Fluorescent and Radionuclide labeling of a synthetic neuroactive glycoside

Nuno Miguel Rodrigues Martins

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Universidade de Lisboa
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Dissertação orientada pela Profª Doutora Isabel Rego dos Santos e Prof. Doutor Alfonso Fernández-Mayolaras Alvarez

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The work described in this thesis was performed in collaboration between two research groups. The first part was developed in the Bioorganic Chemistry Group, Instituto de Química Orgánica General (IQOG) del Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain, supervised by Prof. Dr. Alfonso Fernández-Mayoralas and Dr. Isabel García-Álvarez, under the LLP Erasmus Placement Program. The second part was carried out in the Radiopharmaceutical Sciences Group, Instituto Superior Técnico/Instituto Tecnológico e Nuclear, Sacavém, Portugal, under supervision of Prof. Dr. Isabel Rego dos Santos and Dr. Goreti Ribeiro Morais.

The fluorescent microscopy studies were performed by Dr. Ernesto Doncel-Pérez in the Hospital Nacional de Parapléjicos, Toledo, Spain.

‘Science is facts; just as houses are made of stone, so is science made of facts; but a pile of stones is not a house, and a collection of facts is not necessarily science.’

Jules Henri Poincaré (1854-1912) French mathematician
Acknowledgements

First and foremost, thanks to my family who has given me the opportunity to be a full time student, contributing for the development of my professional/academic skills and in the end turn into a better person.

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Abstract

Clinically, brain and spinal cord injuries are very serious. These injuries can result in permanent, transient sensory or motor defects resulting in serious individual consequences besides social and economic ones. Currently, it is estimated that 90 million people around the world suffer from spinal cord injury (SCI).

One of the main obstacles to central nervous system (CNS) trauma repair is the formation in the lesion area of a growth inhibitory cellular-molecular structure, called 'glial scar'. This inhibitory environment in the injured spinal cord blocks axon growth and precludes nerve repair.

We have previously reported that the new glucoside 3 is a potent inhibitor of glioma and melanoma cells growth. It inhibited human melanoma (A-375 cells) division with an ID$_{50}$ below the micromolar range (0.6 ± 0.3 μM). Thereafter, 3 was found as potential useful in the treatment of SCIs. Considering the potential application of glucoside 3 for SCI repair, there is a great interest to understand its biological behaviour. Aiming to such goal, we designed new 3 derivatives containing molecular probes (fluorophore/radionuclide) suitable for conventional molecular imaging techniques.

This work describes the synthesis, characterization and in vitro biological evaluation of a fluorescent label (NBD) 3 derivative. Primary cultures of astrocytes, from postnatal rats (day 0 -1), were treated with NBD-3 derivative at final concentrations of 3, 15 and 30 μM. Fluorescence microscopy images, obtained after 24 h of treatment, demonstrated the ability of NBD-3 derivative to permeate through the astrocyte cell membrane. Also, the number of astrocytes decreased as the concentration of the synthetic inhibitor increase, showing similar antimitotic activity as the original glucoside 3.

The establishment of a synthetic approach to prepare fluorinated-23 derivative is also reported. This approach will be adopted for the synthesis/radiosynthesis of a fluorine label ($^{19}$F/$^{18}$F) 3 derivative. We also plan to perform biodistribution studies with the $^{18}$F-3 derivative, in order to assess its in vivo tissue distribution/brain uptake.

Key words: Antimitotic, carbohydrate synthesis, fluorescence, SCI, radiosynthesis
Resumo

As lesões do cérebro e da espinal medula conduzem a uma condição clínica muito grave. Estas lesões são suscetíveis de causar defeitos sensoriais ou motores de curta duração ou permanentes, resultando em graves consequências individuais que se refletem tanto a nível social como económico. Atualmente, estima-se que 90 milhões de pessoas em todo o mundo são alvos de algum tipo de lesão da espinal medula (LEM).

Um dos principais entraves ao restabelecimento dos traumatismos associados ao sistema nervoso central (SNC) é a formação na área lesionada de uma estrutura molecular e celular que impede o crescimento, designada de “cicatriz glial”. Este ambiente inibitório no local lesionado da espinal medula bloqueia o crescimento dos axónios, opondo-se à reparação dos nervos.

Anteriormente, nós relatámos que o novo glucósido 3 é um inibidor potente do crescimento de células de glioma e melanoma. Tendo inibido a divisão de células de melanoma humano (A-375) com um $D_{50}$ abaixo da ordem dos micromoles ($0,6 \pm 0,3 \mu M$). Posteriormente, nós descobrimos que o 3 é virtualmente útil no tratamento da LEM. Relativamente a uma possível aplicação do glucósido 3 no tratamento da LEM será uma mais-valia compreender o seu comportamento biológico. Visando tal objetivo foi projetado derivados de 3 portadores de diferentes sondas moleculares (fluoróforo/radionuclídeo) apropriadas para técnicas de imagiologia molecular tradicionais.

O presente projeto descreve a síntese, caracterização e avaliação biológica in vitro do derivativo do 3 contendo a sonda fluorescente. As culturas primárias de astrocitos de ratos (0 – 1 dias pós-natal) foram tratadas com o derivativo do 3 fluorescente em diferentes concentrações finais (3, 15 e 30 μM). Obteve-se imagens por microscopia de fluorescência depois dum período de tratamento de 24 h, o que permitiu concluir que este derivado do 3 é passível de penetrar a membrana celular de astrocitos. Além disto, o número de astrocitos diminuiu à medida que a concentração do inibidor sintético aumentou, demonstrando uma atividade antimitótica semelhante ao glucósido original 3.

Também, é descrito uma via sintética com vista à obtenção do derivativo do 25 contendo flúor. A mesma estratégia de síntese será adotada para radiosíntese do derivativo do 3 contendo flúor ($^{19}F/^{18}F$). Visto isto, planeamos a realização de estudos de biodistribuição com o derivativo do 3 portador do radionuclídeo flúor-18, com o intuito de avaliar o seu comportamento in vivo e averiguar a sua capacidade de atravessar a barreira hematoencefálica (BHE), um requisito essencial para o tratamento da LEM.

Palavras-chave: Antimitótico, síntese de carbohidratos, fluorescência, LEM, radiosíntese
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Abbreviations

2-NBDG 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-α,β-D-glucose
AcOH acetic acid
AHA 6-amino hexanoic acid
atm atmospheres
B boat
BBB blood brain barrier
BF₃(EtO)₂ boron trifluoro diethyl etherate
b.p. boiling point
C chair
calcd. calculated
cm centimeter(s)
CNS central nervous system
conc. concentration
COSY correlation spectroscopy
CT computed tomography
d doublet
dd doublet of doublets
ddd doublet of doublets of doublets
DCC N,N'-dicyclohexylcarbodiimide
DCM dichloromethane
dil. dilute
DMAP N,N-dimethyl-4-aminopyridine
DNA deoxyribonucleic acid
δ chemical shift
EC electron capture
EDC 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ESI electrospray ionization
EtOAc ethyl acetate
EtOH ethanol
eq. equivalent(s)
ε extinction coefficient
[^18F]FDG fluorodeoxyglucose[^18F] or 2-deoxy-2-[^18F]-fluoro-α,β-D-glucose
FC Flash chromatography
FCS fetal calf serum
Φ quantum yield or quantum efficiency
g gram(s)
FRET Forster resonance energy transfer
Fuc fucose
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Gal galactose (or D-galactose)
GalNAc N-acetylgalactosamine (or N-acetyl-D-galactosamine or 2-acetamido-2-deoxy-D-galactose)
GCC gravity column chromatography
Glc glucose (or D-glucose)
GlcNAc N-acetylgulosamine (or N-acetyl-D-gulosamine or 2-acetamido-2-deoxy-D-glucose)
h hour(s)
H half-chair
Hz Hertz
HMBC heteronuclear multiple bond coherence (or correlation)
HPLC high pressure (or performance) liquid chromatography
HSQC heteronuclear single quantum coherence (or correlation)
MRI magnetic resonance imaging
HRMS high resolution mass spectrometry
in inch(es)
ID50 median effective dose
ITC intersystem crossing
J coupling constant
LiOH lithium hydroxide
λabs wavelength of maximum absorption
λem wavelength of maximum emission
M molar (mol.L⁻¹)
M⁺ molecular ion (+ vely charged)
M⁻ molecular ion (- vely charged)
MeOH methanol
m multiplet
mg milligram(s)
M+H⁺ protonated molecular ion
MI molecular imaging
min minute(s)
ml milliliter(s)
mmol millimol(es)
mol mol(es)
m.p. melting point
NBD 7-nitrobenzofurazan or 7-nitrobenz-2-oxa-1,3-diazone
NIR near-infrared
nm nanometer(s)
NMR nuclear magnetic resonance
NR non-radiative
Nuc nucleophile
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\( \eta \) yield

\( \mu \text{L} \) microliter(s)

\( \text{OI} \) optical imaging

\( \text{PET} \) positron emission tomography

\( \text{PG} \) protecting group

\( \text{Ph} \) phenyl

\( \text{ppm} \) parts per million

\( \text{py} \) pyridine

\( q \) quartet

\( \text{rt} \) room temperature

\( R_f \) retention factor

\( s \) singlet

\( S \) skew

\( S_0 \) singlet electronic ground state

\( S_1 \) first singlet excited state

\( \text{sat.} \) saturated

\( \text{SCI} \) spinal cord injury

\( \text{sec} \) second(s)

\( S_{n2} \) nucleophilic substitution

\( \text{SPECT} \) single photon emission computed tomography

\( t \) triplet

\( T_1 \) triplet excited state

\( \text{TBAF} \) tetra-\( n \)-butylammonium fluoride

\( \text{TFA} \) trifluoroacetamido

\( \text{TFP} \) tetrafluorophenol

\( \text{THF} \) tetrahydrofuran

\( \text{TMS} \) tetramethylsilane

\( \text{TLC} \) thin layer chromatography

\( \text{Ts} \) \( p \)-toluenesulfonyl (a.k.a. tosyl)

\( \tau \) excited state

\( \text{US} \) ultrasound

\( \text{UV} \) ultra-violet
Compound naming and numbering

The naming and numbering of the majority of compounds in this thesis is based on the IUPAC recommendations for carbohydrates. Thus, the main carbon backbone is numbered from either the anomic carbon, or in the case of C-glycosyl derivatives, numbered from the terminal end of the aglycone. For the purposes of numbering when presenting experimental data, all well-known groups are listed using common abbreviations, others (e.g. allyl), are numbered from their point of connection with the main carbon chain; priority is given to carbon–carbon bonded sidechains. Compounds incorporating the bicyclic system are named and numbered according to IUPAC recommendations for heterocyclic compounds (see below, as an example, the structure of 2-\([\text{N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino}]-2\text{-deoxy-}\alpha-D\text{-glucose}.\)

\[\text{\includegraphics{compound.png}}\]

Chapter 1. Introduction

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Chapter 1. Introduction

1.1. Glycans, one of the keys of life

Carbohydrates are indispensable to life on Earth. In their simplest form, they serve as a primary energy source for sustaining life (starch, glycogen, dextran) or as structural materials (cellulose, chitin, peptidoglycans). However, in general, carbohydrates do not as simple sugars but as complex molecular conjugates, denominated glycans. Glycans come in many shapes and size, from linear chains (polysaccharides and oligosaccharides) to highly branched molecules bristling with antennae-like arms. Although proteins and nucleic acids, namely DNA have traditionally attracted far more scientific attention, glycans are also key molecules to life. They are ubiquitous in nature, forming the intricate sugar coat that surrounds the cells of virtually every organism and occupying the space between these cells. As part of this so-called extracellular matrix localization, glycans, with their diverse chemical structures, play a crucial role in transmitting important biochemical signals into and between cells, serving as destination labels for some proteins and as mediators of specific cell-cell interactions and interactions between cells and the extracellular matrix (Figure 1.1). Altogether, these sugars guide the cellular communication that is essential for normal cells, tissue development and physiological functions. Specific carbohydrate containing molecules act in cell-cell recognition and adhesion, cell migration during development, blood clotting, immune response and wound healing, just to refer a few of their many roles. In most of these cases, the informational carbohydrate is covalently joined to a protein or a lipid to form a glycoconjugate (proteoglycans, glycoproteins and glycolipids), which is the biologically active molecule. Besides distinct glycoconjugates involved in such processes, it is also the macromolecular system, called ‘glycocalyx’, which is in the focus of glycobiological research.

A deeper knowledge on glycans and on their biological function can provide valuable tools to identify biomarkers for disease risk, disease severity and activity, and preclinical outcome, while also encouraging development of new approaches to diagnosis, prevention, and treatment of diseases or injuries. In this thesis we will design and synthesize new compounds bearing fluorescent or radioactice moieties to help on the in vitro and in vivo biological evaluation of a new glycoside-based drug with potential utility in the treatment of a serious type of central nervous system (CNS) trauma, the spinal cord injury (SCI).
Figure 1.1. Illustration of the complex molecular landscape of the cell surface and extracellular matrix: a great diversity of oligosaccharides with unique structures (represented as colored chain of spheres), components of a variety of glycoproteins or glycolipids on the outer surface of plasma membranes, regulate cell functions, for example interacting with high specificity and affinity with lectins, or viruses receptors, or bacterial toxins, etc, in the extracellular milieu [5].

1.2. Spinal cord injury (SCI)

The spinal cord is a long, thin, tubular bundle of nervous tissue and support cells that extends from the brain (Figure 1.2). The brain and spinal cord together make up the CNS. The spinal cord begins at the Occipital bone and extends down to the space between the first and second lumbar vertebrae; it does not extend the entire length of the vertebral column. It is around 45 cm (18 in) in men and around 43 cm (17 in) long in women. Also, the spinal cord has a varying width, ranging from 1.27 cm (0.5 in) thick in the cervical and lumbar regions to 0.64 cm (0.25 in) thick in the thoracic area. The enclosing bony vertebral column protects the relatively shorter spinal cord. The spinal cord functions primarily in the transmission of neural signals between the brain and the rest of the body but also contains neural circuits that can independently control numerous reflexes and central pattern generators. The spinal cord has three major functions. It serve as a:

  a) conduit for motor information, which travels down the spinal cord;
  b) conduit for sensory information, which travels up the spinal cord;
  c) center for coordinating certain reflexes [6].
Figure 1.2. Spinal cord in vertebral canal: (a) left lateral view showing the spinal cord segments and vertebral bodies/spines; (b) diagram of part of the spinal cord, with the anterior white matter cut away, and one set of segmental nerve roots [7,8].

