SYNTHESIS OF INHIBITORS OF POLYSIALYLTRANSFERASES PST AND STX

Inês Patrícia Félix OLIVEIRA

University of Bradford

2013

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Development of routes to synthesis, preparation and purification of carbohydrate and carbacycle-based potential inhibitors of the polysialyltransferase enzymes PST and STX

Inês Patrícia Félix OLIVEIRA

Submitted for the degree of Master of Philosophy

Institute of Cancer Therapeutics, School of Life Sciences

University of Bradford

2013

ABSTRACT

Inês Patrícia Félix OLIVEIRA

Synthesis of inhibitors of polysialyltransferases PST and STX

Keywords: carbohydrate, cancer, polysialyltransferase, polysialic acid, glycosylation

PolySialic acid (polySia) is a linear carbohydrate homopolymer of α-2,8-linked sialic acids and a posttranslational modification of NCAM (neural cell adhesion molecule), biosynthesized by combined action of two polysialyltransferase enzymes, ST8SiaIV(PST) and ST8SiaII(STX).

PolySia alters NCAM-dependent cell adhesion that is crucial for the CNS development. In adulthood, polySia expression is largely absent persisting only in areas of the brain associated with neuronal plasticity. Significantly, a number of malignant tumours re-express polySia and there is considerable evidence that its presence is related to higher malignancy, invasion and metastasis.

The hypothesis underpinning this project is that inhibition of polySia biosynthesis will prevent (or reduce) tumour cell migration and invasion, thereby reducing the incidence of metastasis, which will lead to higher patient survival.

The work reported in this thesis describes efforts towards the synthesis polysialyltransferase inhibitors that are structural analogues of CMP-Neu5Ac, the natural substrate. Specifically, development of

methodology to synthesise building blocks suitable for conjugation as inhibitors is described.

Quinic acid-based substrate analogues were explored, with a focus on development of chemistry to achieve substitution of C1-OH. Several protected quinic acid-based compounds were synthesized, and deoxygenation of the C1-OH through the use of a Barton-McCombie reaction was accomplished successfully, allowing an attempt to introduce different aliphatic groups at C1 position using the Mukayiama reaction. Synthesis of a cytidine building block, suitable for conjugation to either quinic acid or sialic acid is also reported. In parallel, studies towards the development of sialoside disulfide analogues are described, with novel conditions identified for their synthesis.

ACKNOWLEDGMENTS

I would like to thank the ICT (Institute of Cancer Therapeutics), Dr. Robert Falconer and Prof. Laurence Patterson for the opportunity to carry out this research.

I want to thank especially to Dr. Falconer for all the help and guidance during my placement, for all the advice and support during the hard times, when I felt helpless and it seemed that none of the reactions were working, he pushed me not to give up because chemistry was sometimes like that, sometimes it worked but many times it didn't. I want to thank him as well for the empathy and understanding when I decided not to continue my PhD and to leave at the end of the first year because I wasn't able to adapt to living in Bradford away from my family, because the last thing that I want was to be harmful for the continuation of the PolySia project or to the ICT.

I would like to thank the FCT (Fundação para a Ciência e Tecnologia) in Portugal, who sponsored my grant and enabled me to be part of the ICT and the PolySia project during this year. May they continue to sponsor young scientists in the years to come.

Last but not least I would like to thank my family, boyfriend and friends, especially Rita Santos, who was and is my foundation pillar in Bradford, for all the support and patience during this period of my life, cheering me on and then supporting me when I decided to return to Portugal.

PUBLICATIONS ASSOCIATED WITH THIS WORK

Full papers in peer-reviewed journals

Ribeiro Morais, G.; **Oliveira, I.P.F.**; Humphrey, A.J.; Falconer, R.A. "Identification of an acetyl disulfide derivative in the synthesis of thiosialosides." *Carbohydr. Res.*, **2010**, *345*, 160-162.

Conference abstracts and proceedings

Falconer, R.A.; Al-Saraireh, Y.M.J.; Sutherland, M., Springett, B.R.; **Oliveira, I.P.F.**; Shnyder, S.D.; Patterson, L.H. "Polysialyltransferase inhibition and tumour cell migration" YCR Annual Scientific Meeting, 14 June **2011**, Harrogate, UK. Abstract 12.

Ribeiro Morais, G.; **Oliveira, I.P.F**.; Springett, B.R.; Falconer, R.A. "Identification and utility of an acetyl disulfide derivative in the synthesis of thiosialosides" 18th International Conference on Organic Synthesis, 1-6 August **2010**, University of Bergen, Norway.

Oliveira, I.P.F.; Ribeiro Morais, G.; Springett, B.R.; Falconer, R.A. "Key new observations in the synthesis of thiosialosides" Abstracts of Papers, 240th ACS National Meeting, 22-26 August **2010**, Boston, MA, USA. Abstract CARB-84.

Falconer, R.A.; Al-Saraireh, Y.M.J.; Springett, B.R.; **Oliveira, I.P.F.**; Sutherland, M.; Gill, J.H.; Shnyder, S.D.; Patterson, L.H. "α-2,8-Polysialyltransferase: a target for the development of anti-metastatic agents" YCR Annual Scientific Meeting, 22 June **2010**, Harrogate, UK. Abstract 41.

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LIST OF ABBREVIATIONS

AcBrGluc – acetobromo-α-D-glucose

AcGluc-1SAc – 1-(acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosate

AcGluc-1SSAc–1-(acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranosate

Ac – acetyl

Ac_2O – acetic anhydride

AIBN – azobisisobutyronitrile

AIDS – acquired immunodeficiency syndrome

Alk. – alkylating specie

AMP – adenosine monophosphate

app. - apparent

ATP – adenosine triphosphate

ax – axial

BDNF – brain-derived neurotrofic factor

BnBr - benzylbromide

Boc_2O – di-*tert*-butyl dicarbonate

CAMs – cellular adhesion molecules

Cat. – catalyst

CDP – cytidine diphosphate

CMP – cytidine monophosphate

CNS – central nervous system

CTP – cytidine triphosphate

Cyt-5'TBDMS-Boc2: *N,N*-Di-*tert*-butyldicarbonate- 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine

Cyt-5'TBDMS-Boc1: *N*-*tert*-butyldicarbonate- 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine

Cyt-5'OH-Boc2: Synthesis of *N,N*-Di-*tert*-butyldicarbonate- 2´,3´-*O*-isopropylidene cytidine

Cyt-5'OH-Boc1: *N*-*tert*-butyldicarbonate- 2´,3´-*O*-isopropylidene cytidine

C=C – carbon-carbon double bond

C=O – carbonyl

DCE – 1,2-dichloroethane

DCM – dichloromethane

Desic. agent – dessicating agent

DMAP – 4-dimethylaminopyridine

DMF – dimethylformamide

DMP – 2,2-dimethylaminopropane

DNA – deoxyribonucleic acid

ECM – extracellular matrix

ELISA – enzyme-linked immunosorbent assay

Endo-N – endoneuraminidase

eq – equivalent(s)

eqt – equatorial

EtI – iodo ethane

EtOAc – ethyl acetate

extrac. – extraction

FAK – focal adhesion kinase

FGF – fibroblast growth factor

Filt. – filtration

Fyn – tyrosine-protein kinase Fyn

GNE – UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase

GPI – glycophosphatidylinositol

HPLC – high performance liquid chromatography

hr/hrs – hour/hours

HRMS – high resolution mass spectrometry

Ig – immunoglobuline

iso – isopropylidene

Ki – inhibion constant

Km – Michaelis constant

KSAc – potassium thioacetate

L1 – L1CAM

LDA – lithium diisopropylamide

LRMS – low resolution mass spectrometry

MAP – mitogen activated protein kinase family

mDC – mature dendritic cells

Met. - method

min - minutes

mRNA – messenger ribonucleic acid

MS – mass spectroscopy

MW – molecular weight

NCAM – neural cell adhesion molecule

Neu5Ac – N-acetylneuraminic acid (Sialic acid)

Neu5Ac-2en – Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-glycero-D-talo-non-2-enonate

Neu5Ac-2SAc – Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-S-acetyl-2,3,5-trideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyrosonate

Neu5Ac-2SSAc – Methyl 2-(acetylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyrosonate

Neu5Ac-2SBn – Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-S-benzyl-2,3,5-trideoxy-2-thio-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate

Neu5Ac-2SSBn - Methyl 2-(benzylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate

Neu5Ac-2SEt – Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-S-ethyl-2,3,5-trideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyranosonate

Neu5Ac-2SSEt - Methyl 2-(ethylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyranosonate

neut. – neutralization

NH_4Cl – ammonium chloride

nM – nano molar

NMR – nuclear molecular resonance

ovn. – overnight

PCR – polymerase chain reaction

polySia – polySialic acid

ppm – parts per milion

p-TSOH – *para*-Toluene sulfonic acid monohydrate

Pyr – pyridine

R_f – retardation factor

RT – room temperature

Reag. – reagent(s)

recryst. - recrystalization

s.m. – starting material

Solv. – solvent(s)

Src – src family kinase

TAG-1 – TAG-1 cell adhesion molecule

TBAF – tetrabutylammonium fluoride

TBDMSCl – *tert*-Butyldimethylsilylsilane chloride

TBDPSCl – *tert*-Butyldiphenylsilylsilane chloride

Temp. – Temperature (ºC)

TGFα – tumour growth factor alpha

THF – tetrahydrofuran

TLC – thin layer chromatography

TMSCl - trimethylsilylchloride

TTMSS – tris(trimethylsilyl)silane

UDP – uridine diphosphate

UMP – uridine monophosphate

UTP – uridine triphosphate

UV – ultraviolet

VEGF – vascular endothelial growth factor

w.up – work up

µM – micro molar

η ac. – after column yield

η qt. – quantitative yield

η ov. – overall yield

1. INTRODUCTION

1.1. TUMOURIGENESIS – BIOLOGY, GENETICS AND EMERGING HALLMARKS OF CANCER

Cell multiplication is a process that requires complex regulatory mechanisms that ensure its response to specific body needs at the different times during tissue and organ development.[1,2] Cancer is a disease that results from the breakdown of fundamental regulatory mechanisms of normal cell behaviour which include proliferation,

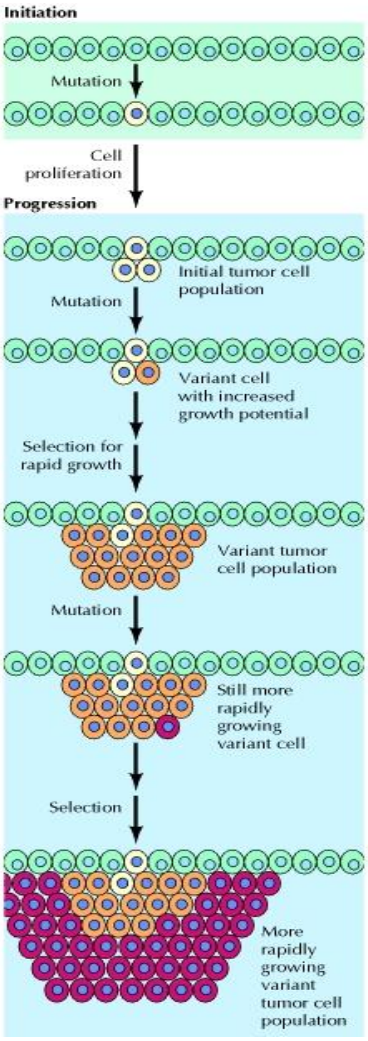


Fig. 1. 1 – Stages of tumour development.[1]

differentiation and survival and are essential for maintaining normal function in a multicellular organism.[1] Loss of regulation triggers uncontrolled growth and division which can ultimately result in formation of a mass, tumour, and spreading of the cancer cells throughout the body, with establishment of secondary areas of growth, a process called metastasis, that can interfere with the function of normal tissues and organs. [1,2]

Tumourigenesis is an accumulation of abnormalities, a multistep process, and reflects the progressive genetic alterations/mutations that drive the gradual transformation of normal somatic cells into highly malignant derivatives. Because of that it's a disease that develops late in life and is associated with the elderly population (**Figure 1.1**).[1,2,3]

Cells need to be in division state so that the mutations pass to progeny cells and lead eventually to cancer, that's why cancers originating from non dividing cells, such as muscle and nerve cells, are not common in adulthood.[2] Studies in experimental human and animal models revealed that specific genes, called oncogenes, are capable of inducing cell transformation and tumour formation. They originated from the activation of proto-oncogenes in normal cells as a result of mutation or suffered DNA rearrangements. Most of the oncogene proteins (e.g. *bcl*-2, a cell death suppressor that prevents apoptosis) function as signalling molecules including polypeptide growth factors, growth receptors and transcription factors. Simultaneously tumour suppressor genes are lost or inactivated in cancer cells, removing the negative control upon proliferation since it's activation after DNA damage leads to cell cycle arrest allowing DNA repair to occur or apoptosis if the damage is excessive (e.g. p*53* gene which is

inactivated and/or mutated in several human cancers such as liver, breast, colon and skin).[1,2,3,4] Although cancer is caused by gene mutations, it cannot be considered a genetic disease by itself, but there are some cases where some individuals have inherited genetic mutations that predispose them to develop some types of cancer.[2]

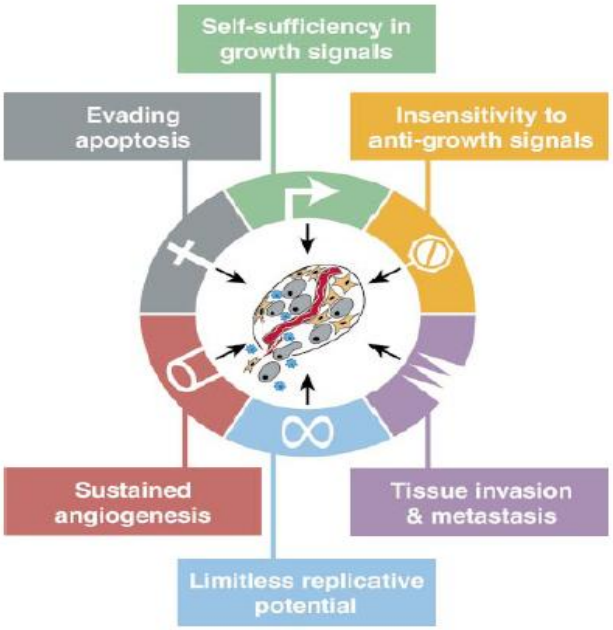


Figure 1.2 – Acquired capabilities of cancer cells. [3]

Recent research revealed some shared acquired capabilities in most types of human cancer as a result of enabling characteristics as genome instability and increased mutability, described previously, and heterotypic interactions in the tumour microenvironment between cancer cells and its normal neighbours (**Figure 1.2 and 1.3**). [1,2,3]

These cancer enhancing interactions between cells include an inflammatory state, that although occurring primarily as an attempt to eradicate altered cells, has tumour promoting effects, through the supply of growth factors, enzymes (that facilitate angiogenesis, invasion and

metastasis) and release of reactive oxygen species that are oxidants mutagenic and carcinogenic. [1,2,3]

Tumour cell characteristics include the features described below (**Figure 1.2**):

- They are not sensitive to antigrowth signals, density-dependent and contact inhibition of cell growth, so they do not become quiescent (G_0 stage) in the cell cycle and fail to differentiate normally and enter a postmitotic stage; [1,2,3]
- They are not dependent on extracellular growth factors because most of them produce their own (e.g. VEGF, vascular endothelial growth factor associated with angiogenesis process and TGFα, tumour growth factor alpha) which leads to an autocrine growth stimulation (continuous autostimulation of cell division) or elicit neighbour cells in their microenvironment to synthesize them, leading to deregulation of cell growth signaling pathways; [1,2,3]
- Most cancer cells are less adhesive, which reflects alterations in cell-cell and cell-matrix interactions and loss of adhesion molecules expression (e.g. NCAM, which is going to be further discussed ahead due to its involvement in this project, E-cadherin and integrins), that relates to morphological and cytoskeletal alterations, involving secretion of extracellular protease enzymes that degrade extracellular matrix components and facilitate invasion of stroma cells, blood vessels and normal epithelial cells, contributing to invasiveness of adjacent tissues

and metastasis in distant sites where nutrients and space are more available; [1,2,3]

- They evade programmed cell death, apoptosis, after DNA damage, hypoxia or oncogene hyperexpression, mainly by loss of proapoptotic regulators (e.g. mutation in tumour suppressor gene p*53*); [1,2,3]
- They have unlimited replication as a result of uncoupling of the cell growth from cell-cell signalling and its environment, defects in DNA-repair machinery and chromosomal abnormalities, such as upregulation of telomerase expression in 85-90% of cancer cells (enzyme required to maintain the ends of eukaryotic chromosomes), because in normal cells these enzymes are progressively less expressed in each cell cycle and eventually there as complete loss of telomeres and consequently cell death; [1,2,3]
- Ability for angiogenesis, through the secretion of growth factors (e.g. VEGF and FGF1/2, fibroblast growth factor) and downregulation of endogenous inhibitors (thrombospondin-1 and β-interferon), allowing the formation of new blood vessels that are going to supply oxygen and nutrients allowing the continuous growth, proliferation and metastasis of cancer cells. The important role of angiogenesis is demonstrated by clear evidence of the use of anti-angiogenic agents to impair tumour growth, with agents already in use in the clinic. [1,2,3]

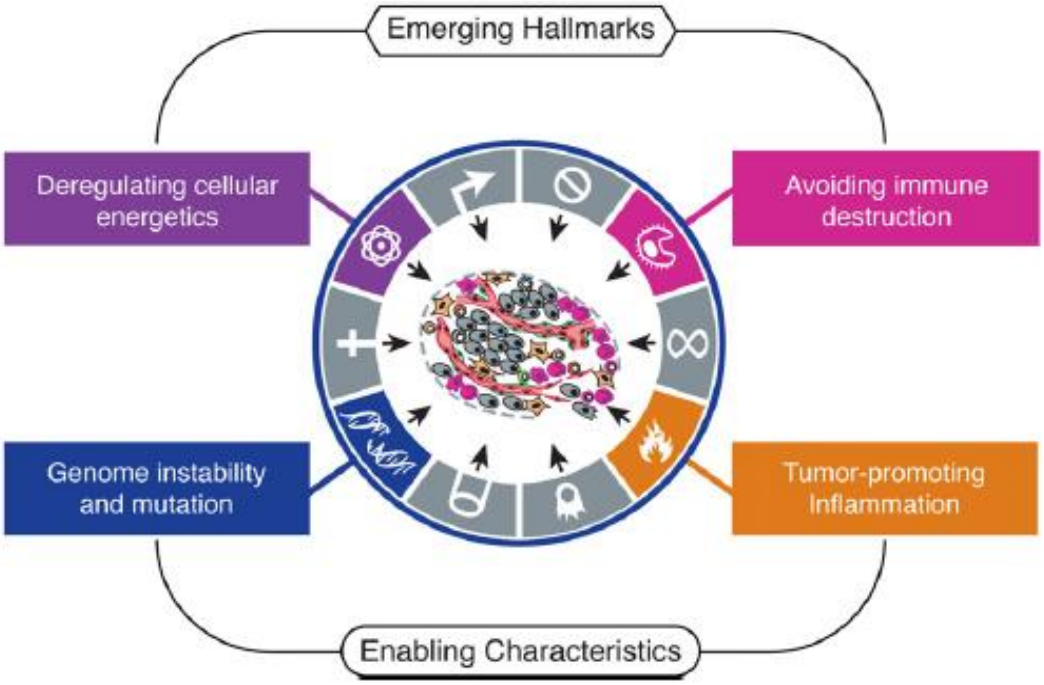


Fig.1.3 Emerging Hallmarks of cancer and Enabling characteristics. [5]

Nowadays there are two more emerging targets that are currently under study in an attempt to try to evaluate their usefulness as cancer therapy targets (**Figure 1.3**): [5,6]

- Alteration of the cellular metabolism essential to respond to increasing energetic needs due to increased growth and proliferation of cancer cells. This characteristic also enables cancer cells to produce energy even in hypoxic conditions because glycolysis is the main energetic process.[5,6]
- Evasion from destruction by the immune system, showing that the body's immune response can have tumour promoting effects, through the inflammation process described previously as an enhancing feature in tumour development, but also tumour growth and progression

inhibition through the possible recognition and elimination of damaged cells. This is supported by the fact that there is an increase of some types of cancers in immunocompromised patients (e.g. AIDS).[5,6]

The physiological changes described previously are therefore considered *hallmarks of cancer* and are all related and dependent on one another in a complex circuitry and progressively acquired during tumour development. These are also key targets for new mechanism-based anticancer drug development (**Figure 1.4**). [3,6]

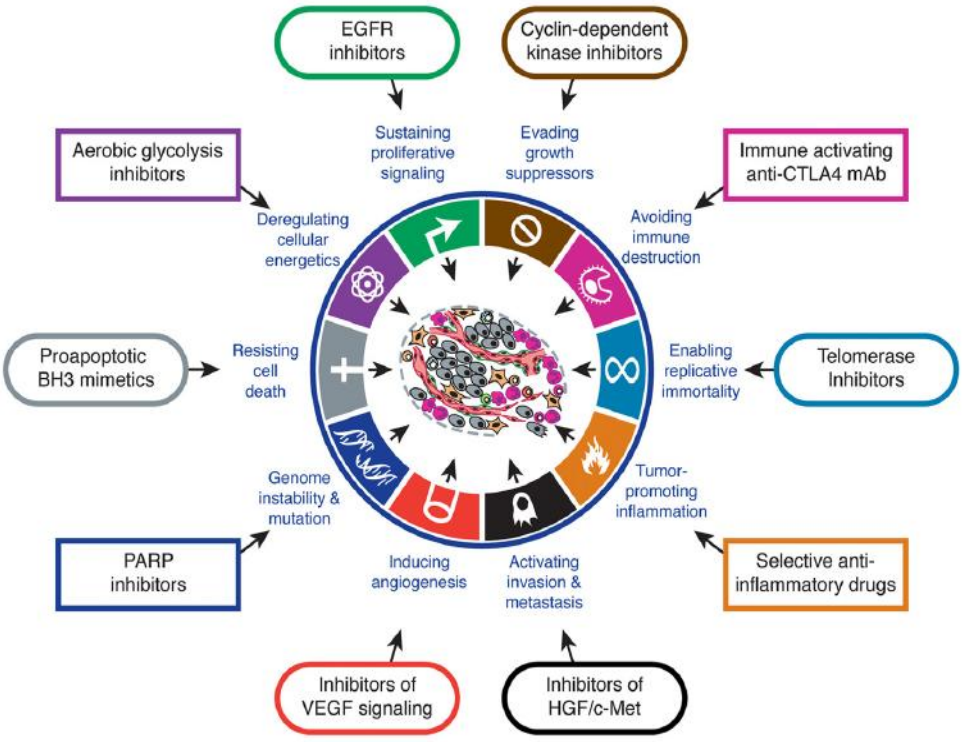


Fig. 1.4 Therapeutic targeting of the hallmarks of cancer. [5]

1.2. CANCER STATISTICS AND THE IMPORTANCE OF NEW THERAPEUTIC STRATEGIES

There are more than a hundred types of cancer, according to the cells from which they originate in the body they can be classified into 3 main groups:

- Carcinomas are the most common type, originating from epithelial cells and account for 90% of all human cancers;[1]
- Sarcomas are the more common solid tumours in animals and originate from connective tissues, such as muscle, bone and cartilage;[1]
- Leukaemias or lymphomas originate from blood-forming cells and cells from the immune system respectively, and their behaviour and response to treatment can vary substantially.[1]

Only malignant tumours are referred to as cancer, because they are capable of invading surrounding normal tissues and spread via the circulatory and lymphatic systems, a process that is named metastasis and is responsible for most (90%) of cancer related deaths due to increased resistance to treatment. Benign tumours are localised to a specific area and can be removed surgically. [1,3]

According to the World Health Organization, 12.66 million people worldwide were diagnosed with cancer in 2008 and 7.56 million died from this disease. [7]

The most common types of cancer, that account for 40% of the world's total are: lung, female breast, colorectal and stomach (**Figure 1.5**) and their

incidence and also geographic distribution depends on factors that include age, sex, race, genetic predisposition and exposure to environmental carcinogens among others.[4,7]

