images representative of three independent experiments.
Figure A.4.11. Some IC$_{50}$ graphs as representative examples.
Figure A.4.12. TPN images of HeLa cells incubated for 1 h with a) 119 (50 μM), b) 119 (50 μM) with β-galactosidase (1 U), c) 120 (50 μM) and d) 119 (50 μM) with β-galactosidase (1 U). λ<sub>exc</sub> = 750 nm. Image representative of three independent experiments.
Figure A.4.13. MS (APCI) analysis of compound 136, which was incubated in HeLa cells at 50 μM for 2 h and subsequently extracted.

Figure A.4.14. MS (APCI) analysis of compound 141, which was incubated in HeLa cells at 50 μM for 2 h and subsequently extracted.
Appendix Chapter 5

Figure A.5.1. $^1$H NMR (400 MHz) of compound 45 (Man-Tb) in a) D$_2$O and b) acetone-d$_6$, showing bad resolution and therefore TFA-d$_6$ was chosen as solvent.

Figure A.5.2. UV-vis spectra of a) Man-Nap ($1 \times 10^{-4}$ M, $\lambda_{\text{max}} = 430$ nm) and b) Man-Tb ($1 \times 10^{-4}$ M, $\lambda_{\text{max}} = 380$ nm) before (black lines) and after the addition of 0.1 equivalents of Con A (red lines), respectively, measured in DPBS (0.1 mM CaCl$_2$ and 0.1 mM MnCl$_2$) at 22 °C. Representative images of three independent experiments.
Appendix

Figure A.5.3. Fitting of three independent titrations of Man-Nap ($1 \times 10^{-5}$ M) with different concentrations of Con A into a Hill plot, giving a good fit for each independent titrations but showing no reproducibility.

Figure A.5.4. Z-stacks confocal images of compound 149 (50 uM) incubated in HeLa cells for 24 h, showing small aggregates located inside the cells (a-c) and large aggregates interacting with the cell membrane (d-f). Images representative of three independent experiments.