A SCI usually begins with a sudden traumatic blow to the spine that fractures or dislocates vertebrae (Figure 1.3 (a)). The damage begins at the moment of injury when displace bone fragments, disc material, or ligaments bruise or tear into spinal cord tissue. Most injuries to the spinal cord do not completely sever it. Instead, an injury is more likely to cause fractures and compression of the vertebrae, which then crush and destroy axons - extensions of nerve cells that carry signals up and down the spinal cord between the brain and the rest of the body. An injury to the spinal cord can damage a few, many, or almost all of these axons. While some injuries will allow almost complete recovery, others will result in complete paralysis [9].

When the spinal cord is severely injured (Figure 1.3 (b)) the extent of the damage will vary significantly, depending on the level at which the injury occurred. In a scenario of:
a) Quadruplegia - damage in the spinal cord occurs in the cervical (neck) area may affect both upper and lower extremities, resulting in complete loss of function, sensation, mobility, as well as other effects of paralysis;
b) Paraplegia - damage in the spinal cord below the cervical area may result in paralysis to one’s legs, trunk, and pelvic area [6].
Brain and spinal cord injuries are a very serious condition and can result in permanent or transient sensory or motor defects \(^{[12,13]}\). Therefore, these injuries have serious individual, social and economic impact. Currently, it is estimated that 90 million people around the world suffer from some form of SCI. In Europe there are approximately 300,000 paraplegics, the average age of whom is 31, while in the United States alone 250,000 people suffer from SCIs (10,000 people each year) \(^{[14]}\). The medical and rehabilitation cost are about 200,000 € the first year and about 30,000 €/year afterwards.

Injury to the adult CNS often results in the transection of nerve fibres and damage to surrounding tissues (Figure 1.4). The distal ends of the severed axons form characteristic dystrophic growth cones that are exposed to the damaged glial environment. During the early phase of injury, myelin-associated inhibitors from intact oligodendrocytes and myelin debris can restrict axon regrowth. Recruitment of inflammatory cells and reactive astrocytes over time leads to the formation of a ‘glial scar’, often accompanied by a fluid-filled cyst. This scarring process is associated with the increased release of chondroitin sulphate proteoglycans, which can further limit regeneration. Altogether, these molecular inhibitors of the CNS glial environment present a hostile environment for axon repair \(^{[15]}\).
The 'glial scar', which is the formation in the lesion area of a growth inhibitory cellular-molecular structure, constitutes one of the main obstacles of CNS trauma repair. This inhibitory environment in the injured spinal cord blocks axon growth and precludes nerve repair \[^{16}\]. ‘Glial scar’ formation at the lesion site is associated to the transation of astrocyte from quiescent to reactive state, resulting in astrocyte proliferation before the axon growth. As a consequence, it is impossible for the axon regrowth through the damage area and restore of synapses.

In the brain exist high concentration of polipeptides with mitotic activity against astrocytes \[^{17}\], then these glial cells rarely divide except when a lesion occurs. It is possible that in normal circumstances the mitotic activity turns blocked by natural inhibitors and after a lesion, occurs a decay of the inhibitor quantity, which permits the mitogens express activity \[^{15}\].
1.3. Natural and synthetic inhibitors of neural cells proliferation

It was described the presence in brain extracts of inhibitors of astroblast and astrocytoma division \[16,18-21\]. The inhibitor had glycic epitopes immunologically related to those of the epidermal growth factor receptor and of blood groups A, H, or Lewis X \[22\]. Based on these observations, Fernández-Mayoralas’ research group in collaboration with Nieto-Sampedro’s group synthesized a family of oligosaccharides with Lewis X-type structure and tested their activity as inhibitors of normal and transformed neural cells division \[23\]. In Figure 1.5, is depicted the structure of one of those oligosaccharides: the tetrascarharide \(\alpha\)-D-GalNAc\(\{1,3\}\)-\(\beta\)-D-Gal\(\{1,4\}\)[\(\alpha\)-L-Fuc\(\{1,3\}\)]-D-GlcNAc \(\text{(1)}\), which inhibited the proliferation of rat C6 glioma cells in culture (ID\(_{50} = 106 \ \mu\text{M}\)) and the growth of brain tumors formed after intracerebral transplantation of C6 cells \[24\].

![Figure 1.5. Structure of the synthetic tetrascarharide \(\alpha\)-D-GalNAc\(\{1,3\}\)-\(\beta\)-D-Gal\(\{1,4\}\)[\(\alpha\)-L-Fuc\(\{1,3\}\)]-D-GlcNAc \(\text{(1)}\).](image_url)

The results obtained with the oligosaccharide \(\text{(1)}\) were the driven force for the synthesis of a second generation of differently substituted mono and disaccharides\[25,26\]. It was shown that an octyl \(N\)-acetylglucosaminide with a pentaerythritol chain at position 6 \(\text{(2)}\) (Figure 1.6) inhibited the growth of a neuroectodermic tumor implanted in rats. This monosaccharide inhibited the division of human glioma U-373 cells in culture, although with a modest ID\(_{50}\) value (43 ± 14 \(\mu\text{M}\))\[26\].

![Figure 1.6. Structure of the synthetic monosaccharide Octyl 2-amino-2-N-acetyl-2-deoxi-6-O-pentaerythritol-\(\alpha\)-D-glucopyranoside \(\text{(2)}\).](image_url)
Based on the results obtained with the monosaccharide 2, new derivatives with improved antimitotic activity were synthesized. These new derivatives were obtained by introducing substituents of different nature, polarity and size at the 1, 2, 3, and 6 positions of the glucosamine backbone. The inhibitory effect of these derivatives on the proliferation of rat (C6), human glioma (U-373) and human melanoma (A-375) cells was also evaluated.

The antimitotic activity of the new compounds on glioma cell lines increased up to 2 orders of magnitude in comparison to the previous monosaccharide 2 (Figure 1.6). Moreover, one of the new glycosides inhibited melanoma division with an ID₅₀ below the micromolar range. Thus, this new glycoside, an oleyl N-acetylglucosaminide with an O-sulphate group at position 6 (3) (Figure 1.7), is a potent inhibitor of glioma and melanoma cells growth [27].

![Structure of the synthetic monosaccharide](image)

**Figure 1.7.** Structure of the synthetic monosaccharide Oleoyl 2-amino-2-N-acetyl-2-deoxi-6-O-(oxosulfonyl)-α-D-glucopyranoside (3) and its median effective dose on inhibition of Rat Glioma (C6), Human Glioma (U-373) and Melanoma (A-375) Cell Cultures.

<table>
<thead>
<tr>
<th></th>
<th>Rat Glioma (C6)</th>
<th>Human Glioma (U-373)</th>
<th>Human Melanoma (A-375)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID₅₀/µM</td>
<td>13.0 ± 3.5</td>
<td>13.0 ± 3.0</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>

The mechanism of growth inhibition by the new compounds is still unknown. An alternative mode of action for the new glycosides to exert their effects could be by altering the biosynthesis of tumor-associated gangliosides [28]. This type of glycosphingolipid has been shown to have crucial regulatory roles in tumor onset and progression [29].

Thereafter, in collaboration with the National Paraplegics Hospital in Toledo Fernández-Mayoralas’s research group have found that glycoside 3 has potential utility in the treatment of SCIs [30].

Regarding a possible application of glycoside 3 for SCI repair, it is important to know if this compound is able to cross the blood brain barrier (BBB). This can be achieved by performing biodistribution studies to gain insights about its *in vivo* behaviour and usefulness for SCI repair. Aiming at this goal, molecular imaging techniques can be explored. Therefore, it will be of great interest to develop an imaging probe derived from 3. To achieve this goal, we decided to synthesize a glycoside 3 derivative bearing a functional group useful for molecular imaging. However, such modification must not modify significantly the original structure of 3.
1.4. Molecular imaging

Molecular imaging may be used for early detection, characterization, and ‘real time’ monitoring of disease as well as investigating the efficacy of drugs. Advances in biology and chemistry have enabled in vitro studies of some important molecular interactions such as genetic events occurring at sub-femtomolar levels. With amplification schemes such as enzymatic reactions, these events may be detectable and design of probes targeted to those biomarkers and their use for in vivo detection of certain events (molecular changes) occurring at picomolar and nanomolar levels. Recent advances in imaging techniques have allowed in vivo detection and quantification of physiologic and pathologic processes with increased molecular sensitivities from micromolar to picomolar level. Among all the molecular imaging modalities we can consider magnetic resonance imaging (MRI), computed tomography (CT), ultrasound (US), bioluminescence, optical imaging (OI) - fluorescence imaging and the nuclear techniques (single photon emission computed tomography (SPECT) and positron emission tomography (PET)) \(^{31,32}\).

1.4.1. SPECT and PET

Presently, there is a consensus among experts in the field that the most sensitive molecular imaging techniques are the radionuclide-based PET and SPECT imaging modalities. PET or SPECT has the sensitivity needed to visualize most interactions between physiological targets such as neurotransmitters and brain receptors, and the respective ligands radionuclide-based imaging modalities are able to visualize specific biomolecules in the picomolar range. However, it must be stressed here, that the choice of a certain imaging modality, MRI, US, OI or PET, depends primarily on the specific question to be addressed.

In recent years, the SPECT and PET technology has also been applied to assist in drug development, whereby understanding drug distribution, establishing dosage regimens of drugs and treatment strategies. Such studies, maybe in the future will provide means to accomplish ‘personalized medicine’ by monitoring individual response to drug delivery.

Several PET radionuclides exist for conjugation and/or incorporation into biomolecules (Table 1.1). However, most of the PET imaging probes applied in neurological research have been labeled either with \(^{11}C\) or \(^{18}F\) \(^{32}\).
Table 1.1. Physical Properties of Commonly Used Positron-Emitting Radionuclides [32].

<table>
<thead>
<tr>
<th>Nuclide</th>
<th>Half-life (min)</th>
<th>Maximum energy (MeV)</th>
<th>Mode of decay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{18}$F</td>
<td>110</td>
<td>0.64</td>
<td>$\beta^+ (97%)$ EC (3%)</td>
</tr>
<tr>
<td>$^{11}$C</td>
<td>20.3</td>
<td>0.97</td>
<td>$\beta^+ (99%)$</td>
</tr>
<tr>
<td>$^{13}$N</td>
<td>10</td>
<td>1.20</td>
<td>$\beta^+ (100%)$</td>
</tr>
<tr>
<td>$^{12}$O</td>
<td>2</td>
<td>1.74</td>
<td>$\beta^+ (100%)$</td>
</tr>
<tr>
<td>$^{76}$Br</td>
<td>972</td>
<td>4.0</td>
<td>$\beta^+ (57%)$ EC (43%)</td>
</tr>
<tr>
<td>$^{124}$I</td>
<td>60 192</td>
<td>2.14</td>
<td>$\beta^+ (25%)$ EC (75%)</td>
</tr>
<tr>
<td>$^{68}$Ga</td>
<td>68.1</td>
<td>1.90</td>
<td>$\beta^+ (89%)$ EC (11%)</td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>762</td>
<td>0.655</td>
<td>$\beta^+ (19%)$ EC (41%) $\beta^+ (40%)$</td>
</tr>
</tbody>
</table>

EC: electron capture

1.4.1.1. PET Methodology

PET imaging agents are radiolabeled with positron emitting radionuclides, which decay by the emission of a positively charged particle, the positron. After being emitted from the nucleus the positron travels a short distance in the surrounding matter or tissue before it annihilates with an electron (Figure 1.8). The distance travelled by the positron before annihilation is known as positron range. The energy of the emitted positrons determines the path range before annihilation and is different for each positron-emitting radionuclide (Table 1.1). The average distance travelled by positron before annihilation increases when the energy of the positron increases, but the special resolution decreases. Annihilation produces two 511 keV photons, which travelled in opposite directions and can be detected by gamma detectors [33].

![Figure 1.8. Principle of PET imaging: $^{18}$F in the sugar molecule, 2-$^{18}$F-fluoro-deoxy-D-glucose (4) ($^{[18]}$FFDG), decays by emitting a positron, a positively charged electron.](image)
1.4.1.2. Radionuclides for PET Imaging

Among the nuclear medicine imaging modalities, PET offers a specific advantage because it employs positron-emitting isotopes, most of them elements as carbon, nitrogen, and oxygen, which are main constituents of biological molecules. The use of this elements allows the syntheses of organic radiopharmaceuticals that are chemically indistinguishable from their nonradioactive counterparts. Apart from a negligible isotopic effect, the radiolabeled pharmaceutical possesses the same physicochemical and biochemical properties as the nonlabeled compound. While the replacement of a carbon-12 or nitrogen-14 atom by carbon-11 or nitrogen-13, respectively, is an isotopic substitution, the radiolabeling with fluorine-18 is not. Although, for biomedical applications fluorine atom is not a constituent of biomolecules, is considered an hydrogen bioisostere and therefore can replace hydrogen atom or hydroxyl group without compromising the desired biological activity.

Of all the positron emitters used in PET, fluorine-18 shows the most favourable physical properties (low positron energy, optimal physical half-life (Table 1.1)). Its half-life of 110 min allows the radiosynthesis of the compounds, longer in vivo investigations, and, most importantly, ‘satellite’ and commercial distribution to clinical PET centers that lack radiochemistry facilities. A prominent representative example of fluorine-18 labeled PET imaging probes synthesized by nonisotopic substitution is 2-[\(^{18}\text{F}\)]fluoro-deoxy-D-glucose (4) ([\(^{18}\text{F}\)]FDG) (Figure 1.8), for studying glucose metabolism \[^{34,35}\]. [\(^{18}\text{F}\)]FDG is the best clinically known and the most successful commercial PET radiopharmaceutical. All experts in the field agree that today there would be no clinical PET imaging without [\(^{18}\text{F}\)]FDG (4) \[^{32}\].

1.4.2. Optical imaging

One of the fundamental reasons to use optical imaging (OI) in biomedical research is the wealth of contrast mechanisms that can be offered when exploiting the physical properties of light (i.e., polarization, interference, etc.), the ability to capitalize on a wide range of light-tissue interactions and corresponding photophysical and photochemical mechanisms and processes at the molecular level (i.e., multiphoton absorption, second-harmonic generation, fluorescence, etc.) \[^{31,36}\].