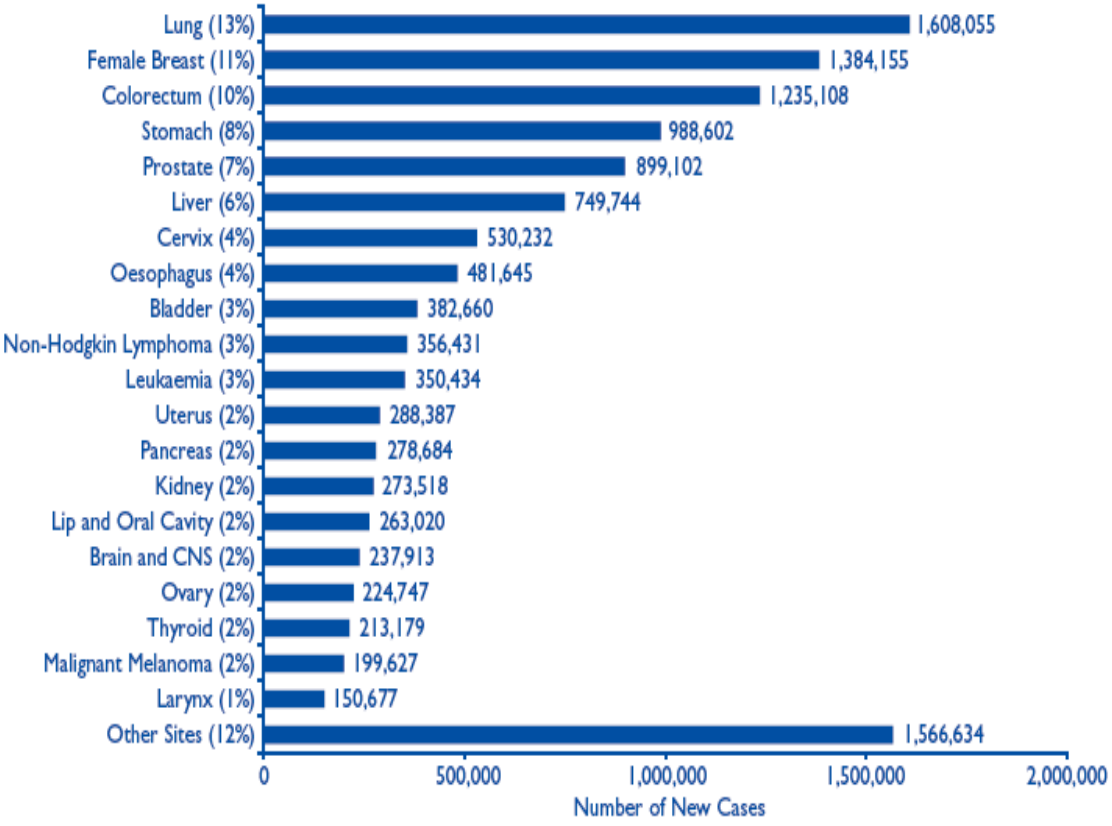


Fig. 1.5 The 20 most commonly diagnosed cancers worldwide, 2008 estimates. [7]

Carcinogens, like radiation and chemicals can damage the DNA and induce mutations, tobacco smoke being the most talked about in the media because of clear evidence relating it to 80 to 90% of lung cancers. But there are other known causes of cancer such as tumour promoters, like hormones, that stimulate growth of some human cancers (e.g. Oestrogen and breast cancer) and viruses (e.g. Human Papilloma Virus and cervical cancer).[1,2,4]

As the world's population is progressively ageing due to increased life expectancy in developed countries which is a result of improved health

assistance, and because cancer is predominantly a disease of the elderly, an increase in the number of cancer cases is expected in coming years.[7]

Ideally, prevention of cancer would be the main approach to this disease or early detection of premalignant stages where the disease is still localised and can be removed surgically or treated with radiation with higher success cure rates and less side effects and systemic toxicity. Once the cancer has spread and micrometastasis are detected there is the need for a systemic approach with the use of chemotherapy drugs, but unfortunately there are problems to overcome with their use because most of them are still not selective to cancer cells (most targets are common to normal cells). They are also often very toxic to the patients, which limits success. The other problem is chemoresistance, because usually when the disease has already spread it is difficult to reach the tumour localization in cytotoxic concentrations (e.g. nervous system and testes) and eventually acquired resistance develops, although there are also some cases of inherent resistance related to the genomic instability of cancer cells that can trigger, for example, the overexpression of transporter molecules that pump out the drugs from the cells. Besides that there can be an adaptation of the cancer microenvironmental cells due to the survival of some cancer cells after the first treatment which allows tumour renewal and clinical relapse, because acting upon one key pathway of tumourigenesis may not disable it completely or may cause a shift to another supporting hallmark characteristic. [5,6]

To overcome these issues, combination chemotherapy is nowadays the main approach, with simultaneous use of drugs with different but complementary mechanisms of action and no overlapping toxicities. [1,4]

The need for better cancer therapeutics demands an improved knowledge of molecular characteristics for the design of more efficient and selective protocols. Here is where molecular diagnostics plays a key role, since it allows the discovery of new therapeutic targets and new genes involved in the origin of the disease. The design of personalised therapeutics could in fact increase the survival rate of patients, and maybe even cure them. [8]

1.3. NEURAL CREST-DERIVED CANCERS AND POLYSIA AS AN EMERGING THERAPEUTIC TARGET

Prevention of tumour dissemination - localized invasion and metastasis - is crucial to patient outcome, since once tumour cells spread from the primary location, morbidity and mortality of cancer increases exponentially. [9]

Localized invasion, which can be seen in cancers such as glioblastoma and bladder cancer requires: partial separation from the primary mass; reorganization of extracellular matrix through actin polymerization and up-regulation of proteolytic enzymes, and consequently migration through it; and survival in adjacent tissues in a syncytial manner. Metastasis, characteristic of lung and breast carcinomas, is similar but individual cells manage to enter the vasculature and reach other organs in distant locations. Motility, of a mass of cells or individual cells, is therefore a rate-limiting process in tumour dissemination. Some cancers such as melanoma are even able to use both mechanisms of tumour dissemination and for that reason it is essential that new cancer therapies target both invasion and metastasis. [9]

Epithelial-to-mesenchymal transition (EMT) and growth-factor induced motility are common characteristics of both processes. EMT allows loosening up of the primary tumour cell mass, but it's difficult to target due to its transient nature, since during metastasis this phenotype is converted to a more mesenchymal state which allows the seeding/survival at the secondary location. Nonetheless there are cells that can move through the ECM by acquiring rounded/amoeboid-like morphology that allows movement of cells without the need for matrix proteolysis, due to the matrix natural porosity. Growth-factor induced motility could be used in the future to make cancer a chronic condition, reducing initial and secondary dissemination, since solid tumours produce both autocrine and paracrine factors that trigger motility signalling cascades, and there are already clinical results using inhibitors for the EGF receptor (epithelial growth factor) although with limited success. [9]

Targeting metastasis *per se* is challenging, since commonly when the disease is diagnosed, metastasis or invasion already happened. Design of clinical trials to determine the efficacy of these new drugs is also a challenge since these drugs are unlikely to be used as single agents (they will be add-on therapies) and monitoring of tumour nodule size it's not applicable, it would be necessary to design a trial that would measure progression-free time and overall survival, which is possibly more expensive and requires follow-up of a larger number of patients. Measurement of a metastatic biomarker may be a possible solution in some cases. Nonetheless agents targeting tumour dissemination would be able to stop cancer spread, enabling effective limiting of tumour progression. Agents may also enhance

the efficacy of anti-proliferative agents, by limiting transitions to metastatic or resistant cell phenotypes. [9]

Neural crest cancers, such as small cell lung cancer, neuroblastomas and gliomas, are some of the most difficult cancers to treat. Neuroblastoma is one of the more common cancers during childhood, with the tumour development process mirroring neuronal development. [4,10] Only leukaemias cause more deaths in children. [4,10] Brain tumours (e.g. glioma, astrocytoma) do not metastasise, but invade the surrounding brain tissue, a feature related to the existence of an anatomic structure, the Blood-Brain Barrier which controls the input and output of substances to and from the brain. It is also related to the difficulty of delivering chemotherapy drugs to that area and due to their limited efficacy and few therapeutic options (lipophilic drugs like nitrosoureas that can easily cross the barrier are more likely to be used). [8,11,12,13]

Due to the enunciated problems and because surgical removal cannot be applied to all cases, the identification of new therapeutic targets taking in consideration the specific characteristics of individual cancers is of paramount importance. Starting from there, researchers revealed that the key hallmark, *invasiveness*, was related to defects in cell interactions due to a specific posttranslational modification of NCAM (neural-cell adhesion molecule) through the addition of a carbohydrate polySia (polysialic acid).

1.3.1. CELL ADHESION DEREGULATION - POLYSIA EXPRESSION ON NCAM AS A BIOMARKER FOR A SERIES OF CANCERS

Polysialic acid (polySia) has been demonstrated to be a biomarker for a series of cancers and presence of polySia in serum is considered to be a disease marker.[14,15,16] It is expressed in cancers derived from the neural crest cells, multipotent vertebrate cells that give rise to several cell lineages, such as melanocytes, peripheral and enteric neurons, glia cells, smooth muscle, craniofacial cartilage and bone during embryogenesis.[8,11,12,13] These tumours include glioblastoma [11,12], rhadomyosarcoma [11,17,18], neuroblastoma [11,13,19,20], medulloblastoma, pancreatic cancer [11,21], pituitary adenoma (where NCAM-polySia is also related to higher growth rate of the tumour) [22], small-cell lung cancer [23] and Wilm's tumour [24]. However, studies also demonstrated that polySia can be found in non-neural crest tumours such as multiple myeloma [11], non-small cell lung cancer, [17] thyroid cancer [14] and colorectal tumours (with nodal invasion) [25] and be responsible for the enhanced migration of cells due to the loss of focal adhesion, cell-to-extracellular matrix adhesion and metastasis. [15,21,26]

To determine the role of polySia in tumour growth and development there are several studies of note in the literature. A selection of these will now be explored.

PolySia is known to promote *in vitro* migration of neuroblastoma cells, and to influence tumour cell growth and differentiation through affecting the

NCAM signalling cascade. This involves the triggering of tyrosine kynase molecules responsible for the adhesion process. It is due to the expression of polySia in neuroblastoma that the scientific community first started to take interest in this interesting phenomenon in cancer. As a result most of the early information available focused on this type of cancer. More recently, a number of studies have emerged referring to the role of polySia in other tumours – its expression is widespread in cell lines for the tumour types previously outlined. [13,15,16,19]

In vivo studies using polySia-expressing xenografts, implanted into nude mice, demonstrated the linkage between polySia expression and the appearance of disseminated lung metastases. These *in vivo* models using polySia-expressing and polySia-negative xenografts clearly demonstrate a clear relationship between polySia-NCAM and tumour invasion and metastasis: the number and extent of metastatic tumour deposits in lung cancer [15], neuroblastoma [13,15,16,19], pituitary cancer (topic explored ahead) [22] and glioma.[12]

Studies have demonstrated that polySia controls NCAM signals at cell-cell contacts to regulate focal adhesion processes in rhabdomyosarcoma and neuroblastoma cell lines – TE671, Kelly and SH-SY5Y (polySia+/NCAM+). Studies in these cells revealed, using scratch assays in the presence of endo-N enzyme, that removal of polySia from NCAM from cell-cell contact sites, reduced focal adhesions in a NCAM-dependent manner through alteration of NCAM signalling pathways. PolySia enhanced cell migration, which is a feature of malignancy in these types of neural crest tumours. [25]

Studies of human pituitary adenomas, a type of cancer that although is mainly benign, can behave aggressively and invade the surrounding tissues, revealed that NCAM and polySia can be found in both normal and tumoural pituitary gland. The question to be answered was if the situation was similar to that observed in other neural crest tumours, polySia-NCAM could be tumour marker for malignant pituitary tumours and be related to growth rate and malignancy. For that purpose an *in vivo* model for spontaneous tumours using SMtTW – Wistar/Furth WF/Ico female rats was used successfully by Daniel et al and high polySia-NCAM levels were observed in tumours with malignant features, such as subcutaneous growth, invasion and metastasis. [14]

It is key to note that through the use of EndoN in several of the studies already mentioned (enzyme that is able to remove polySia from NCAM in a specific and selective manner), and together with the data from studies using *in vitro* siRNA knock-down of the polysialyltransferase enzymes, PST and STX (enzymes that are responsible for the biosynthesis of polySia and that are going to be explored ahead in section **1.3.2.**), came to substantiate that by removing polySia from NCAM, via an enzymatic manner or by removal of polysialyltransferase enzyme expression, both had the same result: the abolition of cell migration.[21]

NCAM-180 the membrane linked isoform of NCAM loses its expression in aggressive forms of colon tumours and correlates with poor prognosis, so it was speculated that polySia-NCAM could also be an oncodevelopmental antigen for colorectal cancer, since it was only observed in tumour tissues and not in control/healthy tissues. There are today studies

using colorectal patients to determine the prevalence of polySia+/NCAM+ and how that affects prognosis. It was observed by Briera et al that lymph node metastasis at surgery was correlated with polySia-NCAM coexpression, which reflected in a poor patient prognosis. [25]

PolySia-NCAM exerts its effects over cell adhesion processes in a very complex manner. For that reason and to help to expand upon some of the information mentioned previously, deregulation of cell adhesion processes and its relation to CAMs (cell adhesion molecules, where NCAM is included) and its relation to polySia will now be considered in more detail.

The deregulation of cell adhesion is therefore a key step in tumour invasion and metastasis, as stable cell-cell interactions are essential for the organization of cells into tissues. The process of cell-cell adhesion is selective, because cells only adhere to specific neighbour cells, a process that is mediated by specific Cell Adhesion Molecules (CAMs) in which NCAM is included. [1,6,20] CAMs are integral membrane proteins responsible for cell-cell and cell-matrix homophilic (same cell type) and heterophilic (different cell type) adhesion and interaction. They are divided into 4 groups – selectins, integrins, immunoglobulin superfamily and cadherins, and they communicate directly with the intercellular signalling pathways (**Figure 1.6**). [2,3]

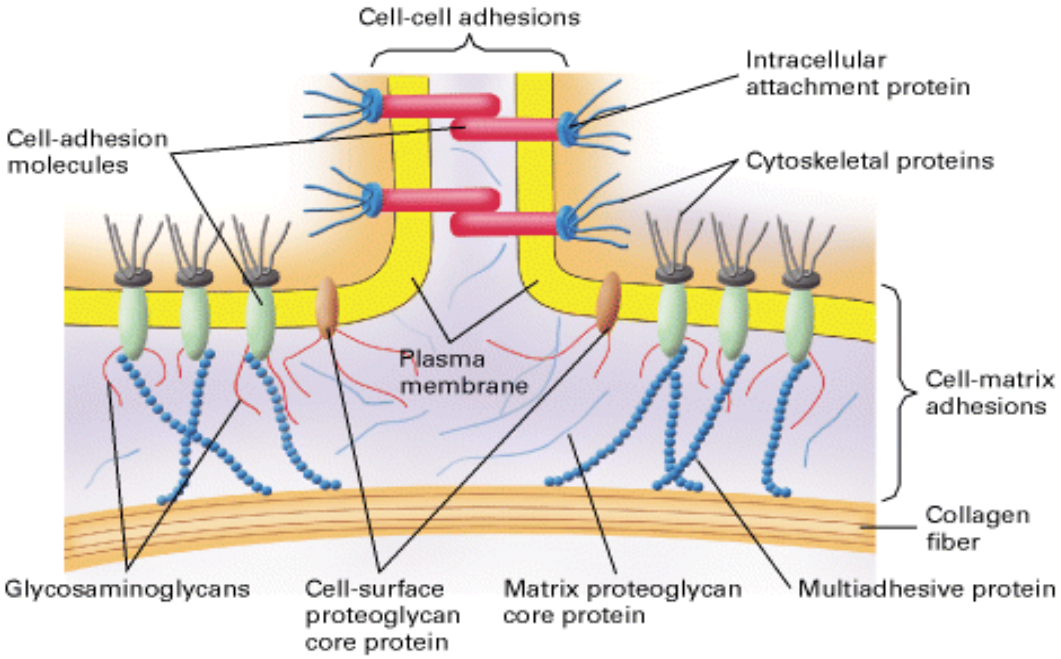


Fig. 1.6 Types of molecules that bind cells to each other and to the extracellular matrix. [2]

NCAM belongs to the immunoglobulin family and plays an important role during development and differentiation of nervous tissue (glia and nerve cells). This cell surface glycoprotein was identified in the 1970s and is encoded by one single gene with 20 major exons and its isoforms are generated by mRNA alternative splicing (**Figure 1.7**). [2,27]The three isoforms of NCAM, differ in length (180, 140 and 120 KDa), protein backbone, mode of attachment to the cell membrane and content of sialic acid residues. [2,3] NCAM 180 is a single pass transmembrane protein and the difference to NCAM 140 is that in the last one the cytoplasmic domain is considerably shorter. NCAM 120 is a GPI-anchored protein without cytoplasmic domain. There is also a secreted form of NCAM that can be formed by enzymatic excision of NCAM 120 GPI-anchor and is related to the formation of truncated NCAM. [18,27,28,29]

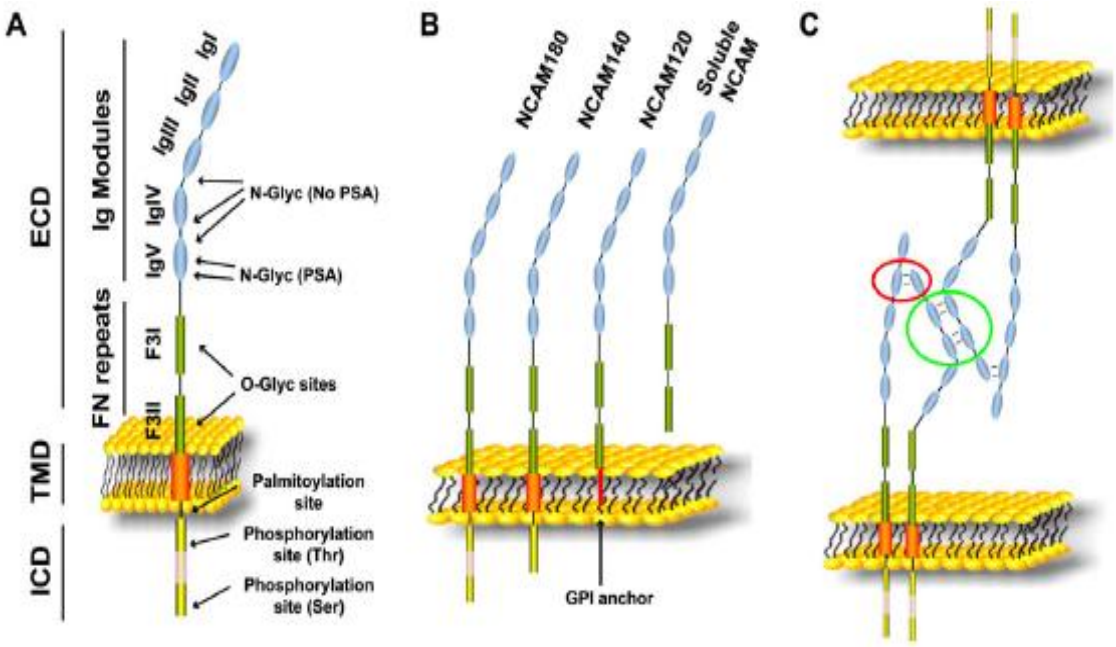


Fig. 1.7 A) NCAM structure; B) NCAM isoforms; C) NCAM cis and trans-interactions.[27]

Human NCAM extracellular motifs responsible for cell interactions are: five immunoglobulin-like domains (IgI to IgV) with six *N*-glycosylation sites, followed by two fibronectin type III-like domains (F3I, which appear to act as the initial recognition site and docking for the polysialyltransferase enzymes that are going to be described later in this section, and F3II) (**Figure 1.7 and 1.9**). [18,27,28,30]

The adhesive properties of NCAM are modulated by addition of α(2→8) chains of sialic acid molecules to the *N*-glycosylation sites to form polySia. (**Figure 1.8**)

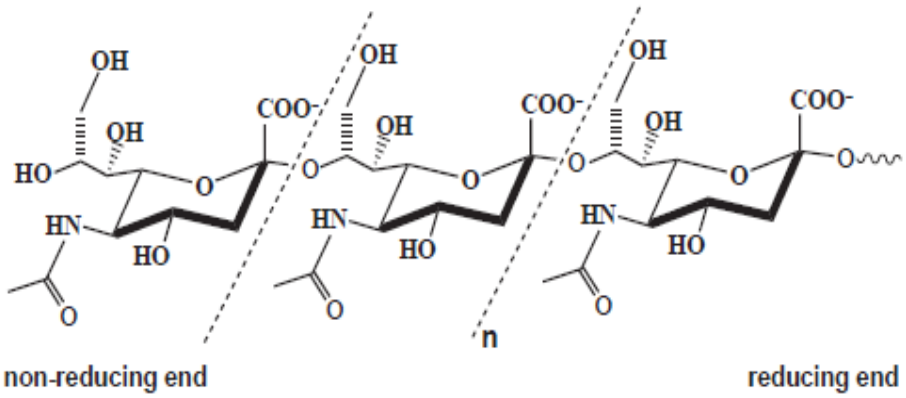


Fig. 1.8 Chemical structure of polysialic acid. [50]

PolySia is a negatively charged sugar, switching NCAM from an adhesive form, in its absence, to a poorly adhesive form in its presence, which enables the modulation of cell interactions and adhesion. NCAM-polySia also triggers cellular signalling cascades. [2,3,18,27,28]

There are an increasing number of studies focussing on the crucial role of glycans (assemblies of oligo and polysaccharide sugars) in tumourigenesis, since glycosylation is a common modification to cell membranes and secreted proteins. This has a major impact in protein folding, stability and function of native proteins, regulating through intracellular and intercellular signalling cascades. This in turn affects the stages of tumour development: proliferation, invasion, metastasis and angiogenesis, and has emerged in the last decades as an important new therapeutic targets for cancer therapy. [27,31]

These sugars can exist in free form, such as hyaluronan, or in the form of glycoconjugates, with lipids, such as glycophingolipids (oligossacharides linked to ceramide) or proteins, as in the case of proteoglycans (heparin

sulphate and chondroitin sulphate) and glycosylphosphatidinylinositol (GPI)-linked proteins. Glycoproteins are very abundant on the cell surface and the linkage can be made through an asparagine amino acid (*N*-linked glycoside) or through serine or threonine amino acids (*O*-linked glycoside). [31]

N-glycosylation of glycoproteins is the prevalent form, being related with protein folding, cell recognition and cell adhesion, and because it is so prevalent it was hard in the beginning for researchers to correlate altered *N*-glycans to the development of some types of tumours. [31]

The majority of glycans exist as membrane-bound glycoconjugates, in the glycocalyx (extracellular structure that coats the plasma membrane of cells and it's formed by glycans and glycoconjugates), and this localisation in the cell membrane is what allows them to be such a crucial part of the cell adhesion (cell-cell and cell-matrix), communication and motility process, since even small changes in glycosylation can affect cell signalling cascade of tyrosine kinase phosphorilations. In fact some of the first-identified tumour-specific antibodies where directed against carbohydrate antigens on tumour glycoproteins. [31,32,33]

One of the main alterations in some types of tumours that were noticed by researchers was the overexpression of glycoconjugates with polysialic acid on cell membrane-located NCAM. (**Figure 1.9**) [31]

Sialic acid (Neu5Ac) is the terminal monosaccharide on glycoconjugates that is most abundant in eukaryotic cells, and besides being involved in cell interactions, as already stated, is also present in the capsule of some bacteria - that cause meningitis facilitating their escape to the immune system response. Sialic acid is also involved in viral infections, since

viruses such as influenza have on their surface a glycoprotein enzyme designated neuraminidase (acetyl-neuraminyl hydrolase) responsible for release of the newly formed virus through removal of sialic acid sugars that exist in the membrane of host cells, therefore increasing the spread of the infection. [34,35]

In fact, studies regarding the role of sialic acid in viral infections and its emerging role as being implicated in therapeutic targets, led to the development of anti-viral therapies that are commercially available nowadays and that became famous at the time of the first outbreak of bird flu, such as zanamivir and oseltamivir, which are neuraminidase inhibitors. [34,36]

The work developed with these inhibitors was also crucial to our own project since they are built from carbacycles derived from quinic acid, and our main objective in this thesis project was to synthesise quinic acid-based analogues to couple to CMP and determine their inhibitory effect towards polysialyltransferases and the biosynthesis of polysialic acid. [37]

The presence of polySia on NCAM in mammalian brains was described for the first time in the 1980's and this glycosylation is the most important of all the post-translational modifications that NCAM can sustain. [38,39,40] It is a linear homopolymer carbohydrate of α(2→8)-linked sialic acid residues that can correspond up to 30% of the NCAM molecular mass and its normal length varies usually up to 100 residues of sialic acid, and is usually attached to *N*-glycosylation sites 5 and 6 on the 5th Ig domain of NCAM. (**Figure 1.9**) [41,42,43,44]