In recent years, fluorescence microscopy and imaging have received particular attention. This is due to the increasing availability of fluorescent proteins, dyes, and probes that enable the noninvasive study of gene expression, protein function, protein-protein interactions, and a large number of cellular processes. In parallel, there is an increasing list of fluorescent imaging techniques that offer microscopic resolutions and video-rate scans, or methods that operate at resolutions beyond the diffraction limit and offer single-molecule sensitivity, yielding unprecedented insights
into biology. On the opposite side of the resolution range, macroscopic fluorescence imaging is gaining momentum as a molecular imaging method for small-animal whole-body tissue interrogations. It has been long known that light can propagate through several centimeters of tissue in the far-red and near-infrared (NIR) \(^{[37,38]}\). However, light becomes diffuse within a few millimeters of propagation in tissues owing to elastic scattering experienced by photons when they interact with various cellular components, such as the membranes and different organelles. Diffusion results in the loss of imaging resolution. Therefore, macroscopic fluorescence imaging largely depends on spatially resolving and quantifying bulk signals from specific fluorescent entities reporting on cellular and molecular activity\(^{[36]}\).

Small fluorescent molecules are indispensable tools for chemical biology, being ubiquitous as biomolecular labels, enzyme substrates, environmental indicators, and cellular staining agents \(^{[39]}\). Choosing a suitable probe to visualize a biochemical or biological process can be daunting, given the countless molecules available either commercially \(^{[40]}\) or through de novo design and synthesis. Fortunately, the plethora of fluorescent probes has an inherent modularity. Attachment of various reactive groups, substrate moieties, chelating components, and other chemical entities to a small number of ‘core’ fluorophores gives rise to the ensemble of extant probes. Overall, these core fluorophores are well-established \(^{[41,42]}\) consisting of molecules with excellent spectral characteristics, high photo- and chemical stabilities, as well as facile syntheses. Probe selection and design can, therefore, be simplified by understanding the properties of these foundational fluorescent compounds.

1.4.2.1. Principles of fluorescence

The process of fluorescence is illustrated in the Jablonski diagram shown in Figure 1.9 (a) \(^{[43]}\). We are more focused on single-photon excitation processes than multiphoton excitation. The fluorescence process begins when a molecule in a singlet electronic ground state (S\(_0\)) absorbs a photon of suitable energy. This promotes an electron to higher energy orbitals, which relaxes quickly to the first singlet excited state (S\(_1\)). The decay of the excited state can occur with photon emission (i.e., fluorescence) or in a non-radiative (NR) fashion. This non-radiative ‘quenching’ of the fluorophore excited state can occur through one of a variety of processes, including bond rotation or vibration, molecular collision \(^{[44]}\), and photoinduced electron transfer (PeT) \(^{[45]}\). The excited state can also undergo forbidden intersystem crossing (ITC) to the triplet excited state (T\(_1\)) and subsequent relaxation either by photon emission (i.e., phosphorescence) or NR decay. ITC efficiency is increased by substitution with, or proximity to, atoms with high atomic number due to spin-orbit coupling—a phenomenon commonly termed the ‘heavy atom effect’ \(^{[46]}\). Another important pathway for decay of the singlet excited state involves Forster resonance energy
transfer (FRET) to an acceptor molecule. This process is distance dependent, and can be used as a ‘spectroscopic ruler’ to measure the proximity of labeled entities\textsuperscript{[47]}. 

1.4.2.2. Fluorophore properties

There are several attributes that are critical to evaluate a fluorophore. The maximal absorption ($\lambda_{\text{max}}$) is related to the energy difference between the $S_0$ and the higher energy levels. The absorptivity of a molecule at $\lambda_{\text{max}}$ is given by the extinction coefficient ($\varepsilon$), defined by the Beer-Lambert-Bouguer law. The maximal emission wavelength ($\lambda_{\text{em}}$) is longer (i.e., lower in energy) than $\lambda_{\text{max}}$ due to energy losses by solvent reorganization or other processes\textsuperscript{[48,49]}. The difference between $\lambda_{\text{max}}$ and $\lambda_{\text{emin}}$ term is therefore termed the ‘Stokes shift’ (Figure 1.9 (b)). Fluorophores with small Stokes shifts are susceptible to self-quenching via energy transfer, therefore limiting the number of labels that can be attached to a biomolecule. The lifetime of the excited state ($\tau$) can range from 0.1 to $> 100$ ns, and is an important parameter for time-resolved measurements and fluorescence polarization applications. Another critical property of a fluorophore is the quantum yield or quantum efficiency ($\Phi$)-essentially the ratio of photons fluoresced to those absorbed.

The product of the extinction coefficient and the quantum yield ($\varepsilon \times \Phi$) is a highly useful parameter for comparing different fluorescent molecules. This term is directly proportional to the brightness of the dye, accounting for both the amount of light absorbed and the quantum efficiency of the fluorophore. Accurate comparisons between dye molecules must include both these parameters. Figure 1.10 plots the major classes of biologically significant fluorescent dyes as $\varepsilon \times \Phi$ against $\lambda_{\text{max}}$\textsuperscript{[50]}. 

![Jablonski Energy Diagram](image)  

\textbf{Figure 1.9.} (a) Jablonski energy diagram. (b) NBD aminoheptanoic acid (in methanol) absorption and emission spectra [51,52].
A notable example of a small heterocyclic fluorophore is 7-nitrobenz-2-oxa-1,3-diazole (NBD) and other related benzoxadiazole compounds. Examples include the amine- or thiol-reactive NBD-Cl \(^{53}\) and the thiol-reactive 7-chlorobenz-2-oxa-1,3-diazole-4-sulfonate (SBD-Cl) \(^{54}\). Primary amine adducts of NBD-Cl (e.g., fluorophore 14 of Figure 1.10) exhibit photophysical properties that belie the size of the molecule. Such derivatives emit in the green portion of the electromagnetic spectrum, with a \(\lambda_{\text{max}} = 465\) nm, \(\lambda_{\text{em}} = 535\) nm, \(\varepsilon = 2.2 \times 10^4\) M\(^{-1}\)cm\(^{-1}\), and \(\Phi = 0.3\) in MeOH \(^{40}\). This lightweight fluorophore allows conjugates with small molecules, such as sugars, to retain biological relevance \(^{55}\). The environmentally-sensitive fluorescence of NBD derivatives can be exploited in a variety of ways \(^{56}\).

The strong fluorescence of NBD, which bears an aminic nitrogen atom in the 7-position, has contributed to the wide use of such compounds as biological test reagents \(^{57,40}\). In the last decade, 2-[\(N\)-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-\(\alpha,\beta\)-D-glucose (2-NBDG) \((5)\) (Figure 1.11) \(^{58,59}\), a fluorescent analogue of D-glucose, has been successfully applied to a wide variety of cells and organs, leading to new findings such as metabolic communication between astrocytes in the brain \(^{60-63}\).
1.5. Research aims

Considering the antimitotic activity of the glycoside 3 and its potential usefulness in the SCI treatment we decided to evaluate its behaviour \textit{in vitro} and \textit{in vivo}. To follow the track of the compounds we decided to use:

a) Fluorescence microscopy and;

b) \textit{in vivo} animal studies.

For this purpose we decided to design, synthesise and characterize new 3 derivatives. Such derivatives would bear either a fluorescent label (Figure 1.12 (6)) or a radionuclide (Figure 1.12 (7)), for fluorescent microscopy and \textit{in vivo} animal studies, respectively.

![Lead compound](image)

\textbf{Figure 1.12}. Glycoside 3 and new 3 derivatives: NBD-C$_6$-3 (6) to use as probe in fluorescence microscopy; [F$^{18}$]-3 (7) to use as probe for biodistribution studies.
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Chapter 2. Results and Discussion

2.1. Synthesis of new 3 derivatives

We envisioned that it would be possible to start from α,β-D-glucosamine and further step by step functionalization of the pyranoid ring synthesize the NBD-C₆-3 (6) and [¹⁸F]-3 (7).

As it is shown in Figure 2.1, our retrosynthetic plan was to obtain the target molecule oleyl 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-6-O-oxosulfonyl-α-D-glucopyranoside, (NBD-C₆-3, compound 6) by amidation of a fluorophore linker acid 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid, NBD-C₅-COOH (8), with the amino group of compound 9, obtained by hydrolysis of the trifluoroacetyl group of precursor 10, which is made from a sulfation reaction of the corresponding glucoside 11. This one is fashioned via Fisher glycolisation from hemiacetal 12, which is obtained by N-trifluoroacylation of α,β-D-glucosamine (13).

![Figure 2.1. Retrosynthetic analysis of NBD-C₆-3 (6).](image)

Concerning the radionuclide-labeled 3 derivative (Figure 2.2), oleyl-2-deoxi-2-N-[¹⁸F]fluoroacetamamido-6-O-oxosulfonyl-α-D-glucopyranoside, [¹⁸F]-3 (7), is obtained in a few steps from common intermediate amine 9 via N-acylation with O-tosyl glycolic unit (14) followed by fluorination reaction. The previous synthetic steps are similar to the ones described for 6.
In order to produce the desired 3 derivatives, 6 and 7, in both synthetic routes a \textit{gluco}-configured starting material with an amine group (C2) was selected between the D-aldoses series. The, 2-amino-2-deoxy-\(\alpha\),\(\beta\)-D-glucopyranose (13) seemed to be the best candidate to start to working. Moreover, 13 is a commercially non-expensive available product among glycosides.

Glucopyranose 13 is a discrete stereoisomer like all free sugars (hydroxyl group in C1). On dissolution in water, the hemiacetal ring reversible opens and reforms, resulting in a monosaccharide-specific equilibrium of \(\alpha\)-pyranose, \(\beta\)-pyranose, \(\alpha\)-furanose, \(\beta\)-furanose and acyclic (open chain) forms. This process is called mutarotation, referring to changes in optical rotation to an equilibrium value when pure anomic forms of monosaccharides are dissolved in water. Although the equilibration is slow at neutral pH, it can be acid or base-catalyzed. Thermodynamic stability of the different forms is of importance for the final molar ratio in a monosaccharide. In general, the acyclic forms are only present in trace amounts, as an example, in Figure 2.3 we show this equilibrium for D-glucose.
Actually, pyranose ring structures can occur in two chair (C), six boat (B), six skew (S), and twelve half-chair (H) conformations (Figure 7.1 in Chapter 7. Appendix). However, due to energy reasons chair conformations (C) strongly dominate. The B, S, and H conformations mainly occur in the presence of double bonds or specific substituents, or in intermediate situations. So, the conformational shape of a pyranose is mainly governed by the relative stability of the two possible chair conformations which are both free of torsional strain, but one of which, in most cases, is clearly energetically unfavoured because of van der Waals interactions of the ring substituents. As is illustrated in Figure 2.4, for 2-amino-2-deoxy-β-D-glucopyranose, transformation of a Haworth representation leads theoretically to two chair conformations, called $^4C_1$ (D) and $^1C_4$(D), with opposing orientations of the substituents (equatorial vs. axial). However, the $^1C_4$ conformation of β-isomer 13 is clearly unfavoured compared to its $^4C_1$ conformation because the van der Waals repulsion of the 1,3 and 2,4-diaxially positioned ring substituents.

Thus, only the $^4C_1$ chair conformation is of importance. It is energetically the most favored chair with the fewest nonbonded interactions, and all OH and NH$_2$ groups and the CH$_2$OH group (driving force) in equatorial position. This conformation is also the relevant one for 2-amino-2-deoxy-α-D-glucopyranose. So, all glucosides we worked on are represented in their cyclic form as a glucopyranoid ring and $^4C_1$ chair conformation.
**Figure 2.4.** Haworth representation of the two isomeric chair conformations of 2-amino-2-deoxy-β-D-glucopyranose, $^{1}C_{4}$ and $^{4}C_{1}$, where the letter C stands for ‘chair’ and the numbers indicate the carbon atoms located above or below the reference plane of the chair, made up by C-2, C-3, C-5 and the ring oxygen.

In addition to intramolecular van der Waals interactions, carbohydrate conformations are determined by some other factors, such as electrostatic interactions as well as intramolecular hydrogen bond formation and especially the anomeric effect (discussed below).

### 2.1.1. Synthesis of fluorescent-labeled 3 derivative

With the purpose to perform in vitro fluorescence microscopy studies of 3, we design the fluorescent-3 derivative (6) (Figure 1.12 and 2.1). We defined one approach towards synthesizing the fluorophore moiety with a linker to further anchor to the sugar backbone via amine group at C-2. So it was selected a 4-nitrobenzofurazan or 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) specie as a fluorescent moiety among the well known small fluorophore variety. Due the strong fluorescence of NBD having an aminic nitrogen atom in the 7-position has contributed to the wide use of such compounds as biological test reagents $^{[40,57]}$. An excellent example of a sugar containing this small fluorophore is 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG )[5] (Figure 1.11), a fluorescent analogue of D-glucose, which has been successfully applied to study a wide variety of cells and organs, leading to new findings such as metabolic communication between astrocytes in the brain $^{[61-63]}$. 
To produce the fluorescent-labeled 3 (6) we must have in mind that the pharmacodynamic and pharmacokinetic properties of 3 should not be altered in a large extend. Taking that into account we opted to not couple the NBD group directly to the sugar ring, like in 2-NBDG (5), but to couple the NBD via a six member hydrocarbon chain spacer.

2.1.1.1. N-acylation (step a)

![Diagram](image)

**Figure 2.5.** Synthesis to obtain (12), step a.

Compound 13 was converted to the N-trifluoroacetyl derivative 12 through a chemospecific acylation in a good yield (73 %). The reaction was carried out in dried MeONa-MeOH to form the free amine from its hydrochloride derivative. By using 1.5 eq. of S-ethyltrifluoroacetyl (SETFA) as the trifluoroacetyl source, an N-acyl substitution occurred. A nucleophilic attack of the amino group of the sugar to the carbonyl group of SETFA takes place resulting an ethyl thiol anion as leaving group.

This reaction was monitored by thin layer chromatography (TLC), in 2:1 EtOAc-MeOH, to ensure the complete consumption of the starting material 13 ($R_f = 0.00$) and the formation of the desired products 12 ($R_f = 0.67$ and 0.56) which gave two spots corresponding to α- and β-isomers, in different ratios (Figure 2.6).