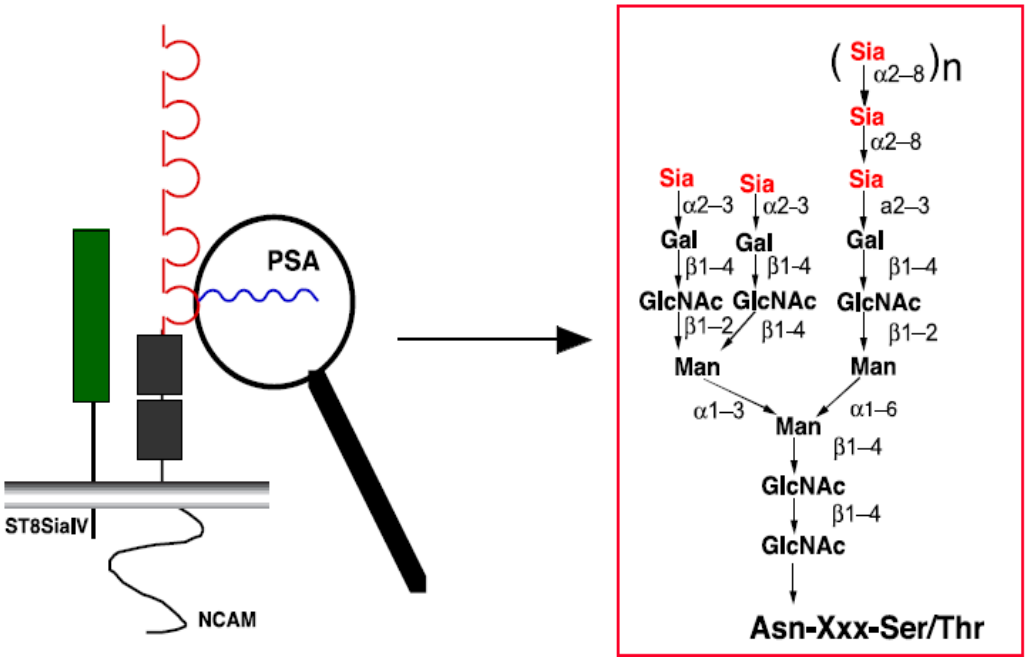


Fig. 1.9 PolySia structure and localization on NCAM.[44]

Polysialylation of NCAM is highly developmentally regulated: expression is very high during embryonic development when neuronal cells need to move to different parts of the body and reaches its peak at the perinatal phase, but soon after birth levels decrease rapidly such that the majority of adult tissues lack this glycan. [45] PolySia is nonetheless still present (albeit in dramatically lower concentrations) in plastic areas of adult brain where there is still neurite outgrowth and regeneration of neuronal cells, such as the olfactory bulb. [45] However, as stated before, polySia expression has been identified in some malignant/aggressive tumours, where it is an oncodevelopmental antigen, related to poor prognosis. [11,18,40,46]

As previously discussed, polySia is able to attenuate NCAM-dependent interactions, and subsequently cell adhesion, since it allows changes in cell position or shape, due to its own structural characteristics. PolySia can mediate cell-cell and cell-matrix adhesiveness, by conditioning

homophilic (NCAM to NCAM, *cis*-interactions if in the same membrane, and involving the IgI-IgI dimer formation, or *trans*-interactions if in opposing membranes, involving the formation of a IgI-IgII *cis* dimer and IgII-IgIII *trans* dimer – "flat-zipper" or IgI-IgIII and IgII-IgII – "compact zipper", **Figure 1.7**) [27,28,47] and heterophilic (NCAM can bind to other members of the Ig family, such as L1 and TAG-1, for example in small-cell lung cancer, where NCAM it's not expressed, and to members of the extracellular matrix, such as glycosaminglycan heparin, chondroitin and heparin sulphate proteoglycans) [11,15] interactions due to its stereochemical properties. These are the large size/bulkiness, which physically impairs attachment of cells, and polyanionic/negatively charged nature due to the presence of the carboxylic functionality that allows it to sustain water molecules and ion species (**Figure 1.10**).[28,48,49,50]

The polyanionic characteristics and large size of the polySia molecule cause the hydrodynamic radius of NCAM extracellular part to double, increasing the inter-membrane space and disrupting the adhesive properties of NCAM and other cell adhesion molecules (as shown in **Figure 1.10**). [21,48,49]

There is now evidence that NCAM polysialylation increases inter-membrane repulsion and abrogates NCAM homophilic adhesion, through disrupting inter-membrane adhesion mediated by C- and E-cadherin. [30,48,49]

PolySia effects are also regulated by triggering NCAM-dependent cell signalling through a cascade of phosphorylations that alter cell adhesion, such as the non-tyrosine kinase receptor Fyn (from the Src family) and the

focal adhesion kinase (FAK) that then activate the downstream ERK/MAP kinase pathways. [15,18]

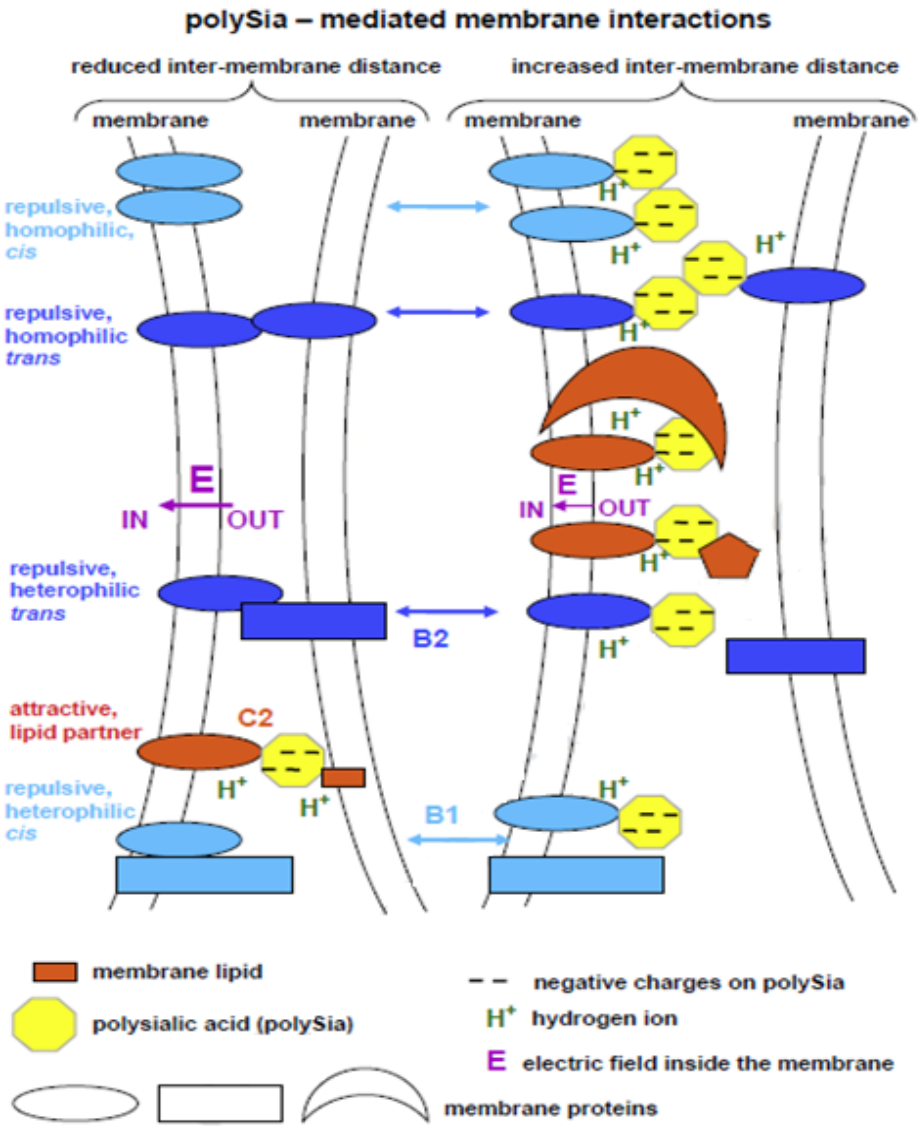


Fig. 1.10 Homophilic and Heterophilic interactions mediated by PolySia.[50]

The use of endoneuraminidase (endo-N) enzyme to remove polySia from where it is attached on the cell surface, both *in vivo* and *in vitro* was a determinant for the discovery of its key role in adhesion, migration and synapse formation, as stated previously. [27,28,43,50,51]

One example where polySia is involved in other cell-cell interactions that do not require the presence of NCAM, is in the case of migration/chemotaxis of mature dendritic cells (mDC cells), where the absence of polySia in neuropilin-2 membrane glycoprotein, also from the immunoglobulin family, is responsible for the decrease in chemotaxis of mDC to the lymph nodes were they are responsible for presenting antigens to the lymphocites, and triggering of an immune response.[52] PolySia can be therefore also a suitable target for modulation of the immune system response and be used as a therapy also in this case. [52]

In relation to its immunogenic/antigen characteristics, there are already available some studies regarding the possible use of polySia in a carbohydrate vaccine towards some types of cancer (namely small-cell lung cancer), in an attempt to trigger an immunological response to cellular polySia in the hope of reducing the occurrence of micro-metastases which is nowadays thought to be the main factor leading to chemoresistance in these tumours. [53,54,55,56] However, there is a natural human immune tolerance to polySia that develops in embryogenesis where its expression is increased and due to its presence in some areas of the brain, which increases the difficulty in constructing a vaccine, which has led to the need of some structural modifications. [53,55,56]

This demonstrates why, as mentioned before, polySia is a good therapeutic target and why the synthesis of inhibitors for the enzymes that biosynthesise polySia is worthwhile and required. This is a topic of increasing interest and as such forms the central core of the project described in this thesis.

Increased polySia expression is related to increased migration and invasion of tumour cells. However, to consider the opposite, a decrease of polysialic expression has also been reported to be involved in some diseases, such as schizophrenia (possibly due to mutations in the polysialyltransferase enzymes). Alterations in its expression appear to be related to neurodegenerative diseases including Alzheimer's and Parkinson's diseases, where there is an increase in NCAM-polySia in the hippocampus region due to disorganization of polySia-immunoreactive fibres. Still regarding the neurodegenerative diseases there is now evidence that oligodendrocytes, cells responsible for myelination, down-regulate polySia synthesis during differentiation as a prerequisite to the normal myelinization process and myelin maintenance which is essential for normal neuronal conductivity. [27,28,57,58, 59]

NCAM-polySia has been considered for promoting neuronal plasticity and repair of brain damage caused by lesions or infections, with beneficial effects in cognitive function, due to the possibility of synaptogenesis and axonal growth. Since it can be found in plastic areas of the brain it's related to the recovery after lesions, learning and memory formation. This happens because polySia by decreasing cell adhesion facilitates neuronal cell plasticity and also because it triggers cell signalling involving BDNF and FGF receptors, promoting long term potentiation, but still numerous questions remain to be answered. Nonetheless there is evidence that a polySia mimetic administrated to mice immediately following spinal cord injury caused neurite outgrowth and lesion recovery. [27,54,60,61]

Ultimately, careful consideration for exclusion of molecules from the brain, by prevention of crossing the blood brain barrier may be important.

In summary, polysialic acid behaves like a molecular switch for cellular behaviour. NCAM facilitates cell adhesion. Once polysialic acid is synthesised on the tumour cell surface, a number of changes are initiated, which results in a cell with enhanced migratory, invasive and metastatic potential. PolySia increases the physical size of NCAM, leading to repulsive forces between adjacent cells. PolySia also binds to other adhesion molecules and causes changes in intracellular signalling, which alters cell behaviour. In short, polySia has excellent potential as a target against tumour dissemination.

1.3.2. BIOSYNTHESIS OF POLYSIA – PST AND STX ENZYMES

In eukaryotic cells there are two closely related enzymes responsible for the synthesis of polySia: two α(2→8) polysialyltransferases named PST (ST8SiaIV) and STX (ST8SiaII). [28,30,37,45] These enzymes catalyse the transfer of sialic acid to an acceptor and are fairly homologous at amino acid level with 59% identity. [62,63, 64, 65]

The α(2→8) polysialyltransferases belong to a gene family of vertebrate sialyltransferases that also includes α(2→3) and α(2→6) sialyltransferases. These enzymes are far more common in normal cells, and therefore much more studied. They are also commercially available for studies. On the other hand, it is more difficult to find α(2→8) enzyme structural information. Among the α(2→8) polysialyltransferases, besides PST (ST8SiaIV) and STX

(ST8SiaII), four others have already been cloned: ST8SiaI, ST8SiaIII, ST8SiaV and ST8SiaVI. Their distribution in tissues also differs and their genes are found in different chromosomes. [28,63,64,65]

ST8SiaII and ST8SiaIV are also characterised by their ability to carry out autopolysialylation, and cells producing polySia often express both enzymes. These enzymes act synergistically for the synthesis of polySia in tumour cells, which means that in most cases even if only one of the enzymes is present, polysialylation of NCAM can still occur *in vitro* and *in vivo*. [62, 63,64,65]

The expression of two similar enzymes has triggered speculation among the researchers as to why there are two, both utilising one single acceptor, CMP-Neu5Ac, and this important question has not yet been answered. Some studies have started to address this question and I will here refer to some key points relating to this issue:

- Their expression profiles are very different, since STX is the predominant enzyme in the embryo and PST persists postnatally, playing the predominant role in adulthood in those limited areas with polySia is still expressed. Studies using Northern blot analysis revealed that in mice, expression of the enzymes was tissue-specific: PST is expressed in lung and heart, and only moderately in skeletal muscle and brain and STX was more expressed in the lung and testes and moderately in the brain. In human tissues PST transcript was found in the spleen, small intestine and leukocytes and almost no STX could be detected. Crucially, it is STX that is the dominant enzyme in tumours; [64,66,67]

- The distribution of chain length of polySia: studies have demonstrated that when PST or STX are expressed alone the chains are shorter than 30 residues and that the presence of longer chains requires their combined presence and combinatory action. The degree of polysialylation is lower with ST8SiaII (about 40 residues) and higher with ST8SiaIV (around 60); [45,65, 68]
- The attachment to the carrier oligossacharide is also slightly different: they both preferably attach to *N*-glycosylation site 6, but STX uses also the *N*-glycosylation site 5; [45,63, 68]
- The enzymes have the capability to accept modified CMP-sialic acid structures to produce modified polySia. Studies involving feeding of cells with modified *N*-acyl mannosamines (which is the precursor from which *N*-acetylneuraminc acid is synthesized, **Figure 1.10**) demonstrated that these synthetic mannosamines (ManLev, Man5Prop, Man5But, Man5Pent), were taken up by the cells and processed by their machinery. Man5Prop, Man5But and Man5Pent (**Figure 1.15**) could inhibit the synthesis of polySia, mainly through selective inhibition of STX (ST8SiaII) *in vitro*. This is also sustained by the fact that in adults a decreased *in vivo* efficiency of PST polysialylation and a higher efficiency of STX have been reported. [38,44,69,70]

Some initial studies described that STX was not able to polysialylate all forms of NCAM*:* only NCAM 180 and NCAM 140, but not the GPI-anchored form. However, recent studies came to show that was not the case,

and that STX and PST have in fact no preference in using different isoforms of NCAM as acceptors. [71]

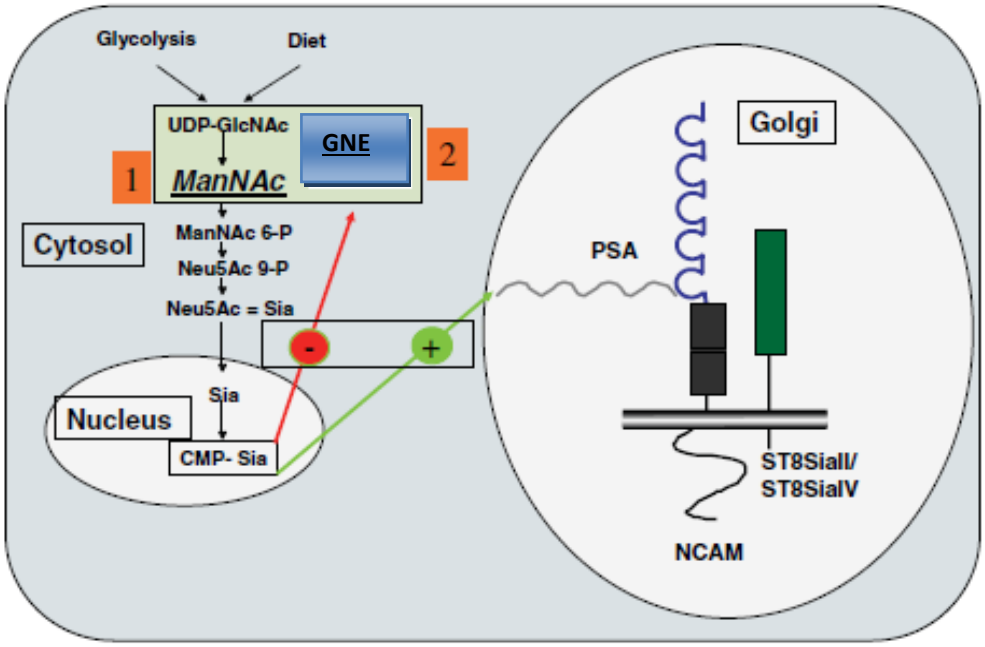


Fig. 1.11 Biosynthesis of PolySia. [39]

Sialic acids are synthesised first in the cytosol of cells. UDP-N-acetylglucosamine (originating from glycolysis or intake from diet, e.g. egg white is rich in sialic acid) is converted by GNE, a bi-functional enzyme, to the mannosamine precursor (ManNAc), which then is converted in three steps to neuraminic acid (Neu5Ac = sialic acid). [39]

Sialic acid is then transported to the nucleus of the cell where it is activated by coupling to a cytidine nucleoside and converted to CMP-Neu5Ac, which is the common glycosyl donor to all sialyltransferase enzymes. The donor substrate is then transported to the Golgi apparatus where by combined the action of polysialyltransferases, PST and STX, allows

the continuous addition of sialic acid to the 5th immunoglobulin domain of NCAM (**Figure 1.9 and 1.11**) and the biosynthesis of polySia. [39,44]

There are now a number of cell lines available for the study of the effect of the presence (polySia+ cells) or absence (polySia- cells) polysialic acid in tumour cells, for the study of polysialyltransferase enzymes (PST-/STX-, PST+/STX+, PST+/STX- and PST-/STX+) – the ICT has a panel of such lines, fully characterised for enzyme and polySia expression. Also, with the development of molecular biology methods, it is now possible to transfect cells (using vectors and plasmids) to express the molecules/proteins/enzymes (PCR method to increase the amount of protein) of interest, and to quantify accurately the amount of proteins present in the cells (through the use of immune assays - Western blot, ELISA), as well as identify the tissue location of a certain protein (immunostainning, [25]) and to determine/identify the proportion of distribution of certain protein biomarker (flow cytometry with the use of specific antibodies for polySia and NCAM, [9]).

To study effects of agents on tumour cell migration, the most common *in vitro* assay is perhaps the scratch assay, to determine if a compound in contact with polySia positive cells is able to inhibit the polysialyltransferase enzymes which can be easily determined visually by the degree of by which the scratch made artificially closes or not. [44] Regarding the *in vivo* models for study of polySia there are several described in the literature involving xenografts in nude mice [11], the use of SMtTW (*in vivo* model of spontaneous tumours malignant and benign in Wistar rats) [22] and the use of NCAM deficient mice. [28] Simultaneously the use of endo-N enzyme to remove polySia from where it is attached, i.e. NCAM, has been used both *in vitro* and

in vivo, and it has allowed for the establishment of the relationship between polySia absence from NCAM and a decrease in tumour cell migration. [28,71]

A surprising fact is that knockout mice for NCAM or polysialyltransferases enzymes do not show phenotype developmental defects. Instead, studies demonstrated that NCAM-polySia or polySia itself have a more important role in maintaining normal CNS function. [30, 72,73,74]

For this project the knowledge that there are only a limited number of proteins that are polysialylated is also important. Besides NCAM only 7 other proteins have been identified: synaptic cell adhesion molecule 1, integrin α-5 subunit, neuropilin-2, α subunit of the voltage-dependent sodium channel, a form of CD36 scavenger receptor found in milk, and the polysialyltransferases themselves, that as mentioned before have the ability of autopolysialylation, but STX is involved to a lesser degree than PST. Studies have shown that polysialylated enzymes tend to add more polySia to NCAM and it is speculated that it improves enzyme-substrate linkage. However this has been disputed, since when this process is abolished, the enzymes are still active even if not polysialylated. There is lot to unravel regarding the mechanism of polysialylation. [30, 72,73,74]

1.4. DRUG DISCOVERY AND POLYSIA – RATIONAL DRUG DESIGN AND MOLECULAR MODELLING

Drug discovery, besides the development of new drugs, aims for the improvement of safety and efficiency of new therapies, allowing not only a cure but also an overall improvement in the quality of life, even if that means

the minimising of suffering through palliative cares, which often happens when dealing with cancer. [75]

As mentioned previously one of the main problems in cancer therapy is still the occurrence of disastrous side effects due to a lack of specificity for tumour cells. That makes the drug discovery and design of new medicines time consuming, since it needs a multi-disciplinary team, including medicinal chemists, molecular biologists, organic chemists, pharmaceutical scientists and geneticists, among others, and it needs above all to be increasingly innovative, with the identification of new therapeutic targets, to respond to the increasing demands of the patients. For that, drug design tends to be progressively more expensive, and from all of the new drugs synthesised every year, only a few are going to be commercialised, and even then that will take several years, drug development is still expensive and time consuming – a single drug can cost up to 1 billion Euros and take at least 10 years to be developed. [75]

As a result, new techniques are arising to try and expedite theses processes, without compromising the safety of the new drugs.[75] When a suitable therapeutic target is identified and validated, drug candidates are chemically synthesised. [76] A target can be classified broadly as usually a protein or biomolecule (e.g. enzyme or receptor) for a drug *in vivo*.

Target validation is the first step of new drug discovery methods and involves physiological, pathological and pharmacological evaluation of the target biomolecule, evaluation that can be made at a molecular, cellular or whole animal level. The next steps include designing a bioassay to measure biological activity, constructing a high-throughput screen to find hit

compounds and evaluation of those hits in terms of stability, pharmacological effects and *in vivo* and *in vitro* toxicity. [76]

In cancer there is an increasing role and importance of personalised therapeutics, as previously mentioned, that accounts for intra and inter-patient tumour heterogeneity, since now there is a better understanding of the biology features of tumours as described in the Hallmarks of cancer (**Figures 1.2, 1.3** and **1.4**). The use of personalised therapeutics has potential advantages over conventional chemotherapies by increasing patient survival and also through the reduction of toxic side effects, taking into account patient's age, overall health, tumour type, stage and grade, among other features, but are more expensive, since they have a limited/specific use and for that their economical return is slower. It was also discovered that targeted therapies have the disadvantage of acquired resistance, since they are taken in long periods of time, which also can lead to less compliance of the patients. Personalized therapies are already available for some types of cancers, such as breast, non-small cell lung cancer and colorectal cancer. For example: in breast cancer, the identification of genes such as BRCA1, BRCA2, CDH1, CHEK2, P53 has recently been under the media spotlight. These findings allow prior warning, possibly decades before any cancer develops, to patients with these genetic predisposing characteristics. This gives the individuals (and their clinicians) the power to make early decisions to prevent/detect the cancer, and gives the oncologist the means to predict the efficacy of a given chemotherapy, which is more tailored to the individual patient. Also, in breast cancer, there has recently been much interest in the specific target HER-2 (epidermal growth factor receptor oncogene). This

tyrosine kinase receptor was proved to be overexpressed in 10-34% of breast cancers and to be related to malignancy – proliferation, migration and metastasis - and to poor prognosis. New therapeutics were developed, anti-HER-2 (inhibitors), such as trastuzumab and lapatinib. Lapatinib is in phase II trials for use in cases of trastuzumab resistance and CNS metastasis), and is showing promising results. [3,5,75,76]

In contrast, previously the discovery of new drugs relied on trial-and-error testing of compounds and also in the use of known natural products which by experience were known to have a specific use. More recently, rational drug design is the core process for drug development. It is based on a substrate of a specific and well described therapeutic target, a molecule involved in a specific molecular or signalling pathway that is specific for a disease, for which a three dimensional structure is known through methods of X-ray crystallography and spectroscopy. Computational chemistry is frequently used, employing molecular modelling which allows identification of a series of compounds that can bind/fit into a specific protein target, predict their action and determine which is the better molecule for that specific target with high affinity and selectivity. This makes the process of drug development less time consuming, since it allows to the discovery of several possible hit compounds for a specific known target, and less dependent of *in vivo* testing, since the behaviour of similar hit compounds are already known. Nonetheless, full understanding of the complex molecular pathways involved in tumour progression is still to be gained. Chemoresistance, acquired or secondary, continues to be a major problem, as is the appearance of unexpected side effects and bioavaibility problems. [75,76]

Rational drug design requires a series of key components, as described in **Figure 1.12**:

- Suitable target with biological rationale that substantiates it (e.g. there is *in vitro* and *in vivo* evidence that polySia-NCAM correlates to increased migration and metastasis in malignant tumours); [75,76]
- Druggable target, which means that the drug molecule will be able to reach it and cause an effect (e.g. with polySia there are already evidence that when unnatural mannosamine precursors of CMP-Neu5Ac are fed to cells they can alter the function of the enzymes responsible for the biosynthesis of polySia causing an inhibitory effect); [75,76]
- Selective target (e.g. polySia is only overexpressed in some types of tumours in adulthood, and because of that side effects resulting from the alteration of polySia in healthy cells should be minor); [75,76]
- Administrable drug (oral, iv or other) in a way that allows for an effective concentration to reach the tumour cells without harming normal/healthy cells (without any major side effects); [75,76]
- Right biomarker which proves reproducibility and validation of the target and drug – studies have demonstrated that polySia is by itself a biomarker of disease and can be easily detected/measured. [75,76]

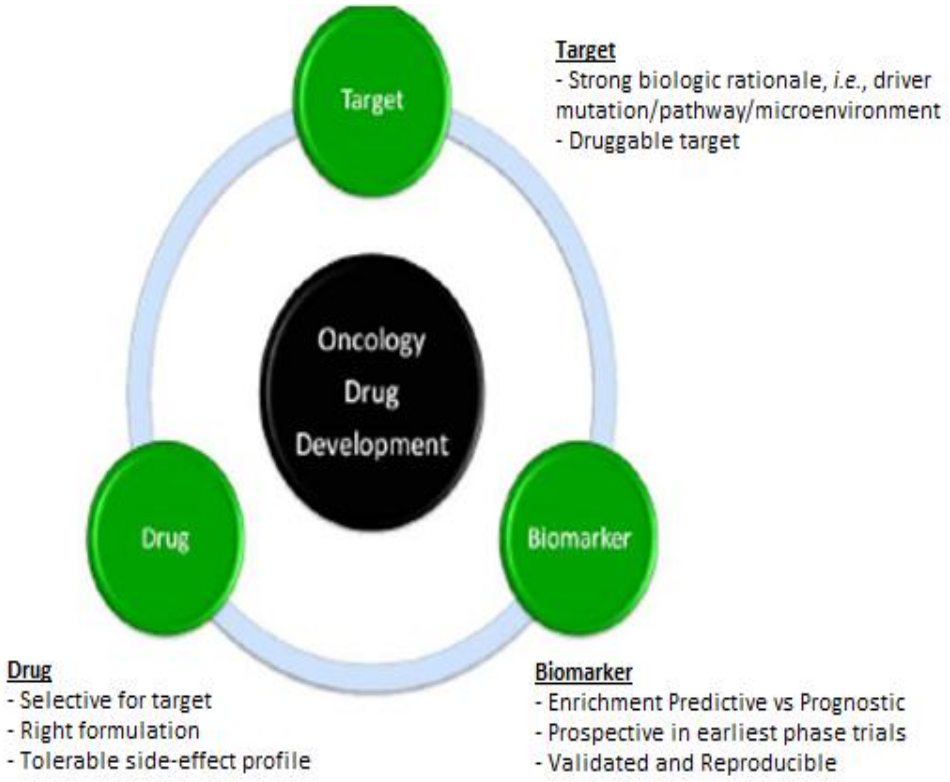


Fig. 1.12 Key components of oncology drug development. [75]

1.4.1. GLYCOSYLTRANSFERASE INHIBITORS

The development of glycosyltransferase inhibitors is an expanding area and has advanced immensely over the past decade. Several enzymes have been reported in the literature due to the increasing understanding of their importance in modulation of cell interactions and signalling pathways. The synthesis of specific inhibitors is therefore essential to the understanding of their influence in biological systems. [77,78]

As previously mentioned, glycosyltransferases catalyse the transfer of a sugar moiety (e.g. Neu5Ac) from an activated nucleotide sugar (e.g.CMP-Neu5Ac) to the hydroxyl group of an acceptor, which can be a growing

oligossacharide (e.g. polySia in NCAM), a lipid or a protein. (**Figure 1.11**) [77,79]

At the present time there are already a number of sialyltransferases available for study and for *in vitro* testing of compounds; these are mainly animal α(2→6) sialyltransferases, but there are some key problems that make rational drug design difficult. Little is known about the 3-D structure of the enzymes and their catalytic mechanism.[77,78,79]

In order to identify potential glycosyltransferase inhibitors, generally three different approaches are used:

- Design of acceptor analogues – there are strict specificities regarding the terminal glycan portion of the acceptor structure and some inhibitors have been synthesised, but show lower inhibitory activity when compared to donor analogues and transition state mimetics; [78,79, 80 ,81]
- Design of donor analogues – CMP-NeuAc, is the donor substrate of all sialyltransferase enzymes and as such analogues of that molecule as inhibitors are of great importance; [77,78, 79 ,80]
- Design of transition state mimetics – e.g. CMP-NeuAc$^{\neq}$ using carbomimetic molecules. Horenstein proposed in the 1990's that the sialylation transfer reaction proceeded through a S_N1-like mechanism, forming an oxacarbenium ion-like intermediate (**Figure 1.13**), with a positive charge in C2 delocalized with the ring oxygen atom. Kinetic studies have shown that the affinity towards an enzyme active site is 10^7 to 10^{15} times higher when in transition state, emphasising the

potential importance of these types of analogues. [77,78, 79 ,80] This approach is often the most challenging chemically.

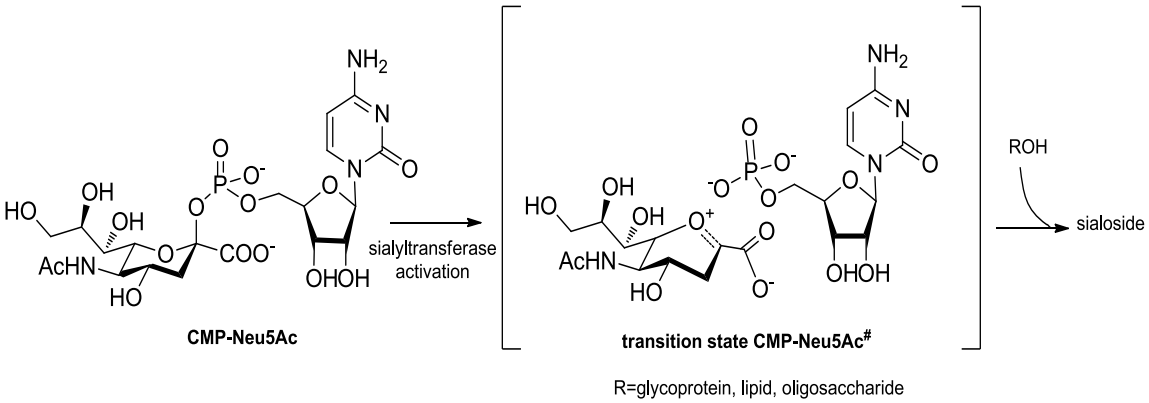


Fig. 1.13 Transition state Neu5Ac$^{\neq}$ proposed mechanism. [79]

There are different types of glycosyltransferase inhibitors, but most of those already synthesised against sialyltransferases lack significant activity. The first approaches focused on modifications to the Neu5Ac core (**Figure 1.14**) of CMP-sialic acid, constructing substituted analogues with: C5 amino group acetylated or glycosylated; acetylated hydroxyl groups, specifically C9; C9 phosphorylation and C8 methylation or sulfatation. Besides the fact that saccharide chemical synthesis is very difficult, sugars often (but not always) possess undesirable physicochemical properties that makes them very difficult to handle and purify (they frequently require preparative HPLC). [79,80,81]

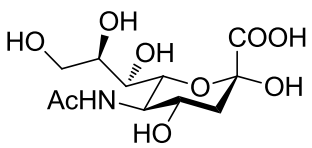


Fig. 1.14 Structure of Neu5Ac. [79]

For that reason, one approach was to start to replace the sugar (in this case sialic acid) core for a carbohydrate mimetic, where the glycosidic oxygen atom of the sugar moiety is substituted by a heteroatom or carbon atom. This often resulted in iminosugars and carbasugars, in the hope of maintaining the shape and function of the natural substrates, maintaining their biological function and ground/transition state, but increasing their affinity and stability towards the enzymes. [77]

In iminosugar analogues, the oxygen atom is substituted by a nitrogen atom and they are potentially important compounds since biological activity tends to be high, making them good candidate drugs for diabetes, cancer and viral infections as glycosyltransferase transition state mimetics inhibitors. Several inhibitors were already synthesized including pyrrolidinols and piperidinols. [77]

1.4.1.1. CARBASUGAR ANALOGUES OF CMP-Neu5Ac

Carbasugars and analogues are a group also designated as carbomimetics or pseudo-sugars. This is the group that has received the most interest for this project since it includes analogues of furanoses/pyranoses where the oxygen atom is substituted by a carbon atom. Besides maintaining affinity towards enzyme, there have been many examples in the literature of promising compounds. [77]

Syntheses of carbasugar-based nucleotide analogues of CMP-Neu5Ac have been reported in the literature. Sialyltransferase enzyme inhibitors of this type were the focus of this project. Our goal was to inhibit

α(2→8) polysialyltransferases PST and STX leading to a decrease in polySia biosynthesis and its expression on NCAM. Such effects were hypothesised to have antimetastatic activity. [77] Schmidt et al. reported the synthesis of structural CMP-Neu5Ac donor analogues and transition state (CMP-Neu5Ac$^{\neq}$) analogues, using phosphorous derivatives, with potent sialyltranferase inhibitory activity (rat liver α(2→6) sialyltransferases are the most commonly used for these kinetic studies) described in **Figure 1.15**.

Here, donor analogues were quinic acid-based analogues and when compared to the *K*m of the natural substrate (*K*m = 46 μM) unmodified quinic acid showed a *K*i = 44 μM. These similar values indicate that the Neu5Ac core is not necessary for inhibitory activity but the nucleotide is. This suggested that the quinic acid core could be a good substitute for the sialic acid core, which has a difficult synthetic chemistry, without loss of activity towards the sialyltransferase enzymes. Compounds that were transition state mimetics, with delocalized positive charge in the core ring as it happens in CMP-Neu5Ac (**Figure 1.13**), showed the highest inhibitory activity in this studies (*K*i = 40 nM, as can be seen in **Figure 1.15**). [78,79,83,84] These types of compounds are often the most challenging synthetically. These compounds were not considered in this work (but were subject of future plans: see Conclusions and Future work).

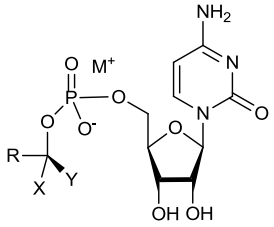
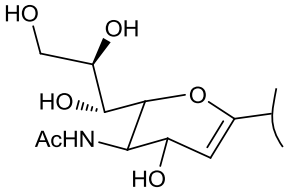
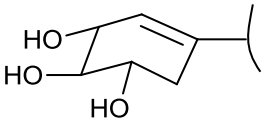
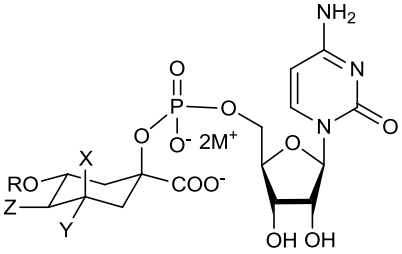
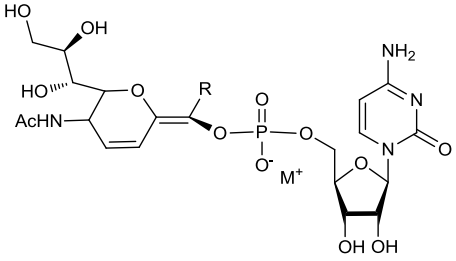
Inhibitors	Rat liver α(2-6)-sialyltransferase Ki (μM)
	R = Ph, X = H, Y = CO_2Na 10 R = Ph, X = CO_2Na, Y = H 7 R = Ph, X = H, Y = -P(O)(OH)(ONa) 0.35-0.20 R = 2-furyl, X = H, Y = -P(O)(OH)(ONa) 1-0.28
R= 	X = -P(O)(OH)(ONa), Y = H 0.35
R= 	X = -P(O)(OH)(ONa), Y = H 1.60 X = H, Y = -P(O)(OH)(ONa) 0.27
	R = Y = H, X = Z = OH (**Quinic acid**) 44 R = X = H, Y = NHAc, Z = OH 84 R =$(CH_2)_2OH$, X= Z = OH, Y =H 20
	R = H 6 R = -P(O)(O)(0Na) 0,04

Fig. 1.15 Biological evaluation of sialyltransferase inhibitors. [77]

In order to block the full transferase potential of these enzymes, there are certain aspects of the donor substrate binding area that potentially need to be fulfilled such as: inclusion of a CMP moiety or a monophosphate residue, tolerates variations in the phosphate linkage and at C4, C5 and C9 positions of Neu5Ac, but stability of the glycosidic bond is crucial. Quinic acid and its derivatives, attached to CMP via the tertiary hydroxyl fulfils these demands, and although the protection of the other hydroxyl groups of quinic acid at C3, C4 and C5 is required, methodologies for that purpose are already available and are simple to perform. This includes the attachment of the CMP aglycone to the modified sialic acid core, via a classical phosphite amide methodology (the formation of the glycosidic linkage is usually the last step of the synthesis process inhibitors of sialyltransferases) (see Scheme 5.1.1). [78,81,83,84,85]

Quinic acid is a chiral natural product that has the advantages of being commercially available, having a carboxylic group that facilitates functionalization, as well as the required axial-equatorial triol pattern that can mimic the sugar moiety of Neu5Ac. It is also important to mention that there is also a study were a macrolide antibiotic with a quinic acid scaffold led to *in vitro* inhibition of tumour cell migration, which came to emphasize its role as the starting material for the synthesis of sialyltransferase inhibitors carbasugars. [86,87,88]

As referred to previously, natural products account for the majority of drugs and drug leads available today in several therapeutic areas (cancer and mainly infection). These natural products include ribosomal peptides, alkaloids and polyketides also referred to as macrolides. [88]

During the study of macrolide antimycobacterials with a quinic acid-scaffold (designed to create pseudo-disaccharides to mimic *mycothiol* already known for its antimicrobial and antifungals effects), it was discovered by Metaferia et al. that some of the synthesized compounds had tumour migration inhibitory effects, superior to migrastin.

Migrastin is a streptomycite-derivative macrolide that has an IC_{50} of 29 µM in breast cancer cells, which led to the interest in developing analogues with higher potenty and selectivity (**Figure 1.16**). [88]

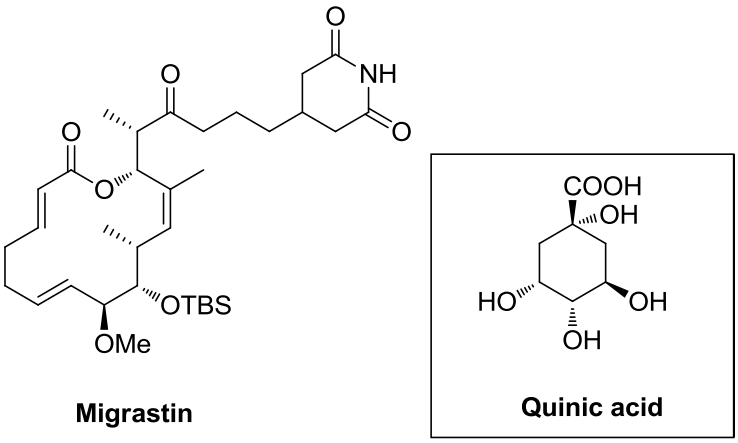


Fig. 1.16 Chemical structure of quinic acid and migrastin antibiotic. [88]

Metaferia et al., when using a quantitative chamber assay and qualitative wound healing assay (also referred to as scratch assay) obtained tumour migration inhibition values (Ki) in the nanomolar range. Further studies made by this group determined that this high tumour migration inhibition effect was a result of blockage of *rac*-mediated lamellipodia formation (actin fibres formation involved in cell migration), similar to the one in migrastin and its analogues. [88]

CMP-analogues derived from a natural sialyltransferase inhibitor CDP (cytidine diphosphate), also possessed good kinetic characteristics and the synthesis of transition state analogues by introduction of a double bond between C2 and C3 of quinic acid, similar to what happens in the transition state of CMP-Neu5Ac (which can be seen in **Figure 1.13**), increased activity three-fold (*K*i = 15 and 10 μM) the binding to α(2→6) rat liver sialyltransferase over CMP-Neu5Ac. [78,83,84]

The cytidine nucleotides proved to have a greater inhibitory ability than other nucleotides such as UTP, UDP, UMP, ATP and AMP, and the number of phosphates and electronegative potential (presence of a negative charge) appear to be related with increasing inhibitory potential. CTP was the nucleotide with the higher inhibitory activity and AMP the one with the least. However, how the phosphate group contribute to the formation of the glycosidic linkage is still not understood. [79]

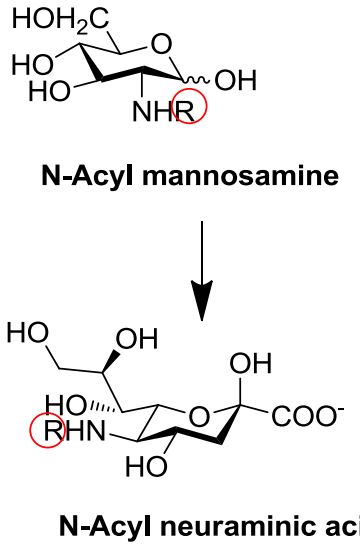
The available studies regarding the synthesis of the previously described structural analogues of CMP-Neu5Ac, whether it was transition state mimetic or not and carbasugar or not, enable researchers access to some important information about what is required for inhibitors to display high affinity to sialyltransferases:

- The more potent inhibitors were transition-state analogues, polar and charged (at least two negative charges close to the glycosylation site);
- Had planarity at the anomeric center, increased distance between CMP and anomeric carbon for bond cleavage;
- Cytidine was the nucleoside.

Nonetheless it still has to be determined whether these compounds have *in vitro* and *in vivo* efficacy, since the above characteristics usually decrease membrane permeability, and so these compounds may not be able to enter the cell and cause any biological inhibitory effect. One of the approaches to overcome this issue could be the use of sialyltransferase prodrug molecules. [79]

1.4.1.2 UNNATURAL MANNOSAMINE PRECURSORS

It is important to highlight once again the fact that a study where after feeding unnatural mannosamine precursors (**Figure 1.17**, Man5But, Man5Prop and Man5Pent, which contain alterations at the C5 amine acetyl group of mannosamine natutal precursor of sialic acid) to cells for the synthesis of polySia, they were incorporated into the corresponding CMP-Neu5Ac molecule, through the action of normal cell machinery. Reports described selective inhibition of STX α(2→8) polysialyltransferase and a resultant decrease in polySia expression on NCAM. [44] Besides CMP, these compounds remain the only reported polySTX inhibitors in the literature.



R:

- acetyl (Ac)	$-COCH_3$
- propanoyl (Prop)	$-COCH_2CH_3$
- butanoyl (But)	$-CO(CH_2)_2CH_3$
- pentanoyl (Pent)	$-CO(CH_2)_3CH_3$

Fig. 1.17 Unnatural mannosamines. [44]

The quantification of their activity, determination of *K*m and *K*i values, can be now determined through radioactive assays. These assays possess safety risks and are relatively expensive.

Other methods that can be used for this purpose include:

- Fluorescence-labelled CMP-Neu5Ac; [77,78,89]
- NADH oxidation; [(77,78,89)]
- UV-labelled acceptor structures, that afterwards are converted to an UV-labelled product; [77,78,89]
- More recently: a non-radioactive glycosyltransferase assay that uses specific phosphatases. The release of inorganic phosphate from this enzymatic reaction is directly proportional to the sugar molecule transferred, and can be detected by colorimetric reagents. This assay is also of interest to our group because of the possibility of being amenable to a

multiwell plate format, allowing the simultaneous screening of several compounds. [77,78,89]

It can be stated that at the present time there are several examples of inhibitors for sialyltransferases, using both sialic acid analogues and carbasugar compounds, but these still require biological testing to investigate whether they can enter the cell and produce therapeutic effects.

On the other hand there are not reported the affinity kinetic studies of these compounds using α(2→8) human polysialyltransferases.

Sialyltransferases are emerging, and will be of use in the design of compounds affecting polySia biosynthesis and its inhibition, which will allow further progress regarding the knowledge of this important new therapeutic target.

2. HYPOTHESIS

Malignant tumours re-express polySia and there is now substantial *in vitro* and *in vivo* evidence that the polysialic acid presence in many tumours is related to higher malignancy, invasion and metastasis.

My hypothesis is that if the expression of polySia could be inhibited in those cancer cells that express it, through the inhibition of polysialyltransferase enzymes, PST and STX, enzymes which are responsible for polySia biosynthesis, then reduced polySia levels in those cancer cells would also reduce tumour cell migration, invasion of surrounding tissues and metastasis in distant locations/organs.

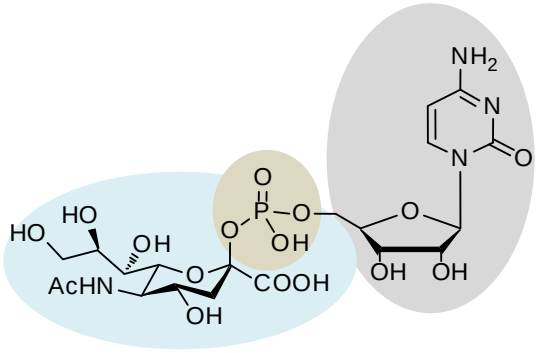
These combined effects would lead ultimately to an improvement of patient outcomes, mainly due to the reduction of cancer malignancy and higher survival of cancer patients.

3. AIMS AND OBJECTIVES

The overall aim of the broader research project, of which the work described in this thesis is a part, was the development of inhibitors of the polysialylation process in tumours.

The aim of this project was to develop chemical methodology towards the synthesis of a series of polysialyltransferase inhibitors which mimic the natural substrate (CMP-Neu5Ac) and that could be used to probe activity in cellular systems. A summary of the overall strategy with regards to modification to the CMP-Neu5Ac substrate is outlined in Scheme 3.1.

Replace or Modify the Core structure:
- replace sugar ring for a carbacycle (e.g. quinic acid or quinic acid analogues)
- replace the N-acyl group
-variation of the anomeric configuration



Replace or Modify the Nucleoside:
- 5-oxo, 5-azido, alkene or alkynes

Replace *O*-glycosidic Linkage:
- *C*-glycosidic, *S*-glycosidic (e.g.sulfide, disulfide, sulphones) or *N*-glycosidic (e.g. triazolines, triazoles)

Scheme 3.1. - Structural modifications for the construction of CMP-Neu5Ac analogues for inhibition of polySia biosynthesis.

Specifically, the aim of the work described in this thesis was the synthesis and development of chemical methodology for building blocks with

suitable chemical functionality for conjugation as potential polysialyltransferase inhibitors. This aim was achieved through the following objectives:

1. Synthesis of building blocks in which the core Neu5Ac sugar structure in CMP-Neu5Ac is replaced by a carbocycle.
    1.1. Synthesis of quinic acid-based analogues, with the aim of C1-OH substitution, to enable conjugation to CMP and/or other moieties.
    1.2. Evaluation of the use of the Barton-McCombie deoxygenation reaction and the Mukaiyama aldol addition, to synthesize C1 substituted quinic acid-based compounds.
        - Evaluation of reaction conditions for optimisation of the Barton-McCombie reaction, to remove the C1 hydroxyl group and insert a hydrogen atom.
        - Investigation of the feasibility of the use of the Mukaiyama reaction and testing of the reaction conditions, for the introduction of aliphatic groups at the C1 position.
2. Synthesis of a suitably protected cytidine building block suitable for coupling to the core structure of sialic acid or carbacycle (e.g. quinic acid analogues) via a phosphate or otherwise.
3. Investigation into methodology for the synthesis of disulfide sialic acid building blocks, and investigation into broader utility of the approach to non-sialic acid-based glycosyltransferase inhibitors.

4. MATERIALS AND GENERAL METHODS

Commercially available reagents were used as received without further purification, all were of reagent grade. Anhydrous solvents were used throughout, unless otherwise stated in the description of the method. Petroleum ether refers to the fraction of petroleum spirit boiling in the range of 60 to 80 °C (Fisher).

Quinic acid (*D*(-)-quinic acid 98%), potassium thioacetate (KSAc batch 1), TTMSS (batch 2), TMSCl, phenylacetaldehyde, $TiCl_4$, TBDMSCl, TBDPSCl, hydrazine acetate, iodoethane, iodine and LDA solution (1.8 M in THF) batches were purchased from Sigma-Aldrich; *N*-acetylneuraminic (sialic) acid was purchased from Dextra laboratories; cytidine, potassium thioacetate (KSAc batch 2) and benzyl bromide were purchased from Alfa Aesar; imidazole and thiocarbonyldiimidazole were purchased from Fluka; TTMSS was purchased from FluoroChem (batch 1).

Flash column chromatography using 9385 silica gel 60 (40-63 μm) from Merck was used for purification of products and analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel 60 F_{254} (Merck), for monitoring of the reactions. Visualization of the TLC plates was carried out using ultraviolet light (254 nm) and/or a basic solution of potassium permanganate ($KMnO_4$) followed by heating. For the sugar compounds sulphuric acid (H_2SO_4) char staining was used. When stated, regarding flash column chromatography and TLC, mixtures of solvents are referred to as percentage volume to volume (v/v) ratios.