After workup of the reaction mixture, the $^1$H NMR spectrum of 12 showed two doublets at 5.15 ppm ($J_{1,2} = 2.9$ Hz) and 4.69 ppm ($J_{1,2} = 8.2$ Hz) in a ratio of 7:3, assigned to the anomic protons, H-1α,β, which appeared downfield shifted with respect to the anomic H-1α,β of the starting material 13, like protons H-2 and H-3. A $J_{1,2}$ value of 2.9 Hz indicates that the dihedral angle between H-1 and H-2 is small thus implying that the anomic hydroxyl group occupies the axial position, corresponding the 5.15 ppm doublet signal to the α-isomer 13. On the other hand, β-isomer 13 with a $J_{1,2}$ value of 8.2 Hz indicated an anomeric group equatorially oriented. $^{13}$C NMR spectrum also showed the presence of C=O and CF$_3$ groups at 159.2 and 119.4 ppm, respectively.
The final reducing saccharide obtained seems to be an anomic glucopyranose mixture with an equilibrium more favorable to α than β-pyranose anomer (7:3). The anomerically bound group (C1-OH) do not follow the same rule as others substituents of a carbohydrate ring. Equatorially positioned substituents, which are, for steric reasons, the most energetically favoured, compared to their axial counterparts, as in every molecule with a chair conformation. The α-configured derivative with the anomic group located in an axial position seemed to be more stable than β isomer, in opposite with what would be predicted from the steric interactions they have with adjacent substituents. The preference of the anomic OH group for the axial position is called the anomic effect, a term introduced by Lemieux in the 1960s [64]. It increases when the electronegativity of the substituent at the anomic center increases, and it decreases going to solvents with a high dielectric constant. The anomic effect can be explained in several ways. However two main explanations have been offered:

a) an explanation based on favorable/unfavorable dipole–dipole interactions between the ring O atom and the anomic OH group (substituent);

b) an explanation based on favorable/unfavorable orbital–orbital interactions between the ring O atom and the anomic OH group (substituent).

As nowadays the second theory (molecular orbital theory) is the most accepted one, this stereoelectronic explanation is depicted in Figure 2.7 for 12. A parallel overlap of the axial molecular orbital with a lone pair of electrons on O5 and the ‘back lobe’ of the σ molecular orbital of the C1–O1 bond (σ* antibonding molecular orbital) enables hyperconjugative delocalization of the electron pair, resulting in a stabilization of the α-pyranose anomer over the β-pyranose anomer.
Figure 2.7. (a) Anomeric effect; favorable/unfavorable orbital–orbital interactions between the ring O atom and the anemic OH group in the α- and β-anomeric forms of 12 in the $^4C_1$ conformation. (b) Newman projection C1-O5 of 12 showing the favored anomer (α) due to $n_{O5} \rightarrow \sigma^*$ delocalization of nonbonding electrons which is possible with an anti-periplanar arrangement of the involved orbitals. A significant effect is obtained when the $\sigma_{C1\alpha1}^*$ orbital is of low energy [64].

This favourable $n_{O5} \rightarrow \sigma^*$ delocalization of nonbonding electrons (‘negative hyperconjugation’) is only possible when the C1-O1 bond is axial, as in α-glycoside. In β-glycoside the non-bonding electrons of the endocyclic oxygen atom are delocalized into the antibonding C1-H1 orbital, which is of higher energy than the C1-O5 $\sigma^*$ orbital. The overlap of the lone pair is therefore more efficient in the α-anomer than in the β-anomer. The $n_{O5} \rightarrow \sigma^*$ delocalization of electrons is also reflected by bond length change, slightly shortening the O5-C1 bond while lengthening the C1-O1 bond [3].

The anomeric effect is the result of many factors. It can also be influenced by the substituent at C-2 (‘neighbor group effect’) due to steric reasons, or dipole-dipole interactions, or H bond formation.
2.1.1.2. O-glycosylation (step b)

Figure 2.8. Synthesis to obtain 11, step b, b’ and b”.

Due to the special reactivity of the anomic position (C-1) of glucopyranose 12, and carbohydrates in general, we decided to focus first on this position before modification of other hydroxyl group of the pyranoid ring. For formation of an oleyl glucoside we used a modern Fisher glycosylation \[65\]. As Figure 2.9 illustrates Fisher glycosylation involved protonation of the free sugar 12 (cyclic hemiacetal) by H$_2$SO$_4$-silica 23%, followed by its reaction with oleyl alcohol to form the corresponding glucoside (cyclic acetal). Protonation of the anomic hydroxyl group initiates departure of water from the saccharide leading to a cationic intermediate, a resonance-stabilized oxocarbenium ion. This species eventually reacts with an oleyl alcohol molecule giving the corresponding glucoside or reacts with water leading the starting material.
However the water formed as the by-product of this type of reaction causes hydrolysis of the formed acetal under the acidic conditions applied and the equilibrium is shifted towards the desired product, when an excess of alcohol is used, under an inert atmosphere and at very high temperature (180 °C). This is possible because oleic alcohol besides reactant served as solvent too. Performing this type of reaction under these conditions is an advantage in comparison with classic Fisher glycosylation. In fact, in Fisher glycosylation acid promoter in solid phase is not used and it is usually limited to the use of low-boiling alcohols, such as methanol, ethanol and short-chain alkyl alcohols.

Fisher glycosylation yields a mixture of stereoisomeric glycosides. In theory, pyrano- and furanosides can be formed, which are in equilibrium via open-chain intermediates. The formation of furanosides is fast, however, under thermodynamic control these will isomerizes to give the more stable α- and β-pyranosides (Figure 2.10).
To ensure the formation of the desired product 11 the reaction was monitored by TLC using EtOAc as eluent (Figure 2.11), which showed the presence of new spots with higher R	extsubscript{f} values, due to the replacement of the -OH at C-1 with an alkyl chain group. After 2 min of reaction, the major product formed was the β-pyranoside 11’ (R	extsubscript{f} = 0.24), the kinetic product. Thereafter more 13 minutes the starting material 12 (R	extsubscript{f} = 0.10) was fully consumed and two more isomers appeared, α-pyranoside 11 (R	extsubscript{f} = 0.48) and 1,2 glycosyl orthoamide 11” (R	extsubscript{f} = 0.52) as by-product. Compound 11 ended to be the major compound, thus the most stable thermodynamic isomer.

![TLC (EtOAc) of O-glycosylation reaction of 12.](image)

The structure of 11 was confirmed by its 	extsuperscript{1}H NMR, which showed the presence of protons from the alkyl chain group at 5.35, 3.71, 3.40 and 2.00 – 0.90 ppm. It also indicated a doublet at 4.91 ppm (J	extsubscript{1,2} = 3.5 Hz) from anomic proton, H-1, which is shifted downfield with respect to the H-1 of the starting material 12.

Characterization of the compound 11” by 	extsuperscript{1}H NMR showed at 5.12 ppm a doublet with a J	extsubscript{1,2} value of 5.0 Hz that indicates a larger dihedral angle between H-1 and H-2 comparing to its isomer 11, however this kind of J	extsubscript{1,2} value implying that the exocyclic oxygen occupies the axial position at C-1. These coupling constant changes indicate a flattening of the pyranose ring near C-1 and C-2 due to the bicyclic ring-fused structure of the 1,2-orthoamido derivative. Additional support for this comes from the signals multiplicity of corresponding protons H-2 and H-3 of this 1,2-glycosyl orthoamide 11” which differs from α- and β-glucopyranosides, 11 and 11’ respectively. Double doublets observed in glucopyranoside are now triplets for the orthoamide derivative, also resulting in a changing of dihedral angle between H-2 and H-3 (Figure 2.12).
Modern Fisher glycosylation afforded 11 in the disappointing yield of 23%. This is the major disadvantage of this method, the resulting poor yields obtained. Nevertheless, a stereoselective glycosylation was achieved, to obtain the α-pyranoside 11, which is favored by the anomeric effect. In Fisher glycosylation is normally obtained the α-pyranoside as the main product of this thermodynamically controlled reaction. Nevertheless, in alkyl glycopyranosides the anomeric effect operates not only along the endocyclic C-1 oxygen bond but also along the exocyclic C-1 oxygen bond. The anomeric effect leading to preferred conformations of the exocyclic alkoxy group is called ‘exo-anomeric effect’. Again it is an anti-periplanar arrangement of a lone pair on the aglycon oxygen and the C1-O5 bond which determines the favored conformation. In axially configured acetals the exo-anomeric effect is less important because it operates in the opposite direction than the ‘endo-anomeric effect’.[3]

In Figure 2.13 (b), three Newman projections are presented for the situation around the anomeric C atom in oleoyl 2-deoxy-2-N-trifluoroacetyl-β-D-glucopyranoside (11′), whereby C1–O_{aglycone} is the axis in the projection from C1 to O_{aglycone}. In the situations (i) and (ii), stabilizing interactions occur between one molecular orbital with a lone pair of electrons on the O_{aglycone} atom and the ‘back lobe’ of the molecular orbital associated with the s-bond between C1 and O5 (σ* antibonding molecular orbital). Therefore, situation (iii) is energetically not favorable. Situation (i) is preferred.
over situation (ii), because in situation (i) lower steric interactions exist between the alkoxy chain group and the substituents at C1 ($\phi \approx -60^\circ$C, O5–C1–Oglycone–Cglycone, IUPAC/IUBMB nomenclature; $\phi \approx +60^\circ$C, H1–C1–Oglycone–Cglycone, non-IUPAC/IUBMB nomenclature). The parallel overlap results in a shortening of the exocyclic C1–O5 bond and a shortening of the endocyclic C1–Oglycone bond. Looking at the three Newman projections for the situation around the anomic C atom in oleoyl 2-deoxy-2-N-trifluoroacetyl-\(\alpha\)-D-glucopyranoside (11) (Figure 2.13 (a)), in the situations (ii) and (iii) stabilizing interactions occur between one molecular orbital with a lone pair of electrons on the Oglycone atom and the ‘back lobe’ of the molecular orbital associated with the s-bond between C1 and O5 ($\sigma^*$ antibonding molecular orbital). Therefore, situation (i) is energetically not favorable. Situation (iii) is preferred over situation (ii), because in situation (iii) lower steric interactions exist between the alkoxy chain group and the substituents at C1 ($\phi \approx +60^\circ$C, O5–C1–Oglycone–Cglycone, IUPAC/IUBMB nomenclature; $\phi \approx -60^\circ$C, H1–C1–Oglycone–Cglycone, non-IUPAC/IUBMB nomenclature). The parallel overlap results in a shortening of the exocyclic C1–O5 bond and a shortening of the endocyclic C1–Oglycone bond.

**Figure 2.13.** Exo-anomeric effect; favorable/unfavorable orbital–orbital interactions around the glycosidic linkage in: (a) \(\alpha\)- and (b) \(\beta\)-anomeric forms of oleoyl 2-deoxy-2-N-trifluoroacetyl-D-glucopyranoside in the \(^1\text{C}_1\) conformation [64].
Summarizing, the subject of the anomeric effect should be approached with some caution as the abundance of ‘effects’ discussed in the literature to date permits us to explain almost everything, but to predict close to nothing.\[^{3}\]

It is worth to note that 1,2-glycosyl orthoamide 11", a common by-product of glycosylation reaction \[^{3}\], can be isomerized into the desired α-isomer 11, thus increasing the overall yield of this glucoside. Using an excess (4 equiv.) of a different acid promoter, boron trifluoro diethyl etherate (BF₃.(EtO)₂), in anhydrous acetonitrile at 100 °C the orthoamide cleavage can be achieved. The reaction was monitored by TLC in EtOAc (Figure 2.14) until all of the orthoamide 11" was consumed, which resulted, besides the formation of 11, on the deprotection in the anomeric position leading to 12 and oleic alcohol.

![Figure 2.14. TLC (EtOAc) of isomeration reaction of 11".](image)

2.1.1.3. O-sulfation (step c)

Sulfation of glucoside 11 at HO-6 afforded the sugar sulfate 10 as the major product in a good yield (64%). The regioselectivity of this reaction is due to the higher reactivity of the primary hydroxyl group at the C-6 position compared with that of the secondary hydroxyl groups at the C-3 and C-4 positions. This sulfation reaction involved the treatment with 2 equivalents of sulfur trioxide pyridine (SO₃.py) at room temperature for 1.5 h followed by neutralization with KOH.
TLC analysis revealed besides the major product 10 (R<sub>f</sub> = 0.12 in 9:1 EtOAc-MeOH) it was also obtained a disulfated glucoside 10' (R<sub>f</sub> = 0.07 in 9:1 EtOAc-MeOH) at positions C-6 and C-3. Characterization of the compound 10 by <sup>1</sup>H NMR confirmed the presence of an oxosulfonyl group at C-6 from the further downfield chemical shift of the H-6a and H-6b peaks, which appear at 4.27 and 4.18 ppm, as doublet of doublets (CH<sub>2</sub> group showed two different multiplet signals instead of one because the protons are diastereotopic). The <sup>1</sup>H NMR spectrum of 10', besides the mentioned resonances, presents also a doublet of doublets assigned to H-3, which is further downfield (4.50 ppm) shifted compared to the H-3 of 10 (3.82 ppm), due of its close proximity to the electronegative oxosulfonyl group at position C-3. These displacements on the chemical shifts were also observed on the <sup>13</sup>C NMR spectra of these compounds.

Other attempts to perform this reaction at 0 and 70 °C proved that the regioselectivity of the reaction increased and decreased significantly at lower and higher temperature, respectively, compared to room temperature. However, at these temperatures lower yields of 10 were obtained: 47 % at 0 °C; 45 % at 70 °C.

2.1.1.4. N-deprotection (step d)

The next step in this synthesis was deprotection of the N-trifluoroacetyl group to obtain the free amine group in position C-2 of the sugar backbone to yield 9. This hydrolysis reaction was accomplished by reacting glucoside 10 with a 4:1:0.5 MeOH-Et<sub>3</sub>N-H<sub>2</sub>O mixture at 50 °C for 48 h. Besides the long reaction time the starting material was not completely consumed (reaction extension incomplete), although providing a good yield of 71 %.
Figure 2.16. Synthesis to obtain 9, step d.

The reaction was monitored by TLC (2:1 EtOAc-MeOH), which revealed one spot that was significantly more polar than that of precursor 10. The structure of glucoside 9 was confirmed also by the disappearance of the C=O and CF₃ groups, which appear at 159.2 and 119.4 ppm, respectively, in the $^{13}$C NMR spectrum of 9. An upfield shift (0.9 ppm) of H-2 signal in the $^1$H NMR spectrum of 9 compared to that of the starting material 10, was also indicative of the expected loss of the trifluoroacetyl group.

HRMS spectrometry, in the positive ion mode (M+H)$^+$, was also performed for compound 9 (signal at m/z = 532.2763) and confirmed its structure.

2.1.1.5. N-acylation (step f)

The next step, after the successful synthesis of the glucoside 9, was to obtain the fluorophore moiety with a linker (Figure 2.17) to further conjugate to the sugar backbone via the amine group at C-2.

![Figure 2.17. Synthesis of the fluorescent 'linker' (8).](image)

This derivative having an NBD group at the end of the alkyl chain was prepared by reacting 6-amino hexanoic acid (AHA) (15) with non-fluorescent NBD-Cl (16) in a 10:1 MeOH-Et₃N mixture at room temperature for 24 h (Figure 2.17). The basic medium resulted in a deprotonation of the switterion 15 into the deprotonated amine, increasing its nucleophilicity. Then, in this bimolecular nucleophilic substitution ($S_N$2)
via a deeper-colored Meisenheimer complex \cite{66,67}, an addition of amino salt (nucleophile) in carbon at position 4 of the NBD molecule and elimination of a leaving group, chloride anion takes place simultaneously (Figure 2.18). This method afforded 8 in an overall yield of 65 % (Rf = 0.48 in 9:1 EtOAc-MeOH) presenting new desired optical properties (fluorescence) as it illustrates Figure 2.19. NBD moiety directly conjugated to nitrogen atom (electrodonor) at position 4 triggers higher electron movement. This phenomenon do not occurs in 16 (non-fluorescent), due to the high electronegativity of chloride atom (electroacceptor).

The structure of compound 8 was confirmed by \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy. HRMS was also done, which gave a m/z of 295.1040 (M + H)^+.

![Figure 2.18. Synthesis of 8 containing a fluorescent moiety via S$_2$2 with the formation of Meisenheimer complex \cite{68}.

![Figure 2.19. TLC (in 9:1 EtOAc-MeOH) of coupling reaction of 8 revealed in ninidrine and observed exposing to: (a) solar light (390 to 750 nm); (b) ultra-violet (UV) light (366 nm).]
The final step was to anchor this ‘fluorescent linker’ (8) to the glucopyranoid ring of 9 in the C-2 position, where the free amino group can couple with the terminal carboxylic group of 8, via an amidation reaction as illustrated in Figure 2.20.

![Chemical structure](image)

**Figure 2.20.** Synthesis to obtain 6, the desired new 3 derivative containing a fluorescent label.

The coupling was performed in the presence of 1-N-[3-(dimethylamino)propyl]-3-N’-ethylcarbodiimide (EDC) in the same proportion as 8, 1.5 equivalents, and catalytic amount (0.5 equiv.) of 4-(dimethylamino)pyridine (DMAP), the ‘Steglich’s base’ in anhydrous tetrahydrofuran (THF) (Figure 2.20). The reaction mixture was stirred at room temperature and monitored by TLC for 24 hours until the starting material 9 was fully consumed to afford the desired fluorescent glucoside 6 (Rf = 0.31 in 4:1 CH₂Cl₂-MeOH) in a good yield (η = 70 %). Support for the presence of the fluorescent moiety at position C-2 comes from ᵃ¹H NMR data, where the signal of H-2 appears 0.89 ppm shifted to lower field than the corresponding signal for the precursor 9. Also a doublet further upfield shifted (4.77 ppm) for the anomeric proton, however the dihedral angle for H-1 and H-2 practically did not change comparing 6 with their precursor 9. In addition ᵃ¹H and ᵃ¹³C NMR characterization shows the correct number of protons and carbons, respectively, for the presence of one NBD conjugate.

Glucoside 6 was also analyzed by HRMS. In the negative ion mode gave a m/z 784.3716 (M⁻) which corresponds to the expected mass of 6.

### 2.1.2. Synthesis of the radionuclide-labeled 3 derivative

With the purpose to perform in vivo biodistribution studies of 3 (Figure 1.12), we have designed the [¹⁸F]-3 (7) (Figures 1.12, 2.2 and 2.21). Difference of 3 and 7 resides in the replacement of one hydrogen atom by fluorine-18 in the N-acetyl group...
at position C-2 of the pyranoid ring. As the steric parameters for fluorine and hydrogen are similar (van der Waal’s radii of fluorine and hydrogen are 1.35 and 1.20 Å, respectively), the replacement of hydrogen by fluorine in a biomolecule induces only minimum steric perturbations [70]. Indeed, fluorine is considered a bioisoster group of the hydrogen atom. Therefore, this replacement is expected to have little influence on the biological activity and in vivo behaviour.

As is shown in Figure 2.21, both inactive (17) and radioactive (7) 3 derivatives have been designed. Synthesis of the inactive compound \( ^{19} \text{F} \) is necessary because is used as surrogate of the \( ^{18} \text{F} \) congener. Commonly, radioactive compounds are characterized by analytical high pressure liquid chromatography (HPLC) comparing their retention times with the retention time of the respective inactive counterparts. Moreover, the inactive compound is also useful for the determination of the specific activity of the active one. In this case, a calibration curve is built with the inactive compound.

![Image](image.png)

Figure 2.21 Structures of fluorinated 3 derivatives, 7 and 17.

2.1.2.1 Preparation of 2-substituted acetic acid derivatives

As depicted in the retrosynthetic analysis of \( ^{18} \text{F}-3 \) (7) (Figure 2.2) it was envisaged to anchor the 2-(O-tosyl)-acetic acid group with the amine group at the C-2 position of the glucopyranoid ring of 9. On the other hand, direct conjugation with the 2-fluoroacetic acid would afford the F-3 (17). Within this purpose, we have tried to prepare both 2-(O-tosyl)-acetic acid (21) and 2-fluoroacetic acid (22) (Figure 2.22). Theirs syntheses was initiated by reacting ethyl 2-hydroxyacetate (18) with \( \rho \)-toluenesulfonyl chloride (1:1.5 eq.) in the presence of triethylamine (1.3 eq.) as base (Figure 2.22, step g). This reaction, carried out at room temperature and overnight, afforded 19 in moderate yield (\( \eta = 55 \% \)). TLC analysis (4:1 \( n \)-hexane-EtOAc) showed one UV reactive spot (\( R_f = 0.27 \)), that was significantly less polar than that of non-UV reactive starting material 18. Identification of 19 was based on the \( ^{1} \text{H} \) NMR spectrum, in which the two doublets at 7.66 and 7.23 ppm and one singlet at the 2.28 ppm of the
tosyl group were observed. $^{13}$C NMR spectrum was also in agreement with the presence of the tosyl group in the compound 19 (145.09 - 127.61 and 21.07 ppm).

Attempt to prepare the respective fluorinated derivative 20, refluxing 19 with tetrabutylammonium fluoride (TBAF) (1:4 eq) in THF at 80 °C for 30 minutes has failed (Figure 2.23, step h). In the literature, the preparation of the ethyl 2-fluoroacetate has been described, however no synthetic methodology was shown.\(^\text{71}\). Monitorization of this nucleophilic substitution reaction by TLC was also difficult due to problems of visualization/revealing the new formed compounds. Also, the low boiling point of 20 (b.p. = 119.3 °C) suggests that it should be purified by distillation, which was difficult with the working amounts (milligram range).

This unsatisfactory result prompted us to try the ester hydrolysis of 19 before to the fluorination reaction. Then, treatment of the newly formed 19 with 1.1 eq. of lithium hydroxide (LiOH) in absolute ethanol (EtOH) during 4 h yielded the desired 21 (η = 92%) (Figure 2.22, step i). Under these conditions the ester group was selectively hydrolyzed. TLC analysis (9:1 CH$_2$Cl$_2$-MeOH) indicated complete consumption of 19, with concomitant formation of a more polar UV-reactive compound 21 ($R_f$ = 0.14). NMR experiments indicated the disappearance of the peaks correspondents to the ethyl group (4.02 and 1.07 ppm) in the $^1$H NMR spectrum and at 61.91 and 14.01 ppm in the $^{13}$C NMR spectrum. Further, this 2-(O-tosyl)-acetic acid (21) will be conjugated to the glucoside as a mean to prepare the respective [$^{18}$F]glucoside derivative 7.

As the synthesis of 20 failed, it was tried to prepare the 2-fluoroacetic acid (22) directly from the carboxylic acid 21, using similar fluorinating conditions (Figure 2.22, step h). Once again, monitorization of this reaction was problematic. However, the boiling point of 22 (b.p. = 165°C) is higher than the b.p. of 20, no purification was tried and compound 22 was used further without purification (see Figure 2.25).

\[ \text{HO-CH}_2\text{-COO} \xrightarrow{\text{p-TsCl (x 1.5)}} \text{TsO-CH}_2\text{-COO} \]

\[ \text{ethyl 2-hydroxyacetate (18)} \quad \text{ethyl 2-(O-tosyl)-acetic acid (19)} \]

\[ \text{Ts = } \begin{array}{c} \text{O} \\
\text{S} \end{array} \]

\[ \text{TsO-CH}_2\text{-COO} \xrightarrow{\text{TBAF (x 4)}} \text{F-CH}_2\text{-COO} \]

\[ \text{THF, 80 °C, 30 min} \]

\[ \text{ethyl 2-fluoroacetate (20)} \]

\[ \text{LIOH (x 1.1)} \]

\[ \text{EtOH, rt, 4h} \]

\[ \text{2-(O-tosyl)-acetic acid (21)} \]

\[ \text{TsO-CH}_2\text{-COO} \xrightarrow{\text{TBAF (x 4)}} \text{F-CH}_2\text{-COO} \]

\[ \text{THF, 80 °C, 30 min} \]

\[ \text{2-fluoroacetic acid (22)} \]

\[ \text{Figure 2.22. Synthesis of 2-substituted acetic acid derivatives.} \]
2.1.2.2. N-Acylation of 23

Having the intermediate 21 prepared, the next step would consist in its coupling to the free amino group at the C-2 position of the glucopyranoid ring of 9, via an amide bond (Figure 2.2). In order to optimize this conjugation reaction, we have selected glucoside 23 as the starting material. This selection was based mainly on the fact that more amount of the precursor of 23 than precursor 9 was available and that would facilitate to experiment different strategies.

Similarly to the synthesis of 9, compound 23 was obtained by base-catalysed hydrolysis of the N-trifluoracetyl in 11 (Figure 2.23, step d) in good yield ($\eta = 74\%$). Initially, in order to test the chemoselectivity of this N-acylation reaction, glucoside 23 reacted with 2-hydroxyacetic acid (24), using DCC and tetrafluorophenol (TFP) (Figure 2.23, step j). Under these conditions, the carboxylic acid function was activated by in situ preparation of the O-tetrafluorophenol ester. As expected, coupling of the carboxylic acid took place selectively on the amine group, although in poor yield ($\eta = 38\%$). TLC analysis in 9:1 CH$_2$Cl$_2$-MeOH showed two less polar spots ($R_f = 0.22$ and 0.33) related to the $\alpha$- and $\beta$-isomers 25. $^1$H NMR spectrum indicated a singlet at 4.01 ppm, corresponding to the methylene group of the 2-hydroxyacetyl unit. Moreover, the spectrum showed the absence of the doublet ($\delta = 3.04$ ppm) attributed to the H-2 in compound 23.

With the purpose to activate the carboxylic function of 21, priori to use, compound 21 was reacted with DCC (1.1 eq.) and TFP (1.1 eq.) (Figure 2.24). Characterization by NMR spectroscopy of the reaction products showed that this reaction was unsuccessful. It was postulated that the hydroxyl group of the activating agent attacks the high electrophilic carbon adjacent to the O-tosyl group (leaving group) instead of the carbon of the carboxylic group. However, formation of dicyclohexylurea could be detected, which suggests that TFP attacked on both electrophilic carbons. Extra efforts were carried out in order to minimize this side-reaction, using TFP as the limiting reagent (0.5 eq). Unfortunately, no improvement was accomplished.
Aiming to improve the yield of 26, it was also tried an approach similar to the one used in the N-acylation of 9 with the NBD-C6COOH (8) (Figure 2.20). So, 21 was conjugated with glucoside 23 using DMAP (0.5 eq.) in combination with the DCC (1.5 eq.) (Figure 2.23, step l). Contrarily to the reaction of 21 with DCC and TFP (activation of the carboxylic function) (Figure 2.23, step k), this strategy intends to enhance the nucleophilicity of the amine group, by using a deprotonating agent. This reaction was
carried out in anhydrous THF, at room temperature for 4 hours. Monitorization by TLC indicated that glucoside 23 was fully consumed to afford the glucoside 26 containing the O-tosyl glycolic unit \( (R_t = 0.30 \text{ in EtOAc}) \), although in a lower yield \( (\eta = 31\%) \) than the one obtained with the \textit{in situ} activation of the carboxylic acid. As expected \(^1\)H NMR spectrum of glucoside 26 obtained from step l is similar to the one obtained in step k.

2.1.2.3. Synthesis of fluorinated 23 derivative

As mentioned above, cold fluorinated derivatives have also to be prepared to act as surrogates of the radioactive congeners \(^{18}\)F). Ideally, synthesis of the fluorobased glucosides would be by the direct conjugation of the 2-fluoracetic acid (22) with the amine group of either the NH2-3 (9) or 23. It was tried to use 22 (see Figure 2.22, step h), without being submitted to work-up, for the conjugation to the amine group of glucoside 23. Therefore, crude mixture of 22 (1.5 eq.) was reacted with glucoside 23 (1 eq.), in the presence of DCC (1.5 eq) and DMAP (0.5 eq.) (Figure 2.25, step n). The reaction was carried out in the same solvent as the fluorination (THF) and at room temperature for 4 hours. A new product was detected on the TLC plate \( (R_t = 0.53 \text{ in 100% EtOAc}) \). However, \(^{19}\)F-NMR experiments of the purified fraction indicated that the isolated product did not contain a fluor atom. Therefore, an alternative strategy would have to be tried, such as the nucleophilic substitution reaction using the corresponding O-tosylated-based glucoside 23 as the precursor (Figure 2.25, step o).

![Diagram](image.png)

\[ 
\text{o-} \text{H}_{\text{N}} \text{H}_{\text{glucosamine (23)}} \]

\[ 
\text{o-} \text{H}_{\text{O-tosyl-glucosamine (26)}} \]

\[ 
\text{o-} \text{F-} \text{glucosamine (28)} \]

\text{Figure 2.25. Attempt (step n) and design route (step o) to obtain the 28.}
2.2. Biological studies using 3 derivatives

2.2.1. Studies using fluorescent-labeled 3 derivative

In order to test the uptake by astrocytes of derivative 3 containing a fluorescent label (6) and the antimitotic activity of this derivative we used primary cultures of astrocytes from postnatal rats (day 0 - 1).