For full characterization of the obtained products, optical rotations were measured using a PerkinElmer 341 polarimeter. The concentration (*c*)

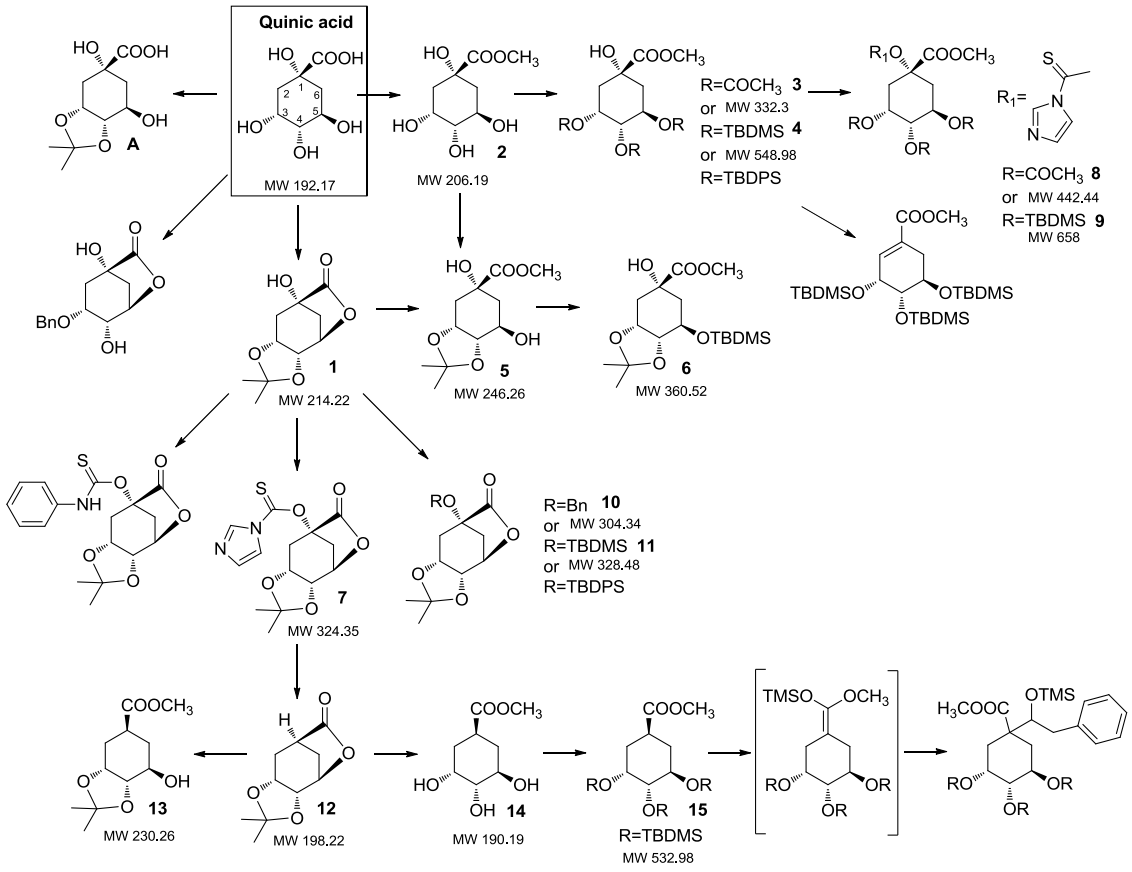
is given in grams per 100 mL and solvents are stated for each compound. Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra were recorded using a Bruker AMX400 (400 MHz) instrument. Chemical shifts are reported in parts per million (ppm) and coupling constants (*J*) are expressed in Hertz (Hz). Carbon Nuclear Magnetic Resonance (^{13}C NMR) was performed on the same instrument operating at 101 MHz. NMR solvents used were as stated for each compound. Routine low resolution mass spectra (LRMS) were generated using a Micromass Quattro Ultima spectrometer in the electrospray ionisation (ESI) or atmospheric solid admission probe (ASAP) modes, and high resolution mass spectrometry (HRMS) was performed at the National Mass Spectrometry Centre (Swansea, UK) using a MAT95 or MAT900 instrument in electrospray ionisation (ESI) mode.

For the majority of compounds reported, the assignment of the carbon numbers does not follow IUPAC rules; the numbering of atoms is assigned in the structure.

5. RESULTS AND DISCUSSION

5.1. QUINIC ACID-BASED ANALOGUES OF CMP-Neu5Ac

Regarding the synthesis of the structural analogues of CMP-Neu5Ac (see Scheme 3.1), by replacement of the core sugar structure by a carbacycle, quinic acid, scheme 5.1.1 describes a summary of the tested reactions and the successfully synthesised compounds (which are numbered). [91]



Scheme 5.1.1. – Quinic acid-based compounds: a summary tested reactions and synthesized compounds.

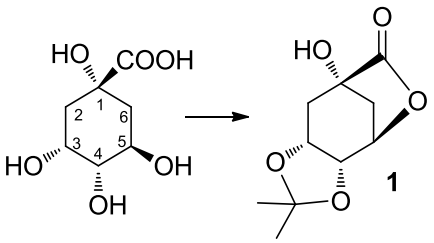
5.1.1. Protection of C3, C4 and C5 hydroxyl groups

The first objective was to protect the C3, C4 and C5 carbons of quinic acid, with the final aim to substitute the C1 tertiary hydroxyl group. Substitution at this position, e.g. by an aliphatic group, was key to being able to manipulate quinic acid in the manner we required, to produce potential inhibitors. Due to the similarities between quinic acid and the sialic acid part of CMP-sialic acid, a suitable linkage/functional group at that position would enable the synthesis of several compounds to be coupled, via a phosphate linkage or otherwise, to a cytidine molecule or other suitable nucleoside. The quinic acid-based analogues would then be evaluated biologically to demonstrate their inhibitory activity towards polysialyltransferases, PST and STX. [92,93,94]

5.1.1.1. Lactone and isopropylidene ring formation

Starting from quinic acid, 3,4-O-isopropylidene-1,5-quinic lactone (**1**) was synthesised successfully using acetone and sulphuric acid as the catalytic donor, to promote the formation of the isopropylidene ring between C3 and C4 and the lactone ring between the carboxyl function and the C5 carbon (see Scheme 5.1.2). These protecting groups ensure that only the free hydroxyl group is at the C1 position, which could be substituted afterwards. Different conditions were already reported in the literature, with conflicting reports of success. An investigation was initiated to obtain the best

synthetic method (see Table 5.1.1 for reaction conditions tested - use of desiccating agent, temperature and reaction time, and results obtained). [92]



Scheme 5.1.2. – Synthesis of 3, 4-O-isopropylidene-1, 5-quinic lactone (**1**) starting from commercially available D-(-)-quinic acid 98%.

Method 1, reported by Barco et al.,[95] proved to be the least successful due to long reaction times and the need for purification, which proved to be difficult due to solubility issues with the crude reaction mixture (see Table 5.1.1, entry 1). Methods 2 [96] and 3 [97] were very similar. Using reflux (see Table 5.1.1, entry 2 and 3), but the use of longer reaction times gave a higher quantitative yield of compound (**1**), without the need for purification via chromatography. This was since the work-up employed (neutralization to pH 6-7 with solid $NaHCO_3$, filtration, solvent evaporation and extraction using water and DCM) completely removed the main impurity, as confirmed by TLC and NMR.

Met.	Solv.	Cat.	Desic. agent	Conditions		Results
				Temp.	Time	
1[a)]	**Acetone**	**H_2SO_4**	-	RT	185 hrs	Impure, difficult purification
2[b)]			Na_2SO_4 (7 eq)	Reflux 80 ºC	4.15 hrs	No purification η qt. = 66%
3[c)]			**Na_2SO_4 (6 eq)**	**Reflux 75 ºC**	**24 hrs**	**No purification** **η qt. = 85-96%**

η qt. = quantitative yield
a) Adapted from Barco et al. (1997). [95]
b) Adapted from Baptistella (2004). [96]
c) Adapted from Abella (2006) better and more simple synthetic method. [97]

Table 5.1.1. – Testing conditions and results, for the synthesis of 3, 4-O-isopropylidene-1, 5-quinic lactone (**1**) starting from commercially available D-(-)-quinic acid 98%.

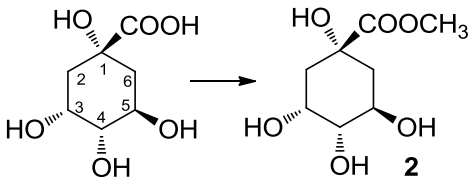
5.1.1.2. Esterification of the acidic function

Simultaneously, starting from quinic acid a second of my main objectives was the esterification of the acid function, to yield compound (**2**) methyl quinate (see Scheme 5.1.3).

Esters are useful in drug compounds since they can improve lipophilicity. A lipophilic drug penetrates cellular membranes much easier than a more hydrophilic compound, due to being more readily transported and reaching the desired target area, namely the highly selective/impermeable blood-brain barrier which needs to be surpassed by drugs that are intended to act in the CNS. [92]

Using methanol as a polar protic solvent, different conditions were investigated to obtain the best synthetic method (see Table 5.1.2 for reaction

conditions tested – catalyst used, temperature and reaction time, and results obtained). [36,92,98]



Scheme 5.1.3. – Synthesis of methyl quinate (**2**) starting from commercially available D-(-)-quinic acid 98%.

Met.	Solv.	Catalyst	Conditions		Results
			Temp.	Time	
1[a)]	**MeOH**	H_2SO_4	RT	ovn. + 96 hrs	No purification step η qt. = 69%
2[b)]		**Amberlite resin IR- 120 H^+**	**Reflux 75 ºC**	**24 hrs**	**No work-up No purification step η qt. = 99%**

η qt. = quantitative yield
a) Adapted from Bianco (2001). [36]
b) Adapted from Frank (1998) better and more simple synthetic method. [98]

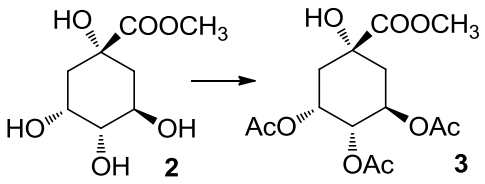
Table 5.1.2. – Testing conditions and results, for the synthesis of methyl quinate (**2**) starting from commercially available D-(-)-quinic acid 98%.

Method 1, which was previously reported by Bianco et al., [36] proved to be the least successful due to longer reaction times and the need for neutralization work-up. This was due to the use of sulphuric acid as catalyst (see Table 5.1.2, entry 1). Method 2, adapted from Frank et al., [98] proved to be the better synthetic method. Amberlite exchange resin IR-120H^+ was used as a catalytic proton donor and reflux, which gave a higher quantitative yield of compound (**2**), with shorter reaction times and without the need of complex

work-up (it was only necessary to filter the resin and evaporate solvent) and purification through a chromatography column, (see Table 5.1.2, entry 2).

5.1.1.3. Trisubstitution of C3, C4 and C5: acetylation and sialylation

Once compound (**2**) methyl quinate (see Scheme 5.1.3) had been synthesized was fine-tuned, the objective was then to devise strategies to protect the hydroxyl groups of C3, C4 and C5. (see Scheme 5.1.4) [92,94,99]



Scheme 5.1.4. – Synthesis of methyl 3, 4, 5-tri-O-acetylquinate (**3**) starting from the previously synthesised methyl quinate (**2**).

5.1.1.3.1. Trisubstitution of C3, C4 and C5: Acetylation

The first strategy employed consisted of the acetylation of those positions and synthesis of 3, 4, 5-tri-*O*-acetylquinate, compound (**3**) (see Scheme 5.1.4). Different conditions have been reported, commonly using pyridine, and an acetyl donor such as acetic anhydride or acetyl chloride. Those conditions were investigated and modified (see Table 5.1.3 for reaction conditions tested – reagents used, temperature and reaction time, and results obtained).

Met.	Reagents	Conditions		Results
		Temp.	Time	
1[a)]	Pyr. Ac_2O (4 eq)	RT	1 hr	No s.m. Difficult purification
2		RT	ovn.	Tetra-acetylated compound (C1, C3, C4 and C5)
3		0 ºC to RT	1.30 hrs	No s.m. Difficult purification
4[b)]	DCM solv. Pyr. (1.5 eq) Acetyl chloride (2 eq)	-20 ºC to RT	1 hr	
5	Pyr. Ac_2O (3 eq)	-40 ºC to RT	2 hrs	
6		0 ºC to RT (Ac_2O added dropwise for 30 min)	2.15 hrs	Still s.m. η ac. = 8%
7	DCM solv. Et_3N Ac_2O (3.1 eq)	**0 ºC to RT (Ac_2O added dropwise for 30 min)**	3.15 hrs	Still s.m. η ac. = 14%
8*	Pyr. Ac_2O (4.5 eq)		3 hrs	No s.m. η ac. = 28%
9*#	**Pyr. Ac_2O (3.5 eq)**		**3.45 hrs**	**No s.m. η ac. = 49%**

η ac. = after column yield
a) Adapted from Bianco (2001).[36]
b) Adapted from Kondo (2005). [100]
* starting material (s.m.) methyl quinate was previously purified through a flash column before setting up the reactions;
# showed to be the better synthetic method.

Table 5.1.3. – Testing conditions for the synthesis of methyl 3, 4, 5-tri-*O*-acetylquinate (**3**) starting from previously synthesized methyl quinate (**2**).

Method 1[36] involving use of pyridine and acetic anhydride as acetyl donor, proved to be unsuccessful, because the reaction time recommended

was too short (1 hour) and the resulting TLC of the crude reaction mixture indicated several products (more than 5), probably resulting from the presence of mono, di, tri and tetra-acetylated compounds plus impurities (see Table 5.1.3, entry 1). This also indicated that fewer equivalents of acetic anhydride should be used to minimize the formation of the undesired tetra-acetylated compound. For that reason and to begin to identify which spot in the TLC corresponded to which acetylated compound, method2 (see Table 5.1.3, entry 2) using the same conditions but overnight, was set up. That enabled the exclusive formation of tetra-acetylated compound, which could now be identified in future analyses. Method3 (see Table 5.1.3, entry 3), demonstrates the first attempt to optimise the reaction time, by decreasing the temperature to 0ºC (ice bath) during the addition of acetic anhydride. Nonetheless, the reaction was still fast, with a similar result as obtained while using method 1.

Method4 (see Table 5.1.3, entry 4), adapted from Kondo et al, [100] uses acetyl chloride as acetyl donor and lower temperatures through the use of ice and salt bath, during the addition of the reagents. These conditions did not alter the reaction time, since starting material couldn't be detected by TLC after 1 hour and none of the spots were prevalent over the others, when comparing to the previously described methods, and still gave a complex mixture difficult to purify.

With the previous results in mind, it was thought that the reaction temperature could be further decreased, and in method 5 (see Table 5.1.3, entry 5) an acetonitrile and dry ice bath was used to reach -40ºC, and the equivalents of acetic anhydride were also decreased. As a result it was

possible to reduce the reaction velocity, but still none of the spots shown in the TLC were prevalent over the others and purification was not successful.

Since decreasing the temperature was not the answer to solve this problem of fast consumption of the starting material, the strategy was turned to how the actual addition of the reagents was made. So in method 6 (see Table 5.1.3, entry 6), the acetic anhydride was added to the cooled (at 0 ºC) solution of compound (**2**) and pyridine, very slowly, dropwise over a period of 30 minutes. In addition, this time the reaction was quenched before the total consumption of starting material to try to minimize the formation of by-products and to determine which spot corresponded to the desired compound, to enable a more accurate control of the reaction in the subsequent attempts. As a result it was possible to isolate and determine for the first time the R*f* of compound (**3**), although with very low yield.

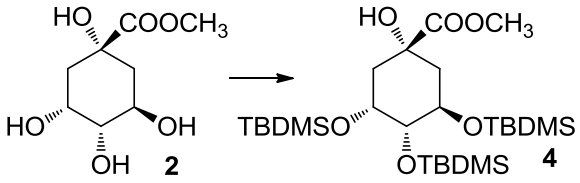
Due to the low yield obtained, it was felt that method 6 still needed further optimisation, and so it was thought that the use of a different base could help to improve the reaction outcome, and in method 7 triethylamine was used (see Table 5.1.3, entry 7) to decrease the presence of impurities, nonetheless the yield obtained for compound (**3**) was still low.

Since changing the base did not improve the resolution of the mixture obtained in method 8 and 9 (see Table 5.1.3, entry 8 and 9) compound (**2**), the starting material was purified by column chromatography in the hope of decreasing the impurities that were making it difficult to purify the reaction products. This did not initially appear to be necessary, given an apparently clean reaction. As a result, method 9 represented an improvement. It was determined that for this type of acetylation reaction, the following was

required: careful purification of the starting material, use of more than 3 but less than 4 equivalents of acetic anhydride in the reaction, dropwise addition of acetic anhydride at 0 ºC, and constant TLC monitoring of the reaction, to try to minimize the by-product formation.

5.1.1.3.2. Trisubstitution of C3, C4 and C5: Silylation

Starting from methyl quinate (**2**) (see Scheme 5.1.3), the second strategy for protection consisted of silylation of the three hydroxyl groups, through the formation of silyl ethers. For that purpose, the following protecting groups were considered TBDMS (*tert*-butyldimethylsilyl- introduced using *tert*-butyldimethylsilylchloride, see Scheme 5.1.5, compound **4**) and TBDPS (tert-butyldiphenylsilyl- introduced using *tert*-butyldiphenylsilyl chloride, see Scheme 5.1.6), mainly because their stability in a basic medium was more suitable. The synthetic method commonly uses: DMF as solvent and imidazole as a catalyst, especially if the silyl compound is bulkier, since there is a need for formation of a more reactive intermediate. The conditions were reproduced and modified to obtain the best synthetic method (see Table 5.1.4 and 5.1.5 for reaction conditions tested – reagents used, temperature and reaction time, and results obtained).



Scheme 5.1.5. – Synthesis of methyl 3,4,5-tri-(*tert*-butyldimethylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate (**4**) starting from previously synthesized methyl quinate (**2**).

Regarding the synthesis of 3,4,5-tri-(*tert*-butyldimethylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate (**4**), method 1, which was previously reported by Abella et al. [101] (see Table 5.1.4, entry 1) the use of a considerable number of equivalents of the TBMDS-Cl reagent (10 eq.) and imidazole (15 eq.), at a temperature of 65 ºC for 22 hrs was reported, but it was concluded that this reaction time was insufficient. As a result, unreacted starting material (**2**) was recovered.

With that in mind, in method 2 (see Table 5.1.4, entry 2) using the same ratios of reagents and a previously purified starting material (**2**), the reaction temperature was increased to 80 ºC and the time to 72 hrs, which proved to be the best conditions to arrive at the desired compound, with an excellent yield of 93% after purification.

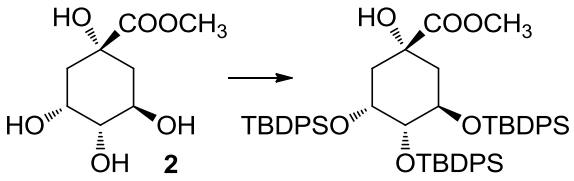
Method 3 (see Table 5.1.4, entry 2) also using purified starting material, reflects the attempt to use a smaller amount of equivalents of TBMDS-Cl (6 eq.) and imidazole (9 eq.) and a shorter reaction time, which led to an average yield of 43% after purification.

Met.	Reagents	Conditions		Results
		Temp.	Time	
1[a)]	**DMF solv.**	Δ 65 ºC	22 hrs	Only s.m. and reagents
2*[#]	**Imidazole (15 eq)** **TBDMSCl (10 eq)**	**Δ 80 ºC**	**72 hrs**	**η ac. = 93%**
3*	DMF solv. Imidazole (9 eq) TBDMSCl (6 eq)		24 hrs	η ac. = 43%

a) Adapted from Abella (2009). [101]
Δ = heating. η ac. = after column yield
* starting material (s.m.) methyl quinate was previously purified through a flash column before setting up the reactions.
# showed to be the better synthetic method.

Table 5.1.4. – Testing conditions for the synthesis of methyl 3,4,5-tri-(*tert*-butyldimethylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate (**4**) starting from previously synthesized methyl quinate (**2**).

Regarding the attempt to synthesise methyl 3,4,5-tri-(*tert*-butyldiphenylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate (see Scheme 5.1.6) starting from methyl quinate (**2**), in method 1 (see Table 5.1.5, entry 1) adapted from Abella et al., [101] firstly it was reacted with TBDPSCl (6 eq.) and imidazole (10 eq.) at 70 ºC for 24 hrs., but after that time TLC indicated no progression of the reaction. Due to this, 2.6 eq. of TBDPSCl were added and the reaction temperature increased to 80 ºC and the reaction stirred for 24 hrs more. As a result only unreacted starting material **2** and reagents were recovered.



Scheme 5.1.6. – Attempt at the synthesis of methyl 3,4,5-tri-(*tert*-butyldiphenylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate starting from previously synthesized methyl quinate (**2**).

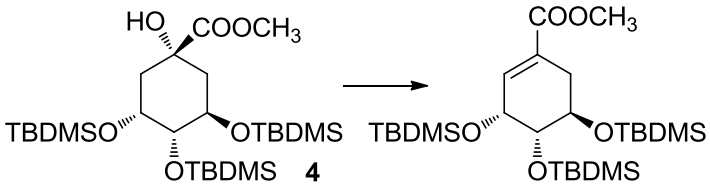
In method 2 (see Table 5.1.5, entry 2) the method that had proved to be the most successful for the synthesis of compound (**4**) was adopted (see Table 5.1.4, entry 2), and as such the same amounts of reagents and conditions were used. Nonetheless, the attempt at the synthesis of the tri-substituted compound with TBDPS was unsuccessful because only di-substituted C3 and C5 compound was isolated after purification. This indicates that the three hydroxyl groups are possibly too hindered for the bulkier TBDPS group to be able to protect the C4 hydroxyl group in the presence of the other two.

After the successful synthesis of compound (**4**), there was an attempt at the synthesis of 3,4,5-tri-*O-tert-(*butyldimethylsilyloxy)-shikimate (see Scheme 5.1.7). The existence of the double bond between C1 and C2, was already reported in the literature for the tri-acetylated compound (**3**) by Bianco et al., [36] and presented a compound of interest due to the elimination of the C1 hydroxyl group and possibility of reduction of the alkene formed to an alkane, with the use of an hydrogen source and an appropriate catalyst (e.g. Pd, Pt or Ni).

Met.	Reagents	Conditions		Results
		Temp.	Time	
1[a)]	DMF solv. Imidazole (10 eq) TBDPSCl (8.6 eq)	Δ 70 ºC + Δ 80 ºC	24 hrs + 24 hrs	Only s.m. and reagents
2	DMF solv. Imidazole (15 eq) TBDPSCl (10 eq)	Δ 80 ºC	72 hrs	Di-substituted compound

a) Adapted from Abella (2009). [101]
Δ = heating. S.m. = starting material.

Table 5.1.5. – Testing conditions for the attempt at the synthesis of methyl 3,4,5-tri--(*tert*-butyldiphenylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate starting from previously synthesized methyl quinate (**2**).



Scheme 5.1.7. – Attempt at the synthesis of methyl 3,4,5-tri-O-*tert*-(butyldimethylsilyloxy)-shikimate starting from previously synthesised 3,4,5-tri-(*tert*-butyldimethylsilyloxy)-1-hidroxy-cyclohexane-1-carboxylate (**4**).

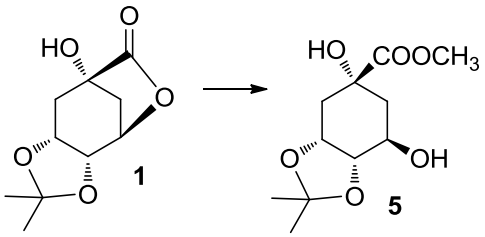
Met.	Reagents	Conditions		Results
		Temp.	Time	
1[a)]	Thionylchloride Pyridine	0 ºC to RT ($SOCl_2$ added dropwise)	7 hrs	-

a) Adapted from Bianco (2001). [36]

Table 5.1.6. – Attempt at the synthesis of methyl 3,4,5-tri-*O-tert-(*butyldimethylsilyloxy)-shikimate starting from previously synthesized 3,4,5-tri-(*tert*-butyldimethylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate (**4**).

In Table 5.1.6 the reaction conditions used are described.. The method adapted from Bianco et al. [36] used thionyl chloride and pyridine, in a based-induced E2 elimination reaction, but although no starting material (**4**) could be isolated after 7 hrs, no desired product was detected: the NMR spectrum indicated the absence of any double bond. Further possibilities include the use of $POCl_3$ (phosphorous oxychloride) or Martin sulfurane dehydrating reagent, reported by Shing et al. [102]; this would be the subject of future work.

5.1.1.4. Selective opening of the C1-C5 lactone ring



Scheme 5.1.8. – Synthesis of methyl 3, 4-O-isopropylidene quinate (**5**) starting from previously synthesized 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

One of the most challenging reactions for the quinic acid-based analogues proved to be that described in Scheme 5.1.8. The reaction aimed for the synthesis of methyl 3, 4-*O*-isopropylidene quinate (**5**) starting from the already successfully synthesised quinide acetal (**1**), through the use of an apparently very common method, described several times in the literature, using methanol as solvent and a sodium methoxide on methanol solution for the opening of the C1-C5 lactone ring, resulting in simultaneous esterification of the carboxyl function of quinic acid.