The cell cultures were supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 IU/mL penicillin, and 50 mg/mL streptomycin (DM-10S) and maintained in a humidified atmosphere of 5% CO₂ at 37 °C. The cells were seeded the day before at 2 × 10⁶ cell per well in microtiter plates (NUNC) and incubated to permit cellular adhesion. After cell adhesion we incubated 24h without FCS for reinitiating cell cycle. Glucoside 6 was added to cell cultures at final concentrations of 3, 15 and 30 μM. A mock experiment without the inhibitor was simultaneously prepared (untreated control). The astrocytes were incubated for 24h with the synthetic inhibitor 6, and fixed with 2% paraformaldehyde. The Hoechst agent was added for nuclei labeling. The fixed cells were washed repeatedly with PBS and fluorescence images captured by InCell apparatus (General Electric).

Fluorescence microscopy images of the cells obtained in presence of glucoside 6 are shown in Figure 2.18. The green fluorescence of fluorescent-labeled 3 (6) became clearly visible inside the cells, the nuclei in blue. As expected from a 3 derivative, the number of astrocytes decrease as the concentration of compound 6 increase. In addition glucoside 6 exhibits similar ID₅₀ value as 3, in other words, it has almost the same inhibitory proliferation power [²⁷]. Thus the results obtained using 6 are reliable information about the original glucoside 3.

![Figure 2.21](image)

**Figure 2.21.** Fluorescence images of astrocyte cells (postnatal rats, day 0 - 1) after 1 day of treatment with compound 6. Concentrations of: (a) 3, (b) 15 and (c) 30 μM.
Chapter 3.
Conclusion and Perspectives

3.1. Conclusions

3.2. Further perspectives
Chapter 3. Conclusions and Perspectives

3.1. Conclusions

This work focused on the preparation of 3 derivatives which would help, by molecular imaging (MI), on the investigation of the biological profile of glucoside 3, providing insights on its utility in the spinal cord injury (SCI) treatment. The new 3 derivatives must contain a fluorescent moiety or a radionuclide, and must be biologically evaluated using astrocyte cultures (fluorescence microscopy) and animals (biodistribution studies), respectively.

One of the keys to achieve such goals was the development of a simple synthetic protocol towards glucosamine (13) derivatization/functionalization, in a multi-step manner, in order to obtain a versatile 3 derivative, the NH2-3 (9). The glucoside 9 has a free amine group in the C-2 position, which can be conjugated to a linker containing a desirable imaging moiety. Since we have the knowledge to produce this versatile 3 intermediate (9), with a broad spectrum of stable latent probes with numerous applications in biochemical and biological research will be possible to perform several studies using different MI techniques.

Preparation of the fluorescent-label 3 derivative, NBD-C6-3 (6) was successfully accomplished. This involved the chemoselective conjugation of the fluorophore unit to the amine group, bearing a spacer (alkyl chain) between the sugar ring and the fluorophore moiety. The structural modifications in 6 had no significant effect in its antimitotic activity in comparison with original glucoside 3. Support for this comes from fluorescence microscopy studies in astrocytes. Also the images provided from this in vitro studies lead to conclude that the ability to permeate through the astrocyte cell membrane was not changed.

Altogether, the results obtained with NBD-C6-3 contributed to the understanding of the role of glucoside 3 in SCI repair, by offering new insights into its biological behavior. So far, the in vitro results obtained still places 3 as a potential drug candidate to help the SCI repair.

In order to optimize the preparation of fluorinated 3 derivative (19F/18F-3), significant synthetic work was carried out with 23. This compound was used, in alternative to compound 9, just for preliminary studies aiming to optimize the best experimental conditions for N-acylation of 9 with two substituted acetic acid
derivatives (21, 22). Attempts to prepare the fluorinated acetic acid derivative (22), that would be further conjugated to the amine group (C-2) of 23, have failed. Therefore, we conclude that preparation of the fluoro-derived glucosides was better achieved via the 2-[(O-tosyl)-acetyl containing glucoside 26, which was prepared successfully. Preparation of the fluorinated 23 analogue (28) is undergoing.

3.2. Further perspectives

Based on the optimized results obtained with 23, it is envisaged to prepare and characterize, in a near future, the fluorinated 3 derivative (7 and 17).

After obtaining compounds 17 and 7, we will perform in vitro and biodistribution studies, using astrocyte cells and healthy mice. We will evaluate the in vitro behaviour (antimitotic activity) of 17, like it was done for the NBD-3 derivative (6). We will also use the radioactive analogue (7) to evaluate and quantify the capability of the compound to cross the cell membranes and its distribution in the cell. This assay will elucidate us if the replacement of the hydrogen atom in 3 by a fluor atom will compromise its antimitotic activity and may also contribute for a better understanding of its mechanism of action.

The assessment of the biodistribution profile will confirm if 7 has the ability to cross the blood-brain barrier, an essential requisite for its usefulness for SCI treatment.

![Synthetic route for the preparation of F-3 (17) and ²¹F-3 (7).](image)
Chapter 4.
Experimental Section

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Chapter 4. Experimental Section

4.1. Reactants and Solvents

All chemicals were of reagent grade or higher and were purchased from commercial suppliers or purified by standard techniques. All anhydrous solvents were prepared by usual drying techniques as reported in Perry et al. \[72\]. Dichloromethane and acetonitrile were distilled from calcium hydride under argon atmosphere at 760 torr. Tetrahydrofuran and toluene were distilled from sodium and benzophenone under argon atmosphere at 760 torr.

Preparation of \( \text{H}_2\text{SO}_4 \) adsorbed under silica (\( \text{H}_2\text{SO}_4 \)-silica 23%) was performed following this procedure: 10 g of silica gel (230-400 mesh) in ethyl ether (50 mL) was added slowly to conc. \( \text{H}_2\text{SO}_4 \) (37-38%) (3 mL) with stirring. The solvent was removed under vacuum, and the silica was dried for 3 h at 60 °C under 2-4 mbar pressure \[73\].

4.2. Materials and Methods

4.2.1. Chromatographic procedure

The progress of reactions and column chromatographic elution profile were routinely followed by analytical thin-layer chromatography (TLC) in tanks (Pobel) with vapor saturation. TLC experiments were performed on plate pre–coated with silica UV254 (60 F 254 Merck silica gel, 0.2 mm). Visualization of the plates was carried out using UV light (254 or 365 nm), and/or iodine container, or a solution of 5% \( \text{H}_2\text{SO}_4 \) in ethanol, or a solution of 2% ninhydrin (2,2-dihydroxyindane-1,3-dione) in ethanol, followed by heating. \( R_f \) values were determined from plates of 7 cm height.

Flash chromatography (FC) was performed using thick-walled columns, employing silica gel 60 (Merck 230-400 mesh, 0.040-0.063 mm). Gravity column chromatography (GCC) was performed by using open columns loaded with silica gel (Merck 70-230 mesh, 0.063-0.200 mm). The eluent used is indicated, and solvent ratios refer to volume-volume relation.
4.2.2. Melting points

Melting points (MP) were determined using a microscope Reicher Jung Thermovar. The mp values are not corrected.

4.2.3. Optical rotations

Optical rotations [α]₀ were recorded on a Perkin-Elmer 241 MC Polarimeter using 1 dm quartz cells, 589 nm wavelength, sodium yellow light, 20 °C temperature. The solvents and the concentrations (g / 100 mL) used are indicated.

4.2.4. Spectroscopic techniques

Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on a Varian Unity 300 or Bruker AM-300 or Inova-300 or Inova-400 NMR spectrometer at the frequencies of 400 or 300 MHz (¹H), 100 or 75 MHz (¹³C) and 282 MHz (¹⁹F) with TopSpin 2.1 or Varian software VNMRJ 2.1. Samples (10 to 60 mg) were dissolved in 0.5 mL CDCl₃ (99.8%, SDS) or CD₃OD (99.8%, Merk), and transferred to NMR microtubes (5 mm, Wilmad LabGlass).

¹H and ¹³C chemical shift values (δ) are reported in parts per million (ppm) and referenced with the residual solvent resonances (CDCl₃: δ₁H = 7.26 ppm, δ₁³C =77.16 ± 0.06 ppm; CD₃OD. δ₁H = 3.31 ppm, δ₁³C =49.00 ± 0.01 ppm [74]) relatively to internal tetramethylsilane (TMS) standard. ¹⁹F chemical shifts were referenced externally to α, α’, α’’ - trifluorotoluene (0.05% in C₆D₆; δ₁⁹F = -63.3 ppm). Coupling constant values (J) are reported in Hertz (Hz), and spin multiplicities of signals are designated by the following abbreviations: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublets of doublets), t (triplet), q (quartet), m (multiplet). The number of protons was deduced by integration.

Protons assignment was based on 2D experiments (¹H–¹H correlation spectroscopy, COSY and ¹H–¹³C heteronuclear single quantum coherence, HSQC and heteronuclear multiple bond coherence, HMBC). 2D NMR (¹H–¹H COSY, ¹H–¹³C HSQC and ¹H–¹³C HMBC) experiments were registered at Inova-300 or Inova-400 NMR spectrometer at the frequencies of 400 or 300 MHz employing the standard pulse sequence. The assignment of
the anomic configuration was based on the determination of the coupling constant between H-1 and H-2 ($J_{1,2}$).

High-resolution mass spectrometry (HRMS) spectra were recorded on an Agilent 6520 Accurate Mass Q-TOF spectrometer with an ESI source. The solid samples (< 1 mg) were dissolved in 9:1 MeOH-H$_2$O. A syringe pump (Harvard Apparatus) was used to introduce the sample via direct infusion (35 µL/min). Nonsulfated oligosaccharide experiments were carried out in positive ionization mode with the electrospray source set to 5 KV and 275 °C. Experiments for sulfated oligosaccharides were carried out in negative ionization mode with the electrospray source set to 2 KV and 200 °C. The automatic gain control was set to $1 \times 10^7$ for full scan MS. The MS data were acquired and processed using Xcalibur 1.3. The data are expressed in mass to charge unit (m/z).

4.2.5. Elementary Analysis

Elementary Analysis was performed in an Heraus CHN-O Rapid analyzer. The data are expressed in percentage (%).

4.2.6. Cell Proliferation Assays

Primary cultures of rat astrocytes from postnatals (day 0 - 1) of two microglia cell lines the BV-2 and N13 cells were supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 IU/ml penicillin, and 50 mg/mL streptomycin (DM-10S) and maintained in a humidified atmosphere of 5% CO$_2$, at 37 °C. The cells were seeded the day before at $2 \times 10^6$ cell per well in microtiter plates (NUNC) and incubated to permit cellular adherence. After cell adhesion we incubated 24h without FCS for reinitiating cell cycle. The glucoside 6 was added to cell cultures at final concentrations of 3, 15 and 30 µM. A mock experiment without the inhibitor was simultaneously prepared (untreated control). The astrocytes were incubated for 24h with compound 6, and fixed with 2% paraformaldehyde. The Hoechst agent was added for nuclei labeling. The fixed cells were washed repeatedly with PBS. The cells were visualized at 40x magnifications and fluorescence images captured by InCell apparatus (General Electric).
4.3. Syntheses

4.3.1. 2-Deoxy-2-trifluoroacetamido-α,β-D-glucopyranose (TFA-glucosamine (12))

In a 100mL flask with a stirring bar and under an argon atmosphere, a suspension of glucosamine (13) (5.13 g, 23.8 mmol) in anhydrous methanol (25 mL) was treated with sodium methoxide in methanol (500 mg of Na were dissolved in 6.0 mL of methanol). The reaction mixture was stirred for 5 minutes, followed by the formation of sodium chloride (white solid) at the bottom. Then, to this mixture S-ethyl thiol trifluoroacetate (4.2 mL, 32.6 mmol) was added and the reaction mixture was stirred at room temperature for 24 h. Thereafter, the solvent was evaporated and TFA-glucosamine (12) (4.78 g, 73%), was crystallized from the acetone and diethyl ether. Glucoside 12 was obtained as mixture of both anomers in a ratio α/β of 7:3.

R_f = 0.67 (α isomer) and 0.56 (β isomer) (TLC eluent: 2:1 EtOAc-MeOH); MP: 180-184 °C. [α]_D: + 54.6° (c 0.5, MeOH);

^1H NMR (300 MHz, CD_3OD): δ 5.15 (d, J = 2.9 Hz, 1Hα), 4.69 (d, J = 8.2 Hz, 1Hβ), 3.9-3.7 (m, 3H, H-2α), 3.8-3.6 (m, <2H, H-2β), 3.53 (dd, <1H, J=10.4, 8.4 Hz, H-4β), 3.04 (dd, 1H, J=10.4, 3.6 Hz, H-3α,β);

^13C NMR (100 MHz, CD_3OD): δ 159.2 (COCF_3), 119.4 (COCF_3), 96.3 (C-1β), 92.0 (C-1α), 78.2 (C-β), 75.3 (C-3β), 73.2 (C-α), 72.5 (C-3α), 72.2 (C-β), 71.9 (C-α), 62.8 (C-β), 62.7 (C-α), 59.1 (C-2β), 56.7 (C-2α).

4.3.2. O-glycosylation of glucopyranose 12

In a 100mL flask, TFA-glucosamine (12) (1.708 g, 6.2 mmol) and oleic alcohol (11.5 mL, 31.0 mmol) were mixed under an argon atmosphere. Then, H_2SO_4-silica 23% (500 mg) was added and the reaction mixture was stirred at 180 °C for 20 min. Thereafter, the reaction mixture was cooled down to room temperature. The crude was submitted to flash
chromatography (5:1 EtOAc-n-hexane, 10:0 → 4:1 EtOAc-MeOH) to give oleyl-TFA-αglucosamine (11) (0.782 g, 24%), oleyl-TFA-βglucosamine (11’) (0.131 g, 4%) and 1,2-glycosyl-orthoamide (11’’) (0.026 g, 0.8%).

4.3.2.1. Oleyl-2-deoxy-2-trifluoroacetoamido-α-D-glucopyranoside (11)

![Chemical Structure of 11](image1.png)

Obtained as white solid; \( R_f = 0.48 \) (TLC eluent: EtOAc). \([\alpha]_D^\circ = +94.5^\circ\) (c 6.4, MeOH).