The first method attempted was (see Table 5.1.7, entry 1 and 2) adapted from Abella et al, [97] using 1.3 eq of 0.5 M NaOMe in MeOH, added slowly drop-wise over 30 min at 0 ºC, and then stirring for 1-2 hrs. (see Table 5.1.7, entry 1) or 20 hrs. (see Table 5.1.7, entry 2).

After that time, TLC didn't indicate any unreacted starting material and so work-up consisted of neutralization of the basic medium using 1 eq. of acetic acid, followed by extraction using $NaHCO_3$/DCM. However, no compound was recovered.

Since this outcome was a surprise, given that this type of reaction is common, I started by modifying the work-up procedure, in an attempt to minimise potential compound losses through that route.

Met.	Solv.	Reag.	Conditions		W.up	Results
			Temp.	Time		
1[a)]	MeOH	NaOMe 0.5 M in MeOH (1.3 eq)	0 ºC to RT (NaOMe added dropwise for 30 min)	1-2 hrs	acetic acid (1 eq) (neut.) $NaHCO_3$/DCM (extrac.)	No s.m. Nothing recovered
2				20 hrs		
3[(b)]				6.30 hrs	acetic acid (1 eq) (neut.) EtOAc/silica gel pad	
4[(c)]				7 hrs	acetic acid (1 eq) (neut.) No W.up	
5[(d)]		NaOMe freshly made(1.3 eq)		5.30 hrs	Amberlite IR-120 H^+	Methyl quinate (**2**)
6		NaOMe 0.5 M in MeOH (1.3 eq)		3.45 hrs	Dowex 50wx2-200 (neut.)	No s.m. Nothing recovered
7					acetic acid (1 eq) (neut.) EtOAc/NH_4Cl (extrac.)	
8[(e)]	MeOH	**NaOMe 0.5 M in MeOH (1.3 eq)**	**0 ºC to RT (NaOMe added dropwise for 30 min)**	3.45 hrs	acetic acid (1 eq) (neut.) DCM/water (extrac.)	No s.m. Nothing recovered
9				ovn.	acetic acid (1 eq) (neut.) No W.up	Acidic form (**A**)
10				24 hrs	Amberlite IR-120 H^+	Methyl quinate (**2**)
11				5.30 hrs	**Amberlite IR-120 H^+ (pH 7)**	η qt. = 40-49%
12[#]	**MeOH + sieves**			**22 hrs**		**η qt. = 62%**
13	MeOH + Na_2SO_4 (5 eq)			48 hrs		η qt. = 8%

η qt. = quantitative yield. S.m. =starting material.
(a) Adapted from Abella (2006). [97] (b) Adapted from Wang (1999). [103]
(c) Adapted from Mohan (2010). [38] (d) Adapted from Schimdt (1998). [86]
(e) Adapted from Shing (2001). [102] # showed to be the better synthetic method

Table 5.1.7. – Testing conditions for the synthesis of 3,4-O-isopropylidene quinate starting from 3,4-O-isopropylidene-1,5-quinic lactone (1).

Entry 3 from Table 5.1.7 uses the same method but a different work-up after neutralization referred in the literature by Wang et al.[134], in which the crude solution was evaporated, dissolved in EtOAc and then filtered through a silica gel pad. However, the result was the same as previously reported for entry 1 and 2.

In entry 4 from Table 5.1.7 the conditions described by Mohan et al. [38] can be found. In this case, column chromatography is performed after neutralization with acetic acid. However, once again, NMR did not show evidence of the desired compound, since although some of the characteristic signals were present, the methyl COO$\underline{CH_3}$ signal was not.

After this result, the characteristics of the NaOMe solution were examined, because sometimes it is better to prepare this from fresh due to its progressive degradation over time, which can affect the outcome of the reaction. Alternative types of neutralization were also sought, less aggressive but that allowed the esterification.

After consulting the literature, method 5 (see Table 5.1.7, entry 5) adapted from Schimdt et al.[86] was identified, this method used a freshly made NaOMe solution and Amberlite exchange resin IR-120H$^+$ for neutralization and ester formation. The resin, as stated previously for the synthesis of methyl quinate (**2**), had the advantage of a simple work-up. Nonetheless only compound (**2**) was recovered, which results from the opening of both isopropylidene ring and the lactone ring, from which we can assume that the resin was too acidic. With that in mind a weaker acid resin was used, as outlined in entry 6, which proved unsuccessful and different extraction pairs

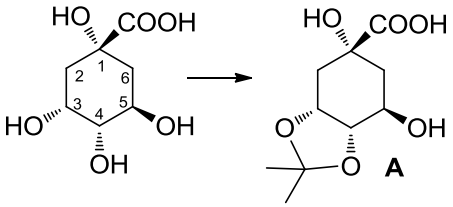
in entry 7 and 8, the last one adapted from Shing et al. [102] , also proving to be unsuccessful.

Reaction times were subsequently increased, as was attempting the reaction without work-up but still with acetic acid as neutralisation agent (entry 9 Table 5.1.7). Finally it was confirmed, as stated in entries 4 and 9, the compound synthesised was in fact the acid form **A** (see Scheme 5.1.9), which indicated that opening of the lactone was occurring, but not esterification.

With that in mind neutralization with Amberlite resin was once again attempted, but with longer reaction times (entry 10 Table 5.1.7). Once again the compound synthesised was methyl quinate (**2**), this time however it was possible to notice that before neutralization, TLC showed a spot with higher R_f than after neutralization. At this point it was concluded that the amount of Amberlite resin was crucial to the success of the reaction, so I attempted to use the smallest amount possible, monitoring progress of the reaction carefully, contrary to literature methods (entry 11, Table 5.1.7). For the first time the desired compound was obtained (**5**) in good yield.

Now that the reaction was finally optimised, my goal was now to try to minimize the difficulties in the work up due to the formation of water molecules in the reaction media, so entry 12 reflects that, with the use of molecular sieves and entry 13, the use of Na_2SO_4. Of the two strategies, the one using molecular sieves proved to be the better synthetic method, improving the yield further to 62%.

5.1.1.5. Selective C3-C4 isopropylidene ring formation



Scheme 5.1.9. – Attempt at the synthesis 3, 4-O-isopropylidene quinic acid (**A**) of starting from commercially available D-(-)-quinic acid 98%.

Since some of the attempted strategies proved unsatisfactory, specifically the exclusive opening of the C1-C5 lactone ring in the quinide acetal (see Scheme 5.1.8 stated previously), a different method, based in the literature method of Wang et al.[103], was attempted to arrive at a suitably protected quinic acid derivative. The method involved the exclusive isopropylidene protection of C3 and C4 (see Scheme 5.1.8), leaving C1 and C5 hydroxyl groups free, and the carboxyl function. The objective was to subsequently protect C5 and convert the carboxyl function to an ester.

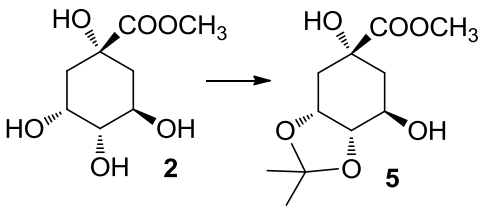
	Reagents	Catalyst	Conditions		Results
			Temp.	Time	
1[a)]	Toluene DMP (4 eq) Na_2SO_4 (2.2 eq)	p-TSOH (0.1 eq)	Reflux 84 ºC	ovn.	-

a) Adapted from Wang (1999). [103]

Table 5.1.8. – Attempt at the synthesis of 3, 4-O-isopropylidene quinic acid (**A**) of starting from D (-)-quinic acid 98%.

The method (see Table 5.1.8) used toluene as solvent, p-toluenesulfonic acid (p-TSOH) as acidic catalyst, DMP (2,2-dimethylaminopropane) to form the isopropylidene ring and Na_2SO_4 as a drying agent. [103] Results were inconclusive, because although NMR of the material recrystalized from the crude reaction mixture, presented as white needle crystals, seemed to correspond to the one from the desired compound (**A**), the mass spectrum did not correspond. The mass spectrum showed a main species with a mass of 246, which corresponds to the esterified compound. This can be a result of an artefact in the NMR that didn't allow the signal of the CH_3, compound (**5**), from the ester to be seen in the first place or in other hand the process of mass spectrometry could reflect the presence of a mixture of both compound (**A**) and compound (**5**).

Since the method for the synthesis of methyl 3,4-*O*-isopropylidene quinate (**5**) starting from the quinide acetal (**1**) previously reported (Scheme 5.1.8, Table 5.1.7) was so difficult to reproduce, due to high sensitivity to the neutralization process, different approaches were tried, starting from previously synthesised methyl quinate (**2**).



Scheme 5.1.10 – Attempt at another synthetic approach for methyl 3,4-*O*-isopropylidene quinate (**5**) starting from previously synthesized methyl quinate (**2**).

The first method, entry 1 Table 5.1.9, consists of an adaptation from a method used for the synthesis of quinide acetal (**1**) (see entry 3, Table 5.1.1) using acetone as solvent, sulphuric acid as catalyst, but at lower temperature and shorter reaction time, to promote isopropylidene ring formation between C3 and C4, without affecting the ester functionality. However, it was concluded that despite using milder conditions the favoured product for this reaction was still quinide acetal (**1**), which meant that the conditions trigger the formation of lactone between the ester group and the C5 free hydroxyl.

With the last result in mind, a weaker acid was considered, and one that is known to maintain the ester functionality, the Amberlite resin IR-120H^+. As can be seen in entry 2 Table 5.1.9, the solution mixture completely degraded, which could be noticed fairly on due to the black colour of the resin and at the same time we noticed the formation of water molecules in the medium.

Facing up the last results, sodium sulphate was additionally employed as a desiccating agent, in addition to carrying out the reaction at room temperature to try to prevent degradation and to try to reduce the formation of by-products (see entry 3 and 4, Table 5.1.9). As a result the reactions were retarded, but the one using Amberlite resin promoted the formation of compound (**A**) (see Tcheme 5.1.9), in which unfortunately the ester was hydrolysed, and in the one using sulphuric acid, once again the product formed was the quinide acetal (**1**).

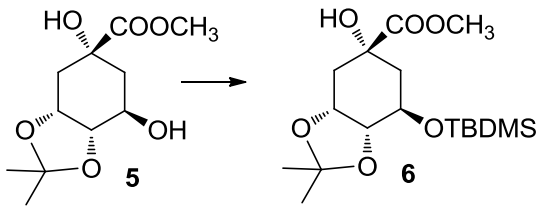
Met.	Reagents	Catalyst	Conditions		Results
			Temp.	Time	
1	Acetone	H_2SO_4	Reflux 65 ºC	1.15 hrs	Quinide acetal (**1**)
2	Acetone	Amberlite IR-120 H^+		29 hrs	Degradation
3	Acetone + Na_2SO_4 (6 eq)	Amberlite IR-120 H^+	RT	46 hrs	Acidic form (**A**)
4	Acetone + Na_2SO_4 (6 eq)	H_2SO_4	RT	30 hrs	Quinide acetal (**1**)
5[a)]	Toluene DMP (4 eq) Na_2SO_4 (4 eq)	p-TSOH (0.1 eq)	Reflux 84 ºC	ovn.	Acidic form (**A**) after recryst.

a) Adapted from Wang (1999). [103]

Table 5.1.9. – Testing conditions for the attempt at the synthesis of 3,4-*O*-isopropylidene quinate (**5**) starting from previously synthesized methyl quinate (**2**).

Although the method for the synthesis of compound (**A**) (Scheme 5.1.9 and Table 5.1.8) proved unsuccessful, a similar method was attempted for the synthesis of compound (**5**). Entry 5 [103] , Table 5.1.9 reflects that the attempt, using toluene, DMP and p-TSOH overnight at reflux, but after recrystalization, the product obtained was compound (**A**), without the ester function. There was insufficient time to repeat this method using shorter reaction times, but this will be the subject of future work, to determine whether compound (**5**) is not an intermediary product of this reaction.

5.1.1.6. C5 protection with TBDMS after C3-C4 isopropylidene ring formation



Scheme 5.1.11 – Synthesis of methyl 5-(*tert*-butyldimethylsilyloxy)-3,4-*O*-isopropylidene quinate (**6**) starting from previously synthesized methyl 3,4-*O*-isopropylidene quinate (**5**).

Although it was not possible to fine-tune the synthesis of compound (**5**), the yields obtained enabled us to nevertheless continue the protection steps, now concentrating on the free C5 hydroxyl group. Our strategy was to protect it with the TBDMS group (see Scheme 5.1.11), due to the already discussed favourable characteristics and easy introduction method, using TBDMS-Cl, and imidazole in DMF. Table 5.1.10 describes the reactions conditions used for the synthesis of methyl 5-(*tert*-butyldimethylsilyloxy)-3, 4-*O*-isopropylidene quinate (**6**).[92]

Entry 1, Table 5.1.10 describes what proved to be the best synthetic method, using 2 eq. of TBDMSCl, 60 ºC and a reaction time of 3.30 hrs. This gave an average yield of 44%.

Entry 2, Table 5.1.10 describes the attempt to improve on the previous yield by using higher amounts of TBDMS-Cl and Imidazole and increased reaction temperature. However, this proved unsuccessful. It is possible that the compound was sensitive to the 10 ºC increase of the temperature and for that reason degraded. In the future it would be desirable to repeat the

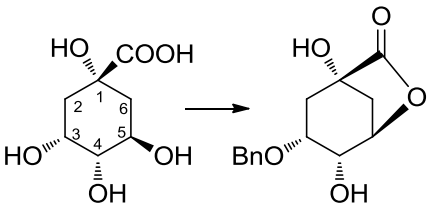
reaction, increasing the equivalents and the reaction time but maintaining the 60 ºC temperature or even decreasing it slightly.

Met.	Reagents	Conditions		Results
		Temp.	Time	
1#	**DMF solv. Imidazole (3 eq) TBDMSCl (2 eq)**	**Δ 60 ºC**	**3.30 hrs**	**η ac. = 44%**
2	DMF solv. Imidazole (4.5 eq) TBDMSCl (3 eq)	Δ 70 ºC	28 hrs	-

Δ = heating. η ac. = after column yield
showed to be the better synthetic method.

Table 5.1.10. – Testing conditions for the synthesis of 5-(*tert*-butyldimethylsilyloxy)-3,4-*O*-isopropylidene quinate (**6**) starting from previously synthesized methyl 3,4-*O*-isopropylidene quinate (**5**).

5.1.1.7. C3 protection with benzyl and C1-C5 protection with lactone



Scheme 5.1.12. – Attempt at the synthesis of 3-benzyloxy-1, 4-dihydroxy-1, 5-quinic lactone starting from commercially available D-(-)-quinic acid 98%.

With persistent difficulties in achieving the protection of the free adjacent hydroxyl groups in quinic acid that were experienced, alternative more robust and reliable procedures were constantly sought after.

A further alternative method considered was as follows: the synthesis of 3-benzyloxy-1, 4-dihydroxy-1, 5-quinic lactone (Scheme 5.1.12). This compound, if successfully synthesised, could be subsequently protected at C4 position, for example using the TBDMS protection method utilised for the synthesis of compound (**6**).

Table 5.1.11 reflects that attempt. Toluene, DMF and p-TSOH were stirred initially with quinic acid, to enable in a first step: formation of the lactone and acetonide rings. Once the dibutyltin oxide is added an intermediate compound is formed with the tin between C3 and C4, which will be determinant for the regioselectivity of nucleophilic substitution reaction after addition of benzyl bromide at C3 position. Despite using a Dean-Stark reflux apparatus, unfortunately the reaction proved difficult to replicate and degradation was the only result obtained. [104,105]

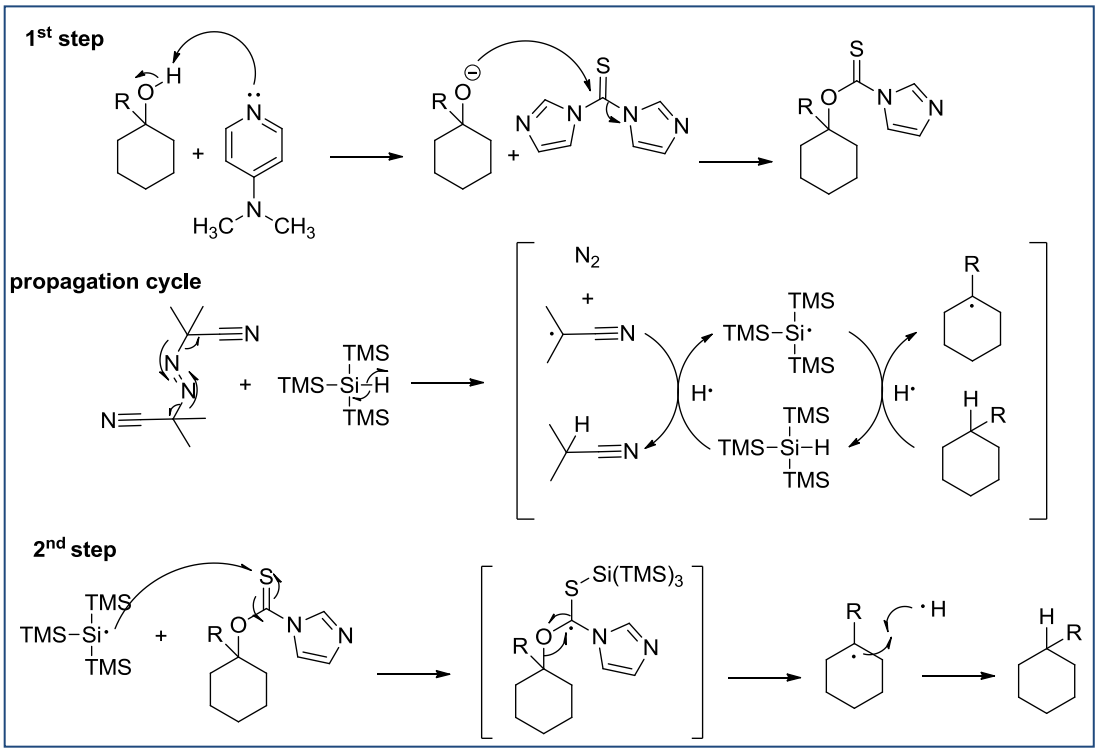
Met.	Reagents	Cat.	Conditions		Results
			Temp.	Time	
1[a)]	Toluene+DMF + Dibutyltin oxide (1.2 eq) + BnBr (1.6 eq)	p-TSOH (0.2 eq)	Dean-Stark reflux 110 ºC	17 hrs + 4.3 hrs + 18 hrs	Degradation

a) Adapted from Hanessian (1997) [104] and Zeng (2009) [105]

Table 5.1.11. – Testing conditions for the attempt at the synthesis of 3-benzyloxy-1,4-dihydroxy-1,5-quinic lactone starting from commercially available D (-)-quinic acid 98%.

5.1.2. Optimisation of the Barton-McCombie reaction, to remove the C1 hydroxyl group and insert a hydrogen atom.

Another of my objectives involved an investigation into the possibility of the use of the Barton McCombie deoxygenation reaction to manipulate C1. This reaction has not previously been applied to quinic acid, and as such would represent a novel means by which to synthesise novel quinic acid-based structures (see reaction mechanism Scheme 5.1.13). [106,107,108,109]



Scheme 5.1.13. - Barton-McCombie radical deoxygenation reaction mechanism schematics. [106,107,108,109]

This reaction allows for the deoxygenation of secondary and tertiary alcohols, through the replacement of a hydroxyl functional group by a hydride, via a radical reaction mechanism. Traditionally tributyltin hydride

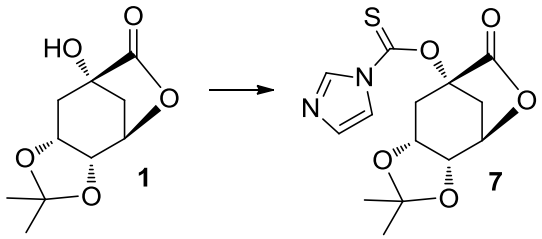
(Bu_3SnH) was used due to it being a good source of hydrogen atoms and its radical stability (the bond between S-Sn is very stable). [109,110,111,112,113]

However, Bu_3SnH is highly toxic, relatively expensive and often presents difficulties in terms of removal from the reaction mixtures. Efforts have been made to find feasible alternatives, such as the corresponding tin oxide, trialkylborane-water complexes and TTMSS (tris(trimethylsilyl)silane). [114,115, 116]

In the first step, the alcohol (in this specific case a tertiary alcohol) is converted to a thiocarbonyl derivative using DMAP as a catalyst. [107,108,110,112,117]

In the second step of the reaction, TTMSS radical formation is initiated by AIBN. The TTMSS radical then abstracts the thioester forming a very stable S-Si bond and the TTMSS-thioester and the alkyl radical. The alkyl radical is capable of abstracting the hydrogen from the newly formed TTMSS molecule, resulting in the formation of the desired deoxygenated product (Scheme 5.1.13).

5.1.2.1. Thiocarbonyl derivative formation – Barton McCombie 1st step optimization



Scheme 5.1.14. – Synthesis of 1-*O*-(thiocarbonyldiimidazolyl)-3, 4-*O*-isopropylidene-1,5-quinic lactone (**7**) starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**) – 1st step of Barton McCombie Radical Deoxygenation.

Initial tests to apply this reaction starting from quinide lactone **1** (see Scheme 5.1.14) to synthesize 1-*O*-(thiocarbonyldiimidazolyl)-3, 4-*O*-isopropylidene-1,5-quinic lactone (**7**) proved promising, so the reaction conditions were studied and optimized, work that is described in Table 5.1.12.

The first method (see entry 1, Table 5.1.12) used DCE as solvent, 1.5 eq of the thiocarbonyldiimidazole, 0.2 eq DMAP and at RT. However the reaction was very slow (160 h) and the yield low. So, in method 2 (see entry 2, Table 5.1.12) the equivalents of thiocarbonylimidazole reagent were increased slightly. As a result a higher yield was obtained, but the reaction at RT was still very slow.

Met.	Reagents	Cat.	Conditions		Results
			Temp.	Time	
1	DCE solv. Thiocarbonyldiimidazole (1.5 eq)	DMAP (0.2 eq)	RT	160 hrs	η ac. = 18%
2	DCE solv. Thiocarbonyldiimidazole (1.7 eq)			96 hrs	η ac. = 66%
3	DCE solv. Thiocarbonyldiimidazole (1.5 eq)		Δ 50 ºC	90 hrs	η ac. = 28%
4	Acetonitrile solv. Thiocarbonyldiimidazole (2 eq)		Reflux 83 ºC	**24 hrs**	η ac. = 63%
5	DCE solv. Thiocarbonyldiimidazole (2 eq)		**Reflux 85ºC**		η ac. = 74-76%
6#	**DCE solv. Thiocarbonyldiimidazole (2 eq)**	**DMAP (0.4 eq)**			**η ac. = 86%**

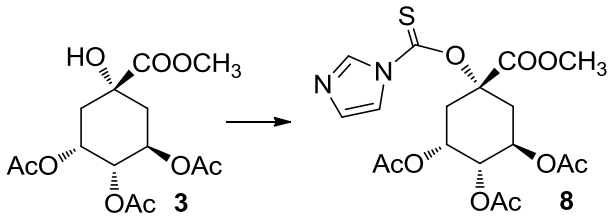
Original conditions adapted from Barton and McCombie (1975). [106]
shown to be the better synthetic method. η ac. = after column yield

Table 5.1.12. – Testing conditions for the synthesis 1-*O*-(thiocarbonyldiimidazolyl)-3,4-*O*-isopropylidene-1,5-quinic lactone (**7**) starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

To improve reaction velocity the temperature of the reaction was increased (see entry 3, Table 5.1.12) to 50ºC, but at that temperature the reaction was still slow and there was not any significant improvement in the yield.

An increase in the amount of thiocarbonyldiimidazole equivalents (2 eq) followed, and further increases in the temperature to reflux conditions. Two reflux reactions were simultaneously tested using different solvents, acetonitrile (see entry 4, Table 12) and DCE (see entry 5, Table 5.1.12). As a result the reaction time could be reduced to 24 h. Higher yields were obtained when using DCE.

Although major improvements had already been made, we additionally investigated whether the quantity of DMAP had any significant effect on the reaction performance. To this end, the same conditions were employed as in entry 5, but with double the amount of DMAP. We observed that there was a further increase in the yield to 86%. These were the optimised conditions: step one was successfully achieved and optimised.



Scheme 5.1.15. – Synthesis of 1-*O*-(thiocarbonyldiimidazolyl)-3, 4, 5-tri-*O*-acetylquinate (**8**) starting from 3, 4, 5-tri-*O*-acetylquinate (**3**) - 1st step of Barton McCombie Radical Deoxygenation**.**

After optimization of the first step of the Barton McCombie reaction attention was focused on applying these conditions to other compounds that were synthesised previously, i.e. compounds that were protected differently to quinide lactone (**1**), but still had the tertiary hydroxyl group at C1 position.