\(^1\)H NMR (300 MHz, CD3OD): \( \delta \) 5.4-5.3 (m, 2H, O(CH\(_2\)_8 CH=CH(CH\(_2\)_7 CH\(_3\))\), 4.90 (d, 1H, J=3.5 Hz, H-1), 3.9-3.8 (m, 2H, H-2, H-6a), 3.7-3.5 (m, 4H, OCH\(_2\), H-6b, H-3, H-5), 3.4-3.3 (m, 2H, OCH\(_2\)' H-4), 2.0-1.9 (m, 4H, O(CH\(_2\))\(_7\)CH=CHCH\(_2\)(CH\(_2\))\(_6\) CH\(_3\)), 1.6-1.5 (m, 2H, OCH\(_2\)CH\(_2\)(CH\(_3\)) CH=CH(CH\(_2\)) CH\(_3\)), 1.3-1.2 (m, 22H, OCH\(_2\)CH\(_2\)(CH\(_2\))\(_2\)CH\(_2\) CH=CHCH\(_2\)(CH\(_2\))\(_2\)CH\(_3\)), 0.86 (t, 3H, CH\(_2\) CH\(_3\)).

4.3.2.2. Oleyl-2-deoxy-2-trifluoroacetoamido-β-D-glucopyranoside (11’)

![Chemical Structure of 11’](image2.png)

Obtained as white solid; \( R_f = 0.24 \) (β isomer) (TLC eluent: EtOAc). \([\alpha]_D^\circ = -0.6^\circ\) (c 5.7, MeOH).

\(^1\)H NMR (300 MHz, CD3OD): \( \delta \) 5.35 (m, 2H, O(CH\(_2\))\(_8\) CH=CH(CH\(_2\)) CH\(_3\)), 5.15 – 5.07 (dd, J=9.3 Hz, 1H, H-1), 3.90 – 3.83 (m, 2H, H-2, H-3), 3.75 – 3.65 (m, 3H, H-6a, OCH\(_2\), H-6b), 3.63 – 3.59 (m, 1H, H-5), 3.43 – 3.33 (m, 2H, OCH\(_2\)' H-4), 2.03 – 2.00 (m, 4H, O(CH\(_2\))\(_7\) CH=CHCH\(_2\)(CH\(_2\)) CH\(_3\)), 1.59 – 1.55 (m, 2H, OCH\(_2\) CH\(_2\)(CH\(_3\)) CH=CH(CH\(_2\)) CH\(_3\)), 1.32 – 1.27 (m, 22H, OCH\(_2\) CH\(_2\)(CH\(_2\))\(_2\) CH=CHCH\(_2\)(CH\(_2\))\(_2\) CH\(_3\)), 0.97 – 0.82 (t, 3H, CH\(_2\) CH\(_3\)).
4.3.2.3. 2-amino-2-deoxy-[1,2-O,N-(1-oleyloxyltrifluoroethylidene)]-α-D-glucopyranoside (11’’)

![Chemical Structure Image]

Obtained as white solid; $R_f = 0.52$ (TLC eluent: EtOAc);

$^1\text{H} \text{ NMR (300 MHz, CD}_2\text{OD):} \delta 5.44 - 5.25 \text{ (m, 2H, O(CH}_2)_2\text{CH}=\text{CHCH}_2\text{CH}_2\text{CH}_2\text{)}$, 5.12 (d, $J = 5.0$ Hz, 1H, H-1), 4.50 (t, $J = 5.5$ Hz, 1H, H-3), 4.26 (t, $J = 5.0$ Hz, 1H, H-2), 4.07 – 4.00 (m, 1H, H-4), 3.95 – 3.86 (m, 1H, H-5), 3.83 – 3.66 (m, 2H, H-6a, H-6b), 3.61 (dd, 1H, OCH$_2$), 3.46 – 3.34 (m, 1H, OCH$_2$), 2.14 – 1.89 (m, 4H, O(CH$_2$)$_2$CH=CHCH$_2$(CH$_2$)$_6$CH$_3$), 1.54 (d, 2H, OCH$_2$CH$_2$(CH$_2$)$_6$CH=CHCH$_2$(CH$_2$)$_6$CH$_3$), 1.30 (s, 22H, OCH$_2$CH$_2$(CH$_2$)$_6$CH=CHCH$_2$(CH$_2$)$_6$CH$_3$), 0.90 (t, 3H, CH$_2$CH$_3$).

4.3.3. Isomerization of 11’’ into 11

In a 25 mL flask with a stirring bar and 4 Å molecular sieves (100 mg), glucoside 11’’ (104.4 g, 0.20 mmol) was reacted with BF$_3$·(EtO)$_2$ (50.0 µL, 0.40 mmol) in anhydrous acetonitrile (1 mL) under an argon atmosphere. The reaction mixture was stirred at 100 °C for 5 min. Thereafter, the reaction mixture was cooled down to room temperature. The crude was submitted to flash chromatography (10:0 → 4:1 EtOAc-MeOH) to give glucoside 11 (65.2 mg, 62%).

4.3.4. Isomerization of 11’ into 11

In a 25 mL flask with a stirring bar and 4 Å molecular sieves (100 mg), glucoside 11’ (162.3 g, 0.26 mmol) and oleic alcohol (1.5 mL, 4.04 mmol) were mixed under an argon atmosphere. Then, H$_2$SO$_4$-silica 23% (30 mg) was added and the reaction mixture was stirred at 180 °C for 30 min. Thereafter, the reaction mixture was cooled down to room temperature. The crude was submitted to flash chromatography (5:1 EtOAc-η-hexane, 10:0 → 4:1 EtOAc-MeOH) to give glucoside 11’ (25.9 mg, 19%).
4.3.5. O-sulfation of glucoside 11

In a 50 mL flask with a stirring bar, glucoside 11 (0.458 g, 0.87 mmol) was reacted with SO₃·py (0.290 g, 1.82 mmol) in pyridine (1 ml) under an argon atmosphere. The reaction mixture was stirred at room temperature for 80 min. After this time, the solvent was concentrated under vacuum till solid residue. Then, 2:1 MeOH-H₂O (6 mL) was added, followed by a few drops of aq. KOH (0.5M) till pH = 7. Once again the solvent was evaporated to dryness. The resulting residue was submitted to flash chromatography (1:0:0.005 → 1:0.5:0.005 EtOAc-MeOH-Et₃N), to give TFA-3 (10) (0.360 g, 64%) and glucoside 10' (0.081 g, 12%).

4.3.5.1. Oleyl-2-deoxy-2-trifluoroacetoamido-6-O-(oxosulfonyl)-α-D-glucopyranoside (10)

Obtained as yellow solid; R₇ = 0.12 (TLC eluent: 9:1 EtOAc-MeOH); [α]D° + 70.3° (c 11.3, MeOH);

¹H NMR (400 MHz, CD₃OD): δ 5.42 - 5.27 (m, 2H, O(CH₂)₈CH=CH(CH₂)₇CH₃), 4.91 (d, J = 3.5 Hz, 1H, H-1), 4.27 (dd, J = 10.9, 2.2 Hz, 1H, H-6a), 4.18 (dd, J = 10.9, 5.1 Hz, 1H, H-6b), 3.90 (dd, J = 10.7, 3.5 Hz, 1H, H-2), 3.82 (dd, J = 8.1, 2.8 Hz, 1H, H-3), 3.76 (dd, J = 4.7, 2.5 Hz, 1H, OCH₂), 3.72 (dd, J = 8.2, 4.5 Hz, 1H, H-5), 3.44 (d, J = 8.6 Hz, 1H, OCH₂), 3.41 - 3.36 (m, 1H, H-4), 2.03 (m, 4H, O(CH₂)₇CH₂CH=CHCH₂(CH₂)₆CH₃), 1.57 (m, 2H, OCH₂CH₂(CH₂)₆CH₃), 1.46 - 1.11 (m, 22H, OCH₂CH₂(CH₂)₅CH₂CH=CHCH₂CH(CH₂)₆CH₃), 0.90 (t, 3H, CH₃CH₂).

4.3.5.2. Oleyl-2-deoxy-2-trifluoroacetoamido-3,6-di(oxosulfonyl)-α-D-glucopyranoside (10')

Obtained as yellow pale solid; R₇ = 0.07 (TLC eluent: 9:1 EtOAc-MeOH); [α]D° + 52.9° (c 10.3, MeOH);

¹H NMR (300 MHz, MeOD) δ 5.42 - 5.29 (m, 2H, O(CH₂)₈CH=CH(CH₂)₇CH₃), 5.11 (d, J = 3.5 Hz, 1H, H-1),
4.59 (dd, J = 10.8, 8.9 Hz, 1H, H-3), 4.30 (dd, J = 10.8, 2.0 Hz, 1H, H-6a), 4.19 (dd, J = 10.8, 5.4 Hz, 1H, H-6b), 3.91 (dd, J = 5.1, 2.7 Hz, 1H, H-2), 3.89 – 3.84 (m, 1H, H-5), 3.76 (m, J = 9.9, 6.1 Hz, 1H, OCH₃), 3.66 – 3.58 (m, 1H, H-4), 3.40 (m, J = 10.0, 6.4 Hz, 1H, OCH₃), 2.03 (d, J = 5.5 Hz, 4H, O(CH₂)₇CH₂=CHCH₂(CH₂)₆CH₃), 1.57 (d, J = 6.6 Hz, 3H, OCH₂CH₂(CH₂)₆CH₃), 1.35 – 1.25 (m, 22H, OCH₂CH₂(CH₂)₂CH₂CH=CHCH₂(CH₂)₆CH₃), 0.90 (t, 3H, CH₃CH₃).

4.3.6. Oleyl-2-amino-2-deoxy-6-O-(oxosulfonyl)-α-D-glucopyranoside (NH₂-3 (9))

In a 10mL flask, glucoside 10 (0.107 g, 0.17 mmol) was stirred with a solution of 4:1:0.5 methanol-triethylamine-water (1 mL) for 48 hours at 50 °C for 48 h. After this time, the solvent was concentrated under vacuum till solid residue. Flash chromatography of the crude (5:1:0.005 → 0:1:0.005 EtOAc-MeOH-Et₃N) afforded NH₂-3 (9) (0.600 g, 71%) as a white solid.

Rf = 0.23 (TLC eluent: 2:1 EtOAc-MeOH); [α]₀: +35.7° (c 8.6, MeOH);

¹H NMR (400 MHz, CD₃OD): δ 5.42 – 5.25 (m, 2H, O(CH₂)₇CH₂=CHCH₂(CH₂)₆CH₃), 4.93 (d, J = 3.7 Hz, 1H, H-1), 4.22 (dd, J = 10.9, 2.1 Hz, 1H, H-6a), 4.16 (dd, J = 11.0, 4.9 Hz, 1H, H-6b), 3.78 – 3.74 (m, 1H, OCH₂), 3.73 – 3.69 (m, 1H, H-5), 3.66 (dd, J = 10.3, 9.0 Hz, 1H, H-3), 3.52 – 3.42 (m, 1H, OCH₂), 3.42 – 3.35 (m, 1H, H-4), 3.04 – 2.96 (m, 1H, H-2), 2.08 – 1.92 (m, 4H, O(CH₂)₇CH₂=CHCH₂(CH₂)₆CH₃), 1.62 (m, 2H, OCH₂CH₂(CH₂)₂CH₂=CHCH₂(CH₂)₆CH₃), 1.42 – 1.19 (m, 22H, OCH₂CH₂(CH₂)₂CH₂CH=CHCH₂(CH₂)₆CH₃), 0.88 (t, 3H, CH₃CH₃);

¹³C NMR (100 MHz, CD₃OD): δ 130.83 (CH=CH), 111.19 (C-1), 97.52 (C-6), 72.21 (C-5), 71.41 (C-3), 69.41 (OCH₂), 67.74 (C-4), 55.98 (C-2), 33.07, 30.93, 30.84, 30.80, 30.77, 30.68, 30.65,
30.61, 30.53, 30.45, 30.41, 30.33, 28.17, (OCH\(_2\)CH\(_2\))\(_2\)CH=CH(CH\(_2\))\(_2\)CH\(_3\), 23.74 (CH\(_2\)CH\(_3\)), 14.46 (CH\(_2\)CH\(_3\));

HRMS (ESI) m/z (calcd for C\(_{24}\)H\(_{46}\)NO\(_2\)SK, 531.2684), found 532.2763 (M+H\(^+\)).

4.3.7. 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid (NBD-C\(_5\)COOH (8))

In a 10mL flask with a stirring bar, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (16) (29.2 mg, 0.15 mmol) was reacted with 6-aminohexanoic acid (15) (26.4 g, 0.19 mmol) in anhydrous methanol (1 mL). Then, anhydrous triethylamine (6.0 \(\mu\)L) was added and the mixture was stirred at room temperature for 24 h. After this time, the solvent was concentrated under vacuum till solid residue. The crude mixture was submitted to flash chromatography (1:0 \(\rightarrow\) 0:1 EtOAc-MeOH), to give NBD-C\(_5\)-COOH (8) (29.7 mg, 65 %) as a brown solid.

R\(_f\) = 0.48 (9:1 TLC eluent: EtOAc-MeOH);

\(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.50 (d, 1H, H\(_{\text{arom}}\)), 6.19 (d, 1H, H\(_{\text{arom}}\)), 3.54 (d, 2H, NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 2.39 (t, 2H, NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 1.83 (dd, 2H, NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 1.72 (dt, 2H, NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 1.55 (dd, 2H, NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH);

\(^13\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 143.45, 136.97 (C\(_{\text{arom}}\)), 43.88 (NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 34.10 (NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 29.87 (NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 28.14 (NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 26.37 (NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH), 24.38 (NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)COOH);

HRMS (ESI) m/z (calcd for C\(_{52}\)H\(_{14}\)N\(_{4}\)O\(_{5}\), 294.0967), found 295.1040 (M+H\(^+\)).
4.3.8. Oleyl–2-[6-(7-nitrobenzofurazan)-aminohexanamide]-2-deoxy-6-O-(oxosulfonyl)-α-D-glucopyranoside (NBD-C₆-3 (6))

In a 10mL flask with a stirring bar, glucoside 9 (90.2 mg, 0.16 mmol) was reacted with compound 8 (72.5 mg, 0.25 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (47.2 mg, 0.25 mmol) and 4-dimethylaminopyridine (10.0 mg, 0.08 mmol) in anhydrous tetrahydrofuran (2 mL). The reaction mixture was stirred at room temperature for 24 h. After this time, the solvent was concentrated under vacuum till solid residue. The crude mixture was submitted to flash chromatography (1:0:0.005 → 0:1:0.005 CH₂Cl₂-MeOH-Et₃N) to give NBD-C₆-3 (6) (95.0 g, 70%) as a brown solid.