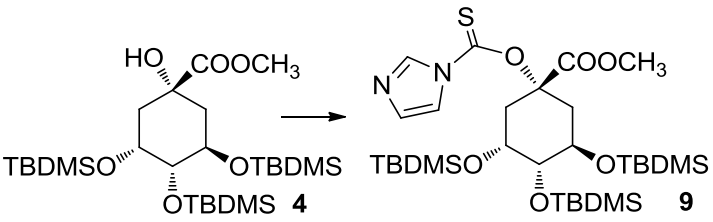
Scheme 5.1.15 shows the synthesis of 1-*O*-(thiocarbonyldiimidazolyl)-3, 4, 5-tri-*O*-acetylquinate (**8**) starting from 3, 4, 5-tri-*O*-acetylquinate (**3**) and Scheme 5.1.16 the synthesis of 1-*O*-(thiocarbonyldiimidazolyl)-3,4,5-tri-*O*-(*tert*-butyldimethylsilyloxy) cyclohexane-1-carboxylate (**9**) starting from 3,4,5-tri-(*tert*-butyldimethylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate (**4**).

Met.	Reagents	Conditions		Results
		Temp.	Time	
1#	**DCE solv. DMAP (1.5 eq) Thiocarbonyldiimidazole (3 eq)**	**Reflux 85 ºC**	**48 hrs**	**η ac. = 14%**
2	DCE solv. DMAP (1 eq) Thiocarbonyldiimidazole (3 eq)		120 hrs	η ac. = 2%

showed to be the better synthetic method. η ac. = after column yield

Table 5.1.13. – Testing conditions for the synthesis of 1-*O*-(thiocarbonyldiimidazolyl)-3, 4, 5-tri-*O*-acetylquinate (**8**) starting from 3, 4, 5-tri-*O*-acetylquinate (**3**).

In entry 1 Table 5.1.13 DCE was used as solvent in a reaction at reflux as for the synthesis of compound (**7**). The amount of DMAP catalyst was increased to 1.5 eq and thiocarbonyldiimidazole to 3 eq. As a result the desired compound (**8**) was obtained after 48 hrs., but only in low yield. The reaction time was increased in an attempt to increase the yield (see entry 2 Table 5.1.13). However, this reaction proved less successful.



Scheme 5.1.16. – Synthesis of 1-*O*-(thiocarbonyldiimidazolyl)-3,4,5-tri-*O*-(*tert*-butyldimethylsilyloxy) cyclohexane-1-carboxylate (**9**) starting from 3,4,5-tri-(*tert*-butyldimethylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate (**4**) - 1st step of Barton McCombie Radical Deoxygenation.

In entry 1 table 5.1.14, the alternative TBDMS-protected derivative was utilised. DCE was used as solvent under reflux once again. The amount of DMAP catalyst was increased to 0.8 eq and thiocarbonyldiimidazole to (**4**) eq. As a result we obtained the desired compound (**9**) after 48 hrs in a higher yield than in compound (**8**). It is possible that compounds (**3**) and (**4**) result in a ring conformation which is less favourable for this type of reaction than is the case for compound (**1**), where this reaction proved very successful.

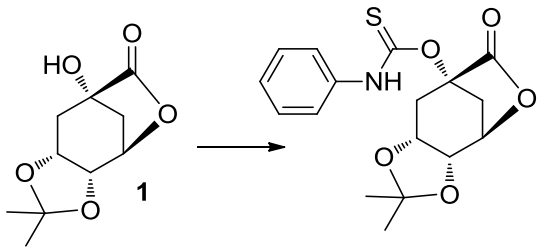
It was thought that perhaps changing the reaction conditions could improve the outcome (entry 2, Table 5.1.14). Firstly, DMF was used as solvent, with sodium hydride as a catalyst. However no progression of the reaction could be detected by TLC. In the end only starting material was recovered and a mixture of compounds which were not separable. Nevertheless the reaction worked very successfully when starting from compound (**4**), and compound (**9**) was synthesized with higher yield than compound (**8**).

In parallel, alternatives to thiocarbonyldiimidazole were explored for the first step of the Barton-McCombie reaction. It was possible that these may have been more favourable for the acetate and TBDMS-protected derivatives. Phenyl isothiocyanate was available in our laboratory, a compound that can be used in this kind of reactions, and so the synthesis of 1-*O*-(phenylthioxocarbamoyl)-3,4-*O*-isopropylidene-1,5-quinic lactone starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**) was attempted (see Scheme 5.1.17) using THF as solvent and sodium hydride as catalyst to promote removal of the hydrogen of the tertiary hydroxyl group at C1 position.

Met.	Reagents	Conditions		Results
		Temp.	Time	
1#	**DCE solv. DMAP (0.8 eq) Thiocarbonyldiimidazole (4 eq)**	**Reflux 85 ºC**	**48 hrs**	**η ac. = 31% (36% s.m. recovered)**
2	DMF solv. NaH (1.3 eq) Thiocarbonyldiimidazole (3 eq)	0 ºC + RT + Δ 45 ºC + Δ 70 ºC	30 min + ovn. + 24 hrs + 24 hrs	52% s.m. recovered Mixture of compound with s.m. not separable

Δ = heating. η ac. = after column yield. S.m. = starting material. # showed to be the better synthetic method.

Table 5.1.14. – Testing conditions for the synthesis of 1-*O*-(thiocarbonyldiimidazolyl)-3,4,5-tri-*O*-(*tert*-butyldimethylsilyloxy)cyclohexane-1-carboxylate (**9**) starting from 3,4,5-tri-(*tert*-butyldimethylsilyloxy)-1-hydroxy-cyclohexane-1-carboxylate (**4**).



Scheme 5.1.17. – Attempt at the synthesis of 1-*O*-(phenylthioxocarbamoyl)-3,4-*O*-isopropylidene-1,5-quinic lactone starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**) - 1st step of Barton McCombie Radical Deoxygenation with different thio compound.

Entry 1 Table 5.1.15 describes the first attempt using 2 equivalents of phenylisothiocyanate and NaH, first at 0 ºC to add the NaH and then at RT, but after considerable time (144 hrs) there was no progression and ultimately only degradation products evident. For this reason, the numbers of equivalents of phenylisothiocyanate were increased (see entry 2 Table

5.1.15) and after addition of NaH at 0 ºC and equilibration of the temperature at RT, the reaction was stirred at 35 ºC. However, once again after 24 hrs the reaction had degraded. It is a possibility that the NaH was too basic, and so an alternative method that proved to be the best one for the synthesis of compound (**7**), using DCE, DMAP as a catalyst and reflux at 85 ºC for 45 hrs was used. However, the poor result was the same. This confirmed that thiocarbonyldiimidazole is the better reagent for this kind of reaction applied to quinic acid chemistry. Other alternatives were not pursued.

Met.	Reagents	Cat.	Conditions		Results
			Temp.	Time	
1	THF solv. Phenylisothiocyanate (2 eq)	NaH (2 eq)	0 ºC to RT	144 hrs	No progression Degradation
2	THF solv. Phenylisothiocyanate (3 eq)		0 ºC to RT + Δ 35 ºC	24 hrs	
3	DCE solv. Phenylisothiocyanate (3 eq)	DMAP (0.2 eq)	Reflux 85 ºC	45 hrs	

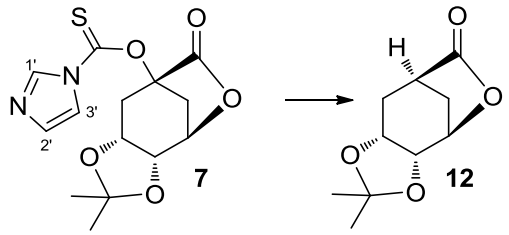
Δ = heating.

Table 5.1.15. – Testing conditions for the attempt at the synthesis of 1-*O*-(phenylthioxocarbamoyl)-3,4-*O*-isopropylidene-1,5-quinic lactone starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

5.1.2.2. Deoxygenation – Barton McCombie 2nd step optimization

The next objective was to attempt the second step of the Barton-McCombie reaction and to synthesise 3,4-*O*-isopropylidene-1,5-lactone cyclohexane (**12**) starting from 1-*O*-(thiocarbonyldiimidazolyl)-3,4-*O*-

isopropylidene-1,5-quinic lactone (**7**) (see Scheme 5.1.18). The reaction was an adaptation from the literature, method originally described by Barton and McCombie, but as described earlier, using TTMSS in place of the more traditionally used and more toxic tin compound (see Scheme 5.1.13, reaction mechanism).



Scheme 5.1.18. – Synthesis of 3,4-*O*-isopropylidene-1,5-lactone cyclohexane (**12**) starting from 1-*O*-(thiocarbonyldiimidazolyl)-3,4-*O*-isopropylidene-1,5-quinic lactone (**7**) – 2nd step of Barton McCombie Radical Deoxigenation reaction.

Entry 1 Table 5.1.16 describes the method first employed, as an adaptation of the published procedure: it used toluene as solvent, TTMSS (2 eq) and AIBN as a catalyst (0.2 eq) for the formation of the radical species by heating the reaction medium at 90 ºC for 20 hrs. This method was tried several times but gave very mixed, inconsistent yields.

For that reason the reaction conditions were first changed by increasing the number of equivalents of AIBN to 0.4 eq (see entry 2 Table 5.1.19). Nonetheless no major improvement in the reaction outcome was accomplished. For that reason method 1 was adopted as standard for future reactions for the synthesis of hydrogenated compound (**12**).

Met.	Reagents	Cat.	Conditions		Results
			Temp.	Time	
1[a)#]	**Toluene solv. TTMSS* (2 eq)**	**AIBN (0.2 eq)**	**Δ 90 ºC**	**20 hrs**	**η ac. = 56-96% (inconsistent)**
2	Toluene solv. TTMSS* (2 eq)	AIBN (0.4 eq)	Δ 90 ºC	24 hrs	η ac. = 59%

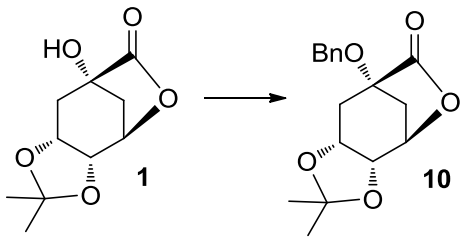
a) Adapted from original methodology by Barton and McCombie (1975). [106]
Δ = heating. η ac. = after column yield
showed to be the better synthetic method.
* TTMSS is only added to the reaction mixture (s.m.+solvent+cat.) after the system is purged for 5 min with N_2.

Table 5.1.16. – Conditions for synthesis of 3,4-*O*-isopropylidene-1,5-lactone cyclohexane (**12**) starting from 1-*O*-(thiocarbonyldiimidazolyl)-3,4-*O*-isopropylidene-1,5-quinic lactone (**7**).

Since compound (**12**) was successfully synthesised, removing the tertiary hydroxyl group and substituting it by a hydrogen atom, one of the key goals of the project was accomplished. It would be possible to attempt to react different substituents to position C1 while maintaining the ester functionality. This represents the first example of a Barton-McCombie type reaction with quinic acid.

5.1.3. Nucleophilic substitution reactions at C1 hydroxyl group

Simultaneously to the work described so far, some simple nucleophilic substitution reactions at the C1 hydroxyl group were attempted, starting from compound (**1**). We used for those reactions benzyl bromide (see Scheme 5.1.19), TBDMSCl (see Scheme 5.1.20) and TBDPhSCl (see Scheme 5.1.21), as potentially useful protecting groups to allow manipulation of the remainder of the ring.



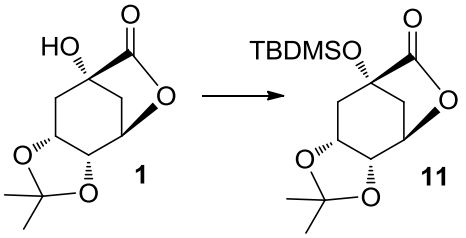
Scheme 5.1.19. – Synthesis of 1-*O*-benzyl-3,4-*O*-isopropylidene-1,5-quinic lactone (**10**) starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

Met.	Reagents	Cat.	Conditions		Results
			Temp.	Time	
1[a)]	**DMF solv. BnBr (1.2 eq)**	**NaH (2 eq)**	**0ºC to RT (NaH added dropwise for 30 min)**	**19 hrs**	**η ac. = 51%**

a) Adapted from Schawrdt (2010) [118] . η ac. = after column yield.

Table 5.1.17. – Testing conditions for the synthesis of 1-*O*-benzyl-3,4-*O*-isopropylidene-1,5-quinic lactone (**10**) starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

For the synthesis of 1-*O*-benzyl-3,4-*O*-isopropylidene-1,5-quinic lactone (**10**), starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**) we used a previously described method from Schawrdt et al. [118] using DMF as solvent, BnBr as nucleophile, and NaH as a catalyst to remove the hydrogen atom from the C1 hydroxyl. After 19 hrs, a moderate yield of 51% after column was successfully obtained for compound (**10**) (see Table 5.1.17). The synthesis of 1-(*tert*-butyldimethylsilyloxy)-3,4-O-isopropylidene-1,5-quinic lactone (**11**) starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**) was then attempted.



Scheme 5.1.20. – Synthesis of 1-(*tert*-butyldimethylsilyloxy)-3,4-O-isopropylidene-1,5-quinic lactone (**11**) starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

Also, an adaptation from the method described by Prazeres et al. [119] using DCM as solvent, pyridine as a catalyst and TBDMSCl as a nucleophile was utilised (see entry 1 Table 5.1.18). After 20 hrs. compound (**11**) was successfully prepared in a good yield of 57%.

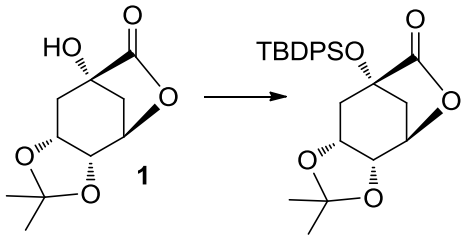
Met.	Reagents	Conditions		Results
		Temp.	Time	
1[a)#]	**DCM solv. Pyridine (2 eq) TBDMSCl (3 eq)**	**0 ºC to RT**	**20 hrs**	**η qt. = 57%**
2	DMF solv. Imidazole (8 eq) TBDMSCl (3 eq)	RT	48 hrs	η ac. = 11%

a)Adapted from Prazeres(2008). [119] η ac. = after column yield. η qt. = quantitative yield.
showed to be the better synthetic method.

Table 5.1.18. – Testing conditions for the synthesis of 1-(*tert*-butyldimethylsilyloxy)-3,4-*O*-isopropylidene-1,5-quinic lactone (**11**) starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

In the hope of improving the reaction yield further, the reaction conditions were changed (see entry 2, Table 5.1.18) to DMF solvent, to using imidazole and TBDMSCl at RT for 48 hrs. However, the obtained yield was lower (11% after column) than obtained previously.

The same method was then applied to synthesise 1-(*tert*-butyldiphenylsilyloxy)-3,4-*O*-isopropylidene-1,5-quinic lactone starting from 3,4-*O*-isopropylidene-1,5-quinic lactone (**1**) (see Table 5.1.19), but there was no progression of the reaction and only starting material was recovered. This led to the conclusion that position C1 is insufficiently accessible when using bigger substitution groups such as TBDPS.



Scheme 5.1.21. – Attempt at the synthesis of 1-(*tert*-butyldiphenylsilyloxy)-3,4-*O*-isopropylidene-1,5-quinic lactone starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

Met.	Reagents	Conditions		Results
		Temp.	Time	
1[a)]	DCM solv. Pyridine (1.7 eq) TBDPSCl (3 eq)	0 ºC to RT	20 hrs	-

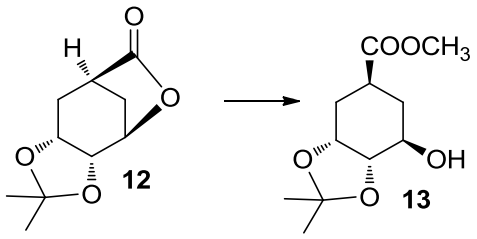
a) Adapted from Prazeres(2008). [119]

Table 5.1.19. – Conditions for the attempt at the synthesis of 1-(*tert*-butyldiphenylsilyloxy)-3,4-*O*-isopropylidene-1,5-quinic lactone starting from 3,4-O-isopropylidene-1,5-quinic lactone (**1**).

5.1.4. Selective opening of the lactone C1-C5 after deoxygenation of C1

Starting from compound (**12**), 3,4-*O*-isopropylidene-1,5-lactone cyclohexane, synthesis of 3,4-*O*-isopropylidene-cyclohexane-1-carboxylate (**13**) was attempted (see Scheme 5.1.22). The method used was reported previously for the synthesis of compound (**15**) and is commonly used for the opening of lactone rings and regeneration of the ester group, through the use of a methanolic solution of NaOMe solution.

Entry 1 Table 5.1.20 describes the method used where the reaction mixture was stirred for 5 hrs with NaOMe and then neutralized with the smallest amount possible the Amberlite IR-120 H^+ resin. As described in previous reactions of this type (already described in this thesis for the synthesis of compound (**5**)) both rings, i.e. the lactone and also the isopropylidene, opened giving compound (**14**) (see Scheme 5.1.23).



Scheme 5.1.22. – Synthesis of 3,4-O-isopropylidene-cyclohexane-1-carboxylate (**13**) starting from 3,4-O-isopropylidene-1,5-lactone cyclohexane (**12**).

Owing to the result of the previous reaction, the reaction time was reduced (see entry 2 Table 5.1.20) and the smallest possible amount of resin was calculated. As a result, the desired compound was successfully obtained (**13**), but as expected, due to the inconsistency of the reaction the yield after

purification was very low (only 17%), and the reaction was, once again, difficult to reproduce. However, this was a promising starting point for future reaction optimisation.

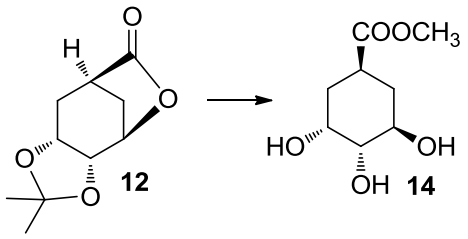
Met.	Solv.	Reag.	Conditions		w.up	Results
			Temp.	Time		
1	**MeOH**	**NaOMe 0.5 M in MeOH (1.3 eq)**	**0 ºC to RT (NaOMe added dropwise for 30 min)**	5 hrs	**Amberlite IR-120 H+ (pH 7)**	(**14**) Opening of the isopropylidene ring
2[#]				**3 hrs**		**η ac. = 17%**

showed to be the better synthetic method. η ac. = after column yield.

Table 5.1.20. – Conditions for synthesis of 3,4-O-isopropylidene-cyclohexane-1-carboxylate (13) starting from 3,4-O-isopropylidene-1,5-lactone cyclohexane (12).

5.1.5. Non-selective opening of both lactone C1-C5 and C3-C4 isopropylidene rings after deoxygenation of C1

With the previous results in mind, the synthesis of compound (**14**), formed during the process of attempting the synthesis of compound (**13**), was considered**.** It was concluded that the opening of both rings during the reaction with NaOMe in MeOH could actually be an advantage, since then it could be possible to protect the three hydroxyls (we previously successfully synthesise compound (**4**), tri-substituted with TBDMS at C3, C4 and C5) for further derivation.



Scheme 5.1.23. – Synthesis of 3,4,5-O-tri-hydroxy-cyclohexane-1-carboxylate (**14**) starting from 3,4-O-isopropylidene-1,5-lactone cyclohexane (**12**).

For that reason my purpose was now to synthesise 3,4,5-*O*-tri-hydroxy-cyclohexane-1-carboxylate (**14**) starting from 3,4-*O*-isopropylidene-1,5-lactone cyclohexane (**12**) without any concerns about the amount of Amberlite IR-120 H^+ resin used in the process (see Scheme 5.1.23).

Table 5.1.21 describes the method used, it closely resembles the one described for compound (**13**) in Table 5.1.20, the only difference being the excess of resin used and the final pH (acidic).

The method was successful and after column chromatography, a yield of compound (**14**) of around 70% was obtained. In addition, the reaction proved very reproducible, which is in contrast to the reaction for the exclusive opening of the C1-C5 lactone ring.

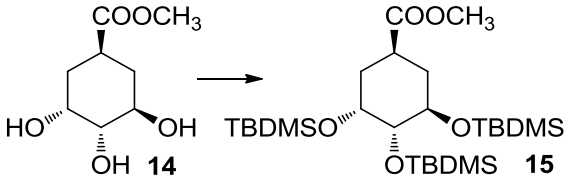
Met.	Solv.	Reag.	Conditions		w.up	Results
			Temp.	Time		
1	**MeOH**	**NaOMe 0.5 M in MeOH (1.3 eq)**	**0 ºC to RT (NaOMe added dropwise for 30 min)**	**3-4 hrs**	**Amberlite IR-120 H+ (pH 3-4) excess**	**η ac. = 65-70%**

η ac. = after column yield.

Table 5.1.21. – Conditions for synthesis of 3,4,5-O-tri-hydroxy-cyclohexane-1-carboxylate (**14**) starting from 3,4-O-isopropylidene-1,5-lactone cyclohexane (**12**).

5.1.5.1. Tri-silylation of C3, C4 and C5

The next step, as previously mentioned, was to protect the three hydroxyl groups and synthesise 3,4,5-tri-*O*-(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate (**15**) starting from 3,4,5-*O*-tri-hydroxy-cyclohexane-1-carboxylate (**14**) (see Scheme 5.1.24). [92,99]



Scheme 5.1.24. – Synthesis of 3,4,5-tri-O-(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate (**15**) starting from 3,4,5-O-tri-hydroxy-cyclohexane-1-carboxylate (**14**).

Entry 1 Table 5.1.22 describes the first method used and was an adaptation from the one used for the synthesis of compound (**4**). DMF was used as solvent, with imidazole and TBDMSCl, heating at 80 ºC for 24 hrs. Unfortunately only the di-substituted compound at C3 and C5 positions was recovered. It was reasoned that a longer reaction time was required, but

instead the solvent for the reaction was exchanged for pyridine (see entry 2 Table 5.1.22).

Met.	Reagents	Conditions		Results
		Temp.	Time	
1	DMF solv. Imidazole (9 eq) TBDMSCl (6 eq)	**Δ 80 ºC**	**24 hrs**	Di-substituted compound C3 and C5
2#	**Pyridine solv. Imidazole (9 eq) TBDMSCl (6 eq)**			**η ac. = 78%**

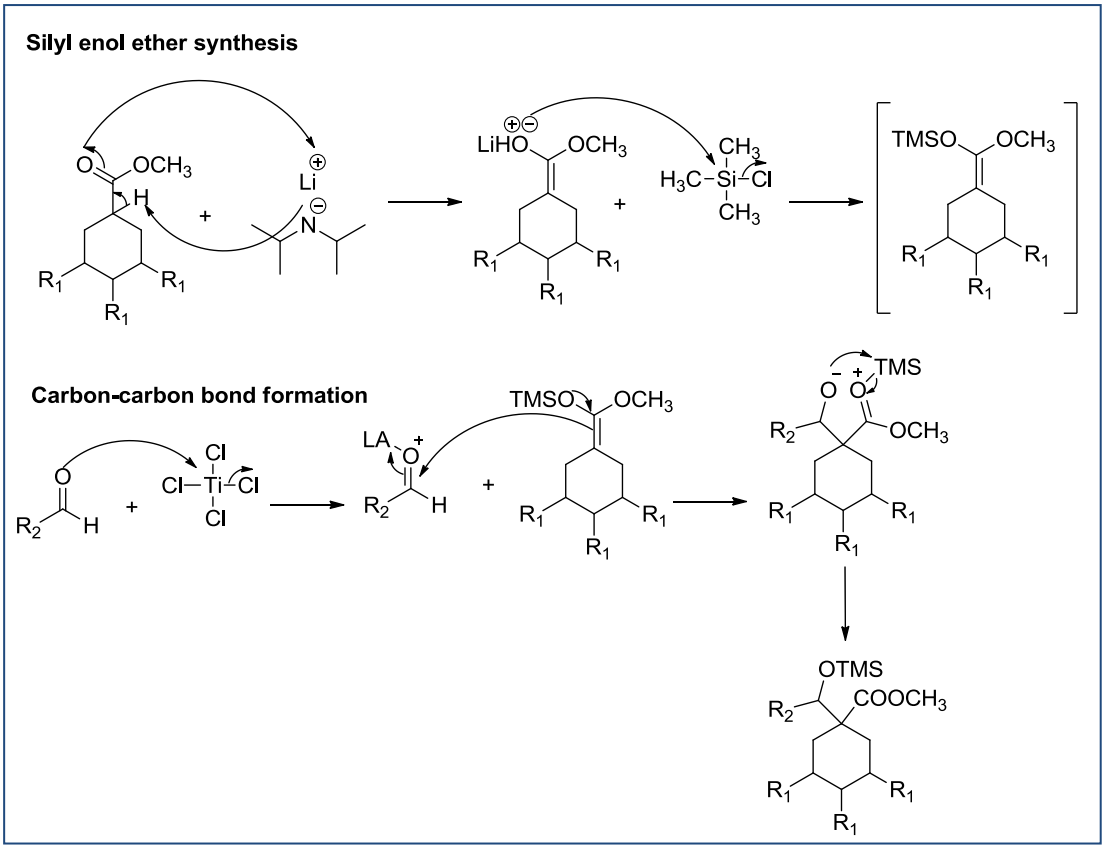
showed to be the better synthetic method. η ac. = after column yield.