Rᵣ = 0.31 (TLC eluent: 4:1 CH₂Cl₂-MeOH); [α]₀ = +39.4° (c 11.3, MeOH);

¹H NMR (300 MHz, CD₃OD): δ 8.51 (d, J = 8.8 Hz, 1H, Hₐrom), 6.35 (d, J = 8.9 Hz, 1H, Hₐrom), 5.30 (m, 2H, O(CH₂)₂CH=CH(CH₂)₇CH₃), 4.77 (d, J = 3.6 Hz, 1H, H-1), 4.26 (dd, J = 10.7, 2.0 Hz, 1H, H-6a), 4.17 (dd, J = 10.8, 5.3 Hz, 1H, H-6b), 3.89 (dd, J = 10.8, 3.6 Hz, 1H, H-2), 3.81 – 3.72 (m, 1H, H-5), 3.71 – 3.68 (m, 1H, OCH₂), 3.65 (d, J = 3.0 Hz, 1H, H-4), 3.53 (s, 2H, NHCH₂CH₂CH₂CH₂CH₂CO), 3.42 (d, J = 9.5 Hz, 1H, H-3), 3.39 – 3.33 (m, 1H, OCH₂), 2.29 (t, 2H, NHCH₂CH₂CH₂CH₂CH₂CO), 1.98 (s, 4H, O(CH₂)₂CH₂CH=CHCH₂(CH₂)₇CH₃), 1.87 – 1.76 (m, 2H, NHCH₂CH₂CH₂CH₂CH₂CO), 1.70 (dd, 2H, NHCH₂CH₂CH₂CH₂CH₂CO), 1.54 (m, 2H, NHCH₂CH₂CH₂CH₂CH₂CO), 1.50 (m, 2H, OCH₂CH₂CH₂CH₂CH₂CH=CH(CH₂)₇CH₃), 1.28 (m, 22H, OCH₂CH₂CH₂CH₃), 0.89 (t, 3H, CH₂CH₃);

¹³C NMR (100 MHz, CD₃OD): δ 175.07 (CO), 144.54, 144.48, 137.53, 130.28 (Cₐrom), 130.20 (CH=CH), 129.61 (C-1), 97.15 (C-6), 71.28 (C-5), 70.98 (C-3), 70.65 (OCH₂), 67.81 (C-2), 67.04 (C-4), 35.55, 32.43, 31.88, 31.06, 29.94, 28.86, 28.14, 27.49, 26.95, 26.37, 26.28 26.18, (OCH₂(CH₂)₇CH=CH(CH₂)₇CH₃, NH(CH₂)₅CO), 22.56 (CH₂CH₃), 13.30 (CH₂CH₃);

HRMS (ESI) m/z (calcd for C₉₆H₸₈N₅O₂₁S, 784.371), found 784.3716 (M⁺).
4.3.9. Ethyl 2-(O-tosyl)-acetate (21)

![Chemical Structure](image)

A solution of ethyl 2-hydroxyacetate (1.404 g, 13.0 mmol) and triethylamine (2.40 mL, 17.5 mmol) in dichloromethane (20 mL) was stirred for 10 min at room temperature. Thereafter, 4-toluenesulfonyl chloride, (3.810 g, 20.0 mmol) was added and the reaction mixture was stirred for more 24 h. The reaction mixture was extracted with sat. sol. sodium bicarbonate (70 mL) and dichloromethane (3 × 20 mL). The organic phase was dried over magnesium sulfate, was filtered and the filtrate was evaporated under vacuum. The resulting residue was submitted to column chromatographic on silica gel (9:1 → 4:1 petroleum ether-EtOAc) to give 21 (1.550 g, 55%) as a colorless liquid.

Rf = 0.27 (4:1 petroleum ether-EtOAc);

1H NMR (300 MHz, CDCl₃) δ 7.66 (d, 1H, H_arom), 7.23 (d, 1H, H_arom), 4.45 (d, 2H, OCH₂COCH₂CH₃), 3.98 (m, 2H, OCH₂CH₃), 2.28 (s, 1H, PhCH₃), 1.22 – 0.97 (t, 2H, OCH₂CH₃);

13C NMR (75 MHz, CDCl₃) δ 165.57 (CO), 145.09, 132.12, 129.60, 127.61 (C_arom), 64.52 (OCH₂CO), 61.38 (OCH₂CH₃), 21.07 (PhCH₃), 13.61 (OCH₂CH₃).

4.3.10. Synthesis of 2-(O-tosyl)-acetic acid (17)

![Chemical Structure](image)

A solution of 21 (836.4 mg, 3.24 mmol) and lithium hydroxide (143.4 mg, 17.5 mmol) in ethanol (20 mL) was stirred for 4 h at room temperature. Thereafter, the reaction mixture was evaporated till solid residue. Column chromatography on silica gel (9:1:0.001 → 4:1:0.001 CH₂Cl₂-MeOH-AcOH) of the crude mixture gave 17 (770.2 mg, 95 %) as a white solid.

Rf = 0.14 (9:1 CH₂Cl₂-MeOH);

1H NMR (300 MHz, CDCl₃) δ 7.71 (d, 2H, H_arom), 7.23 (d, 2H, H_arom), 4.36 (s, 2H, OCH₂COOH), 2.33 (s, 3H, PhCH₃);

13C NMR (75 MHz, CDCl₃) δ 173.14 (CO), 144.95, 131.92, 129.77, 128.00 (C_arom), 66.99 (OCH₂CO), 21.32 (PhCH₃).
4.3.11. Oleyl-2-amino-2-deoxy-α-D-glucopyranoside (23)

In a 25 mL flask, oleyl-TFA-glucosamine (11) (226.8 mg, 0.25 mmol) was in stirred with a mixture of 4:1:0.5 methanol-triethylamine-water (10 mL for 48 hours at 50 °C. After this time, the solvent was concentrated till solid residue. The resulting residue was submitted to column chromatographic on silica gel (9:1 → 4:1 CH₂Cl₂-MeOH) to give oleyl-NH₂-glucosamine (23) (140.6 mg, 74%) as a white solid.

Rₛ = 0.16 and 0.07 for α- and β-isomers (TLC eluent: EtOAc);

¹H NMR (300 MHz, CD₃OD): δ, 5.45 – 5.25 (m, 2H, O(CH₂)₈CH=CH(CH₂)₇CH₃), 4.60 (d, J = 3.4 Hz, 1H, H-1α), 4.39 (d, J = 10.1 Hz, 1H, H-1β), 4.11 – 3.60 (m, 5H, H-6a, H-6b, OCH₂, H-3, H-5), 3.49 – 3.33 (m, 2H, OCH₂, H-4), 3.06-2.97 (dd, J = 9.2 Hz, 1H, H-2), 2.10 – 1.91 (m, 4H, O(CH₂)₇CH₂CH=CHCH₂(CH₆)₃CH₃), 1.69 – 1.56 (m, 2H, OCH₂CH₂(CH₂)₆CH=CH(CH₂)₇CH₃), 1.53 – 1.09 (m, 22H, OCH₂CH₂(CH₂)₂CH₂CH=CHCH₂(CH₂)₆CH₃), 0.95 – 0.77 (t, 3H, CH₂CH₃).


In a 25 mL flask, oleyl-NH₂-Glucosamine (23) (100.2 mg, 0.23 mmol) was reacted with 2-hydroxyacetic acid (22.5 mg, 0.26 mmol), 2,3,5,6-tetrafluorophenol (44.5 mg, 0.26 mmol) and N,N'-dicyclohexylcarbodiimide (53.8 mg, 0.26 mmol) in tetrahydrofuran (10 mL). The reaction mixture was stirred at room temperature for 20 h. After this time, the solvent was concentrated till solid residue. The crude was submitted to column chromatographic on silica gel (9:1 → 4:1 CH₂Cl₂-MeOH) to give oleyl-OH-glucosamine (25) (43.1 mg, 38%) as a white solid.

Rₛ = 0.22 and 0.33 for α- and β-isomers (TLC eluent: 9:1 CH₂Cl₂-MeOH);
**4.3.13. Oleyl-2- (O-tosyl)acetamido-2-deoxy-α-D-glucopyranoside (26)**

**Method A:** In a 25 mL flask, oleyl-NH₂-Glucosamine (23) (42.5 mg, 0.10 mmol) was reacted with 17 (70.1 mg, 0.29 mmol), 2,3,5,6-tetrafluorophenol (30.7 mg, 0.15 mmol) and N,N'-dicyclohexylcarbodiimide (64.4 mg, 0.30 mmol) in tetrahydrofuran (10 mL).

The reaction mixture was stirred at room temperature for 4 h. After this time, the solvent was concentrated and the residue was submitted to column chromatography on silica gel (1:0 → 9:1 EtOAc-MeOH) to give **oleyl-O-tosyl-glucosamine (26)** (26.6 mg, 41%).

**Method B:** In a 25 mL flask, 23 (44.2 mg, 0.10 mmol) was reacted with 17 (47.1 mg, 0.21 mmol), 4-dimethylaminopyridine (6.3 mg, 0.05 mmol) and N,N'-dicyclohexylcarbodiimide (43.4 mg, 0.21 mmol) in tetrahydrofuran (10 mL). The reaction mixture was stirred at room temperature for 4 h. After this time, the solvent was concentrated till solid residue, and the residue was submitted to column chromatographic silica gel (1:0 → 9:1 EtOAc-MeOH) to give 26 (20.1 mg, 31%).

as a white solid; Rᵣ = 0.31 (TLC eluent: EtOAc);

**1H NMR (300 MHz, CDCl₃):** δ 7.79 (d, 2H, H₂α), 7.36 (d, 3H, H₃α), 6.85 (d, J = 9.2 Hz, 1H, H-2), 5.45 - 5.25 (m, 2H, O(CH₂)₈ CH=CH(CH₂)₇ CH₃), 5.07 (d, J = 8.1 Hz, 1H, H-1β), 4.72 (d, J = 3.3 Hz, 1H, H-1α), 4.55 - 4.41 (m, 2H, H-6a, H-6b), 4.15 - 3.50 (m, 5H, OCH₂, H-3, NHCOCH₂, H-5), 3.49 - 3.33 (m, 2H, OCH₂, H-4), 2.44 (d, 3H, PhCH₃), 2.11 - 1.82 (m, 4H, O(CH₂)₇ CH₂CH=CHCH₂(CH₂)₆ CH₃), 1.62 - 1.44 (m, 2H, OCH₂ CH₂CH(CH₂)₂ CH=CH(CH₂)₇ CH₃), 1.42 - 1.16 (m, 22H, OCH₂ CH₂CH₂CH₂CH=CHCH₂(CH₂)₂ CH₃), 0.96 - 0.77 (t, 3H, CH₂).
Chapter 5. Budget and Safety

5.1. Estimated project budget

5.2. Safety and Environmental context
Chapter 5. Budget and Safety

This project was developed taking into account its own sustainability, in other words, the ability of this project to maintain its operations, services and benefits during its projected life time. Always making an extra effort to encompass the design, manufacture and use of efficient, effective, safe and more environmentally benign chemical products and processes within the time and resources available. To contribute for this philosophy was estimated a research work budget (5.1) and a brief safety and environmental impact analysis (5.2).

5.1. Estimated project budget

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<th>Solvents</th>
<th>Products</th>
<th>Providers</th>
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### 5.2. Safety and Environmental context

Laboratory operators were equipped with white lab coat (cotton) and lab eyeglasses in the laboratory area. Sometimes were used gloves to manipulate solvents (latex gloves for acetone, nitrile gloves for other solvents). All operations were made inside of the fume hood. Except for washing glass materials that it was done in the washbasin after these were emptied. To manage with silica-gel powder was used a proper N95 particulate respirator/surgical mask (meets NIOSH 42 CFR 84 N95 requirements).
After purchase or partial use of chemicals from their own bottles/containers, they were stored in a fire and explosion-resistant cabinet or fridge well sectioned or in different cabinets according to acid, base, solid reagents and solvents. All disposal containers of chemical waste (made only glass or polyethylene) were labeled with respective date and contents. Organic waste bottles were capped, however, it were capped loosely to avoid a pressure buildup inside the bottle, and stored inside a fumehood until leave the laboratory to the waste storage area at the time the bottle is full. Before any chemicals or wastes stored together it was confirmed their compatible between each other. Solid residues were placed in a proper disposal container for further treatment. All organic solvent residues were separated into halogenated and non-halogenated solvents and then placed in a proper disposal container for further treatment. Before further treatment EtOAc and n-hexane were recovered by redistillation process to reuse. Resulting inorganic salts from synthesis, like NaCl, were diluted in water and thrown to the washbasin.

Every time was possible it was used petroleum ether instead of n-hexane (mainly because the price difference) and EtOH instead of MeOH (mainly because the toxicity difference).

This type of research requires a greater effort to make the treatment of wastes (solvents, secondary products), since nowadays there are no “green” alternatives that fit the requisites of this type of work (for example solvents must be organic, non-polar and well dried).
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Chapter 6. Bibliography

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7.2. Synthesis of new IG20 derivatives  67
Chapter 7. Appendix

7.1. Structures of pyranose ring interconversions

Figure 7.1. Map and structures of pyranose ring interconversions between chair (C), boat (B), skew (S) and half-chair (H) conformations [Stoddart JF, *Stereochemistry of Carbohydrates*, 1971, Wiley-Interscience, New York].
7.2. Synthesis of new IG20 derivatives

(a) SETFA, MeONa-MeOH, RT, 24 h; (b) oleic alcohol, H$_2$SO$_4$-silica, 180 °C, 20 min; (b') BTFDE, H$_2$SO$_4$-silica, 100 °C, 5 min; (b'') oleic alcohol, H$_2$SO$_4$-silica, 180 °C, 30 min; (c) SO$_3$py, pyridine, RT, 1 h; (d) MeOH-Et$_2$N-H$_2$O 4:1:0.5, 50 °C, 48 h; (e) MeOH-Et$_2$N 10:1, RT, 24 h; (f) NBD-C$_6$H$_2$COOH, EDC, DMAP, THF, RT, 24 h; (g) p-TsCl, Et$_3$N, CH$_2$Cl$_2$, RT, 16 h; (i) LiOH, EtOH, RT, 3 h; (j) 2-hydroxyacetic acid, DCC, TFP, THF, RT, 24 h; (k) DCC, TFP, THF, RT, 4 h; (l) DCC, DMAP, THF, RT, 4 h;