Table 5.1.22. – Conditions for synthesis of 3,4,5-tri-O-(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate (**15**) starting from 3,4,5-O-tri-hydroxy-cyclohexane-1-carboxylate (**14**).

As a result, and in keeping with the other reaction conditions, it was possible to obtain, with the same 24 hrs. reaction time, and after column chromatography, a yield of compound (**15**) of 78%.

5.1.6. Mukaiyama reaction - testing of the reaction conditions, for the introduction of aliphatic groups at the C1 position

Having now successfully synthesized compound (**15**), we were closer to one of our major goals, because at this stage we had now successfully synthesised the starting material required to attempt the *Mukaiyama Aldol Addition Reaction* (see Scheme 5.1.25 and 5.1.27). [120,121,122,123,124,125, 126]

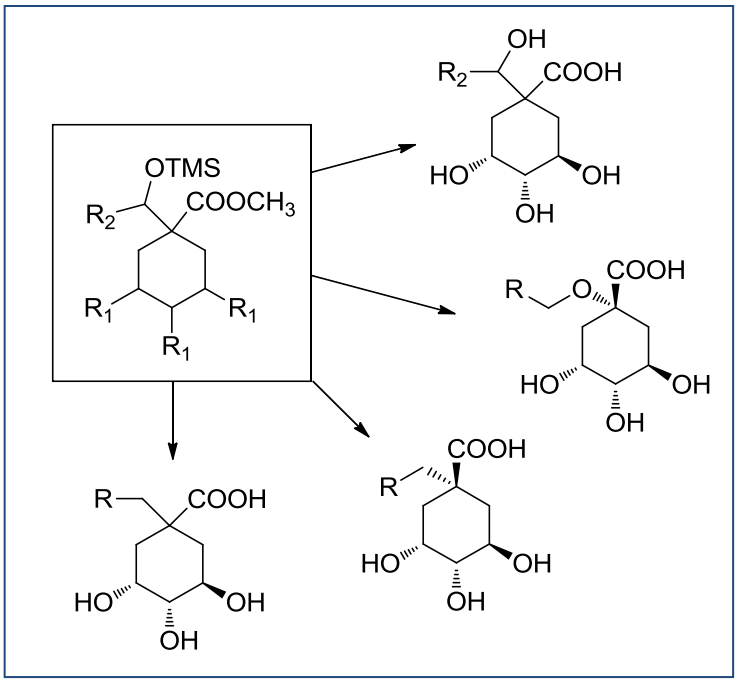


Scheme 5.1.25. – Mukaiyama aldol addition reaction mechanism. [119,126]

This reaction is a powerful tool for carbon-carbon bond formation and as such is widely used in asymmetric and total synthesis. It has the advantage of using mild conditions and a directed donor and acceptor, but it is an indirect process and many factors play a key role in the stereochemistry of the product (*synclinical* or *antiperiplanar* 1:1 or 3:1), although today there are enantioselective methods. [120,121,122,123,124,125, 126]

Nonetheless a successful outcome to using this reaction in the quinic acid compounds with tertiary hydrogen at C1 position, would lead to being able to introduce at that position, while maintaining the ester functionality, a series of aliphatic substituents (see Scheme 5.1.26 for some examples of quinic acid C1 substituted compounds that we aim to synthesise).

This would represent novel methodology, not previously applied to compounds of this type.



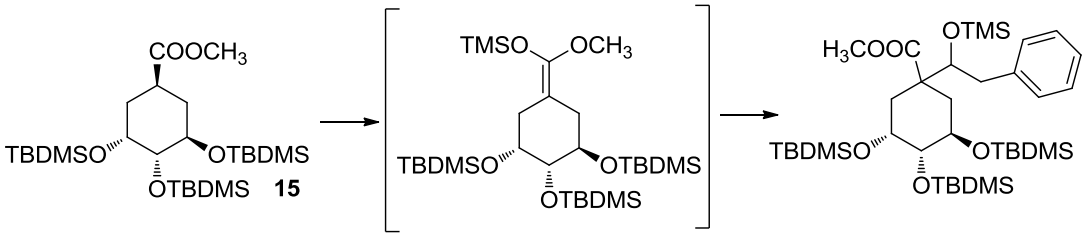
Scheme 5.1.26. - Quinic acid C1 substituted compounds.

There are two synthetic routes, the Lewis acid-mediated and the Lewis base-mediated methods. The traditional route uses a stoichiometric or catalytic amount of a Lewis acid (LA) such as titanium chloride, boron trifluoride diethyl etherate ($BF_3.Et_2O$) and tin chloride ($SnCl_4$).

The first step consists of the synthesis of an intermediate silyl enol ether, through the use of LDA (to abstract the tertiary proton) and TMSCl. The second step consists of the formation of the new carbon-carbon bond, with the activation of the aldehyde or ketone electrophile by the Lewis acid, through an open transition state (see scheme 5.1.25 for the reaction mechanism schematics).

The synthesis of 1-(benzyl-2-trimethylsilyloxy)-3,4,5-tri-*O*-(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate starting from 3,4,5-tri-*O*-

(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate (**15**) was attempted (see Scheme 5.1.27). The method used is an adaptation from that originally used by Mukaiyama et al. [119,126]



Scheme 5.1.27. – Attempt at the synthesis of 1-(benzyl-2-trimethylsilyloxy)-3,4,5-tri-O-(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate starting from 3,4,5-tri-O-(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate (15) – Mukaiyama Aldol Addition Reaction.

Entry 1 Table 5.1.23 describes the reaction conditions used in the first attempt. The reaction has 2 steps, as stated before. In the first step, compound (**15**) was dissolved in THF and LDA is added. After 15 min, TMSCl was added and the reaction was stirred for 15 min, everything at 0 ºC. In the second step, a solution of DCM, $TiCl_4$ and phenylacetaldehyde was first prepared (phenylacetaldehyde was the aldehyde chosen as a model to test the reaction) at -78 ºC. This solution was then added to the mixture prepared in the first step and stirred for 1 hr. unfortunately only unreacted starting material was recovered.

Following this result, the reaction times of all steps were increased (see entry 2 Table 5.1.23), but unfortunately this did not affect the reaction outcome and once again only unreacted starting material was recovered.

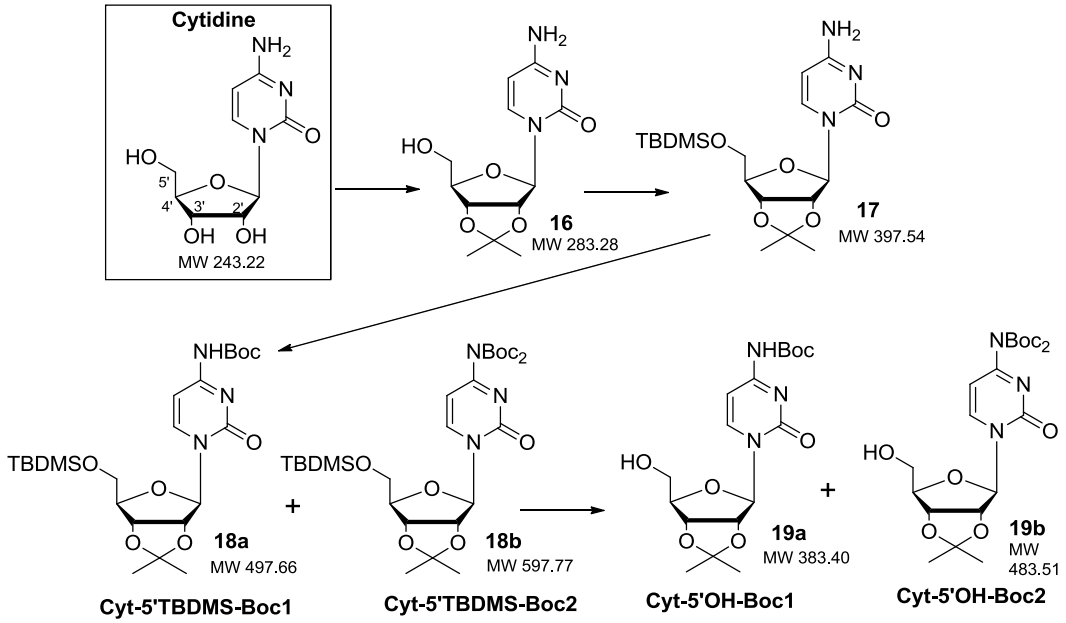
Met.	Reagents	Conditions		Results
		Temp.	Time	
1[a)]	THFsolv. + LDA 1.8 M in THF(1.1 eq) + TMSCl (1 eq)	0 ºC	15 min (s.m.+THF+LDA) + 15 min (+TMSCl)	Only unreacted s.m.
	DCM solv. + TiCl4 1 M in DCM (1 eq) + Phenylacetaldehyde (1 eq)	-78 ºC	1 hr (+previous mixture)	
2[a)]	THFsolv. + LDA 1.8 M in THF(1.1 eq) + TMSCl (1 eq)	0 ºC	30 min (s.m.+THF+LDA) + 1 hr (+TMSCl)	
	DCM solv. + TiCl4 1 M in DCM (1 eq) + Phenylacetaldehyde (1 eq)	-78ºC	1.30 hrs (+previous mixture)	

a) Adapted from Mukaiyama (1973). [119,126]

Table 5.1.23. – Testing conditions for the attempt at the synthesis of 1-(benzyl-2-trimethylsilyloxy)-3,4,5-tri-O-(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate starting from 3,4,5-tri-O-(*tert*-butyldimethylsilyloxy)-cyclohexane-1-carboxylate (**15**).

As future work, the plan would be to alter the equivalents of the reagents used and closely monitor the reaction at the end of the first step, to confirm that the intermediate is formed. A detailed investigation would need to be instigated to ascertain if the problem is in the first step, in the second step or both, and act accordingly.

5.2. CYTIDINE MODIFICATIONS SUITABLE FOR COUPLING TO QUINIC ACID AND SIALIC ACID-BASED ANALOGUES OF CMP-Neu5Ac VIA PHOSPHATE OR OTHER SUITABLE LINKAGES



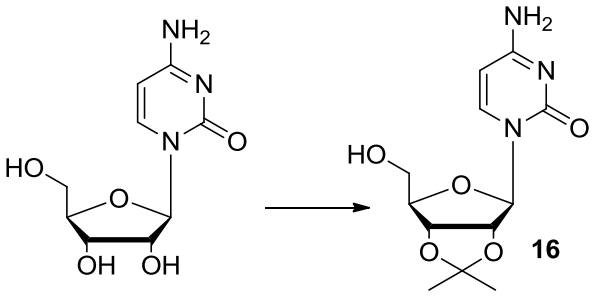
Scheme 5.2.1. – Cytidine: tested reactions and synthesized compounds.

In parallel to my efforts towards the synthesis of quinic acid-based analogues, it was necessary to be engaged in the preparation of suitably protected cytidine derivatives. As referred to previously, one of the aims of this project is coupling, through a suitable linkage, of the quinic acid compounds, synthesized and described is the previous section, to a cytidine nucleotide, so that it resembles the natural substrate of the sialyltransferase enzymes, CMP-Neu5Ac. In that way they would have hopefully serve as effective polyST inhibitors.

The quinic acid analogue compounds, alone or coupled, through different linkages, to cytidine or other nucleotide, would be evaluated through biological assays to determine their possible use as inhibitors and their affinity towards the enzymes.

Cytidine was chosen as the first molecule to be coupled, since it is part of the natural substrate (CMP-sialic acid) for the enzymes responsible for the synthesis of polySia, namely PST and STX. Due to a shortage of time, only the cytidine chemistry and optimization of the reaction conditions for that molecule (synthesized compounds – see Scheme 5.2.1 for the schematics of all the reactions and synthesized compounds) is presented here.

Preparation of the cytidine molecule involves a series of protection steps. [92]



Scheme 5.2.2. – Synthesis of 2',3'-O-isopropylidene cytidine (**16**) starting from commercially available cytidine**.**

The first step consists of the protection of the diol at positions C2' and C3' with a isopropylidene ring (similar to what was made before with quinic acid to protect adjacent positions C3 and C4).

Synthesis of 2',3'-*O*-isopropylidene cytidine (**16**) starting from commercially available cytidine (see Scheme 5.2.2) was achieved using acetone as the solvent and as reagent for the formation of the isopropylidene

ring, with the help of p-TSOH as a catalyst that deprotonates the hydroxyl groups, refluxing at 80 ºC for 2 hrs.

These conditions were replicated successfully and always with very good yields of compound (**16**), and therefore the methodology did not require further optimization (see Table 5.2.1).

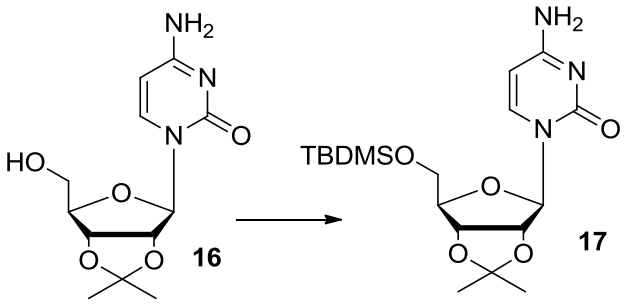
Met.	Solv.	Cat.	Conditions		Results
			Temp.	Time	
1	**Acetone**	**p-TsOH (9.3 eq)**	**Reflux 80 ºC**	**2 hrs**	**η ac. = 93-97%**

η ac. = after column yield.

Table 5.2.1. – Testing conditions for the synthesis of 2',3'-O-isopropylidene cytidine (**16**) starting from cytidine.

The second protection step of the cytidine molecule, consists of the protection of the O5' hydroxyl group. The common methodology, is to protect it using TBDMSCl.

Synthesis of 2',3'-*O*-isopropylidene-5'-*tert*-butyldimethylsilyloxy cytidine (**17**) starting from 2',3'-*O*-isopropylidene cytidine (**16**) (see Scheme 5.2.3) was obtained using DMF as the solvent, 2 eq. of imidazole to 1 eq. TBDMSCl at RT for 45 min, which was so far the standard procedure (see entry 1 Table 5.2.2). However the obtained yield using those conditions was very low.



Scheme 5.2.3. – Synthesis of 2',3'-O-isopropylidene-5'-*tert*-butyldimethylsilyloxy cytidine (**17**) starting from 2',3'-O-isopropylidene cytidine (**16**).

For that reason the amount of imidazole and TBDMSCl used was increased (see entry 2, Table 5.2.2) as well as the reaction time to 1.30 hrs. As a result the yield improved, albeit slightly.

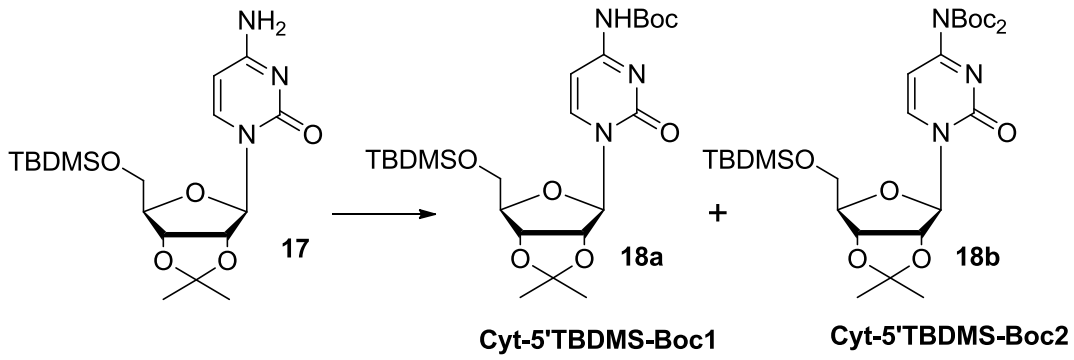
Met.	Solvent	Reagents	Conditions		Results
			Temp.	Time	
1	**DMF**	Imidazole (2 eq) TBDMSCl (1 eq)	**RT**	45 min	η ac. < 5%
2		Imidazole (2.5 eq) TBDMSCl (1.1 eq)		1.30 hrs	η ac. =13%
3#		**Imidazole (5 eq) TBDMSCl (2.2 eq)**		**1 hr**	**η ac. = 97%**

showed to be the better synthetic method. η ac. = after column yield.

Table 5.2.2. – Testing conditions for the synthesis of 2',3'-O-isopropylidene-5'-*tert*-butyldimethylsilyloxy cytidine (**17**) starting from 2',3'-O-isopropylidene cytidine (**16**).

With that in mind the amount of reagents were further increased (see entry 3 Table 5.2.2) and after stirring for 1 hr. it was possible to obtain compound (**17**) with an excellent yield of 97%, after column chromatography.

After optimization of the previous step, protection of the amine group follows. The method uses Boc_2O as the protection agent, THF as the solvent and DMAP as the catalyst, at RT overnight.



Scheme 5.2.4. – Synthesis of N-*tert*-butyldicarbonate 2´,3´-O-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc1, **18a**) and N,N-Di-*tert*-butyldicarbonate 2´,3´-O-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc2, **18b**) starting from 2',3'-O-isopropylidene-5'-*tert*-butyldimethylsilyloxy cytidine (**17**).

The method starting from 2',3'-*O*-isopropylidene-5'-*tert*-butyldimethylsilyloxy cytidine (**17**) leads to the synthesis of two compounds *N*-*tert*-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc1, **18a**, substituted with one Boc molecule) and *N,N*-Di-*tert*-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsi lyloxy cytidine (Cyt-5'TBDMS-Boc2, **18b**, substituted with two Boc molecules) (see Scheme 5.2.4). The ratio of the two compounds may vary but it is preferable to have compound **18b** as the major compound, because in protection steps ahead, protection with two Boc appeared more stable.

Entry 1 Table 5.2.3 describes the reaction to give an overall yield of 79% and Boc1:Boc2 ratio of 44:56, compound (**18b**) as the major one, as determined by NMR spectroscopy.

In the hope of improving further the overall yield and the yield of compound (**18b**), the amount of Boc_2O reagent and DMAP were increased to see whether it had any effect on the reaction outcome (see entry 2 Table 5.2.3). As a result a lower overall yield was obtained, but a higher ratio of compound (**18b**), Boc1:Boc2 ratio of 28:72.

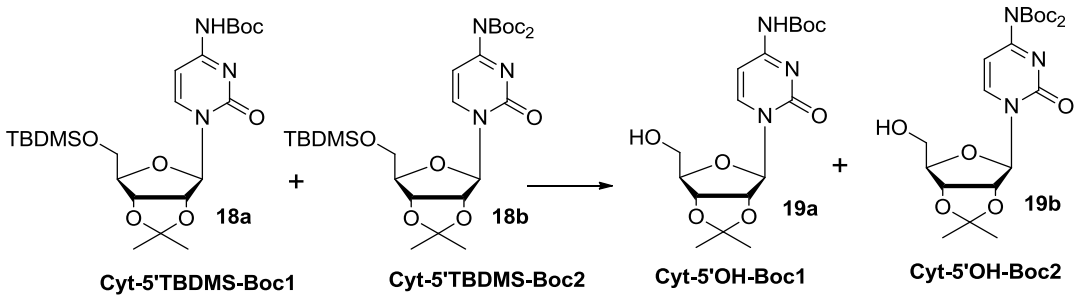
For the next step the mixture of both compounds (**18a**) and (**18b**) was used. Although it was preferable to have compound (**18b**), it is not essential.

Met.	Reagents	Catalyst	Conditions		Results
			Temp.	Time	
1	THF solv. Boc_2O (1.7 eq)	DMAP (0.95 eq)	RT	ovn.	η ac.**18a** = 35% η ac.**18b** = 44% **η ov. = 79%**
2	THF solv. Boc_2O (2 eq)	DMAP (1 eq)			η ac.**18a** = 19% η ac.**18b = 50%** η ov. = 69%

η ac. = after column yield.; η ov. = overall yield.

Table 5.2.3. – Testing conditions for the synthesis of N-*tert*-butyldicarbonate 2´,3´-O-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc1, **18a**) and N,N-Di-*tert*-butyldicarbonate 2´,3´-O-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc2, **18b**) starting from 2',3'-O-isopropylidene-5'-*tert*-butyldimethylsilyloxy cytidine (**17**).

The last step to be described in this section regarding the protection steps of cytidine is the one that follows and consists of the removal of the TBDMS group from the C5' position and regeneration of the hydroxyl group.



Scheme 5.2.5. – Synthesis of N-*tert*-butyldicarbonate 2´,3´-O-isopropylidene cytidine (Cyt-5'OH-Boc1, **19a**) and N,N-Di-*tert*-butyldicarbonate 2´,3´-O-isopropylidene cytidine (Cyt-5'OH-Boc2, **19b**) starting from N-*tert*-butyldicarbonate 2´,3´-O-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc1, **18a**) and N,N-Di-*tert*-butyldicarbonate 2´,3´-O-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc2, **18b**).

Starting from a mixture of *N-tert*-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc1, **18a**) and *N,N*-Di-*tert*-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc2, **18b**) compounds *N-tert*-butyldicarbonate 2´,3´-*O*-isopropylidene cytidine (Cyt-5'OH-Boc1, **19a**) and *N,N*-Di-*tert*-butyldicarbonate 2´,3´-*O*-isopropylidene cytidine (Cyt-5'OH-Boc2, **19b**) (see Scheme 5.2.5) were successfully synthesised, using the method described in Table 5.2.4, that uses THF as solvent, TBAF as the deprotection agent at RT for 2 hrs. As a mixture of compounds was used as starting material, as expected we obtained a mixture of compounds, i.e. one compound with one Boc and two Boc groups. As can be seen in Table 5.2.4 compound (**19a**), with only one Boc, is the major compound, because as mentioned previously, the mono Boc is slightly less stable to the remaining conditions. In any case, the overall yield was very high and the mixture could be used as the starting material for the next step.

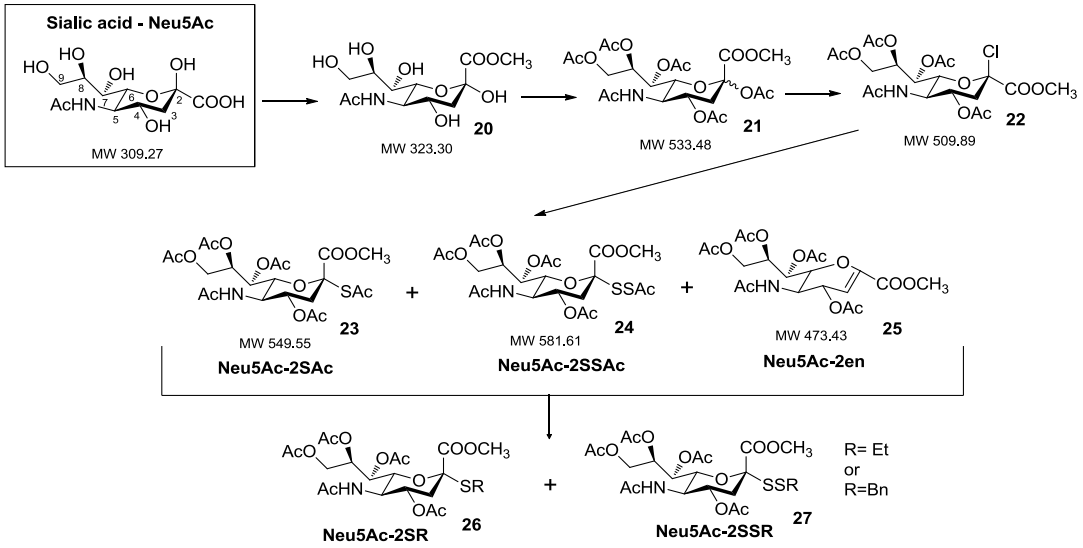
Met.	Solv.	Reag.	Conditions		Results
			Temp.	Time	
1	THF	TBAF 1 M in THF (1.2 eq) Added dropwise	RT	2 hrs	η ac.**19a** = 54% η ac.**19b** = 43% **η ov. = 97%**

η ac. = after column yield. η ov. = overall yield.

Table 5.2.4. – Conditions for the synthesis of N-*tert*-butyldicarbonate 2´,3´-O-isopropylidene cytidine (Cyt-5'OH-Boc1, **19a**) and N,N-Di-*tert*-butyldicarbonate 2´,3´-O-isopropylidene cytidine (Cyt-5'OH-Boc2, 19b) starting from mixture of N-*tert*-butyldicarbonate 2´,3´-O-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc1, **18a**) and N,N-Di-*tert*-butyldicarbonate 2´,3´-O-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (Cyt-5'TBDMS-Boc2, **18b**).

The next step involved, the replacement of an iodine atom at C5' position. This reaction had to be made in a schedule that allows for the immediate coupling step as soon as the iodine compound is synthesized. Unfortunately due to insufficient time, the reaction scheme could not be further explored. The advantage was that the compound was stable and could be stored ready for future use.

5.3. SIALIC ACID-BASED ANALOGUES: DEVELOPMENT OF METHODOLOGY FOR THE SYNTHESIS OF SIALO-DISULFIDES



Scheme 5.3.1. – Sialic acid: tested reactions and synthesized compounds.

This section describes one final area of activity in which I was engaged: glycosyl disulfides, and in particular sialic acid-based disulfides. The wider group already had studied in a very detailed manner the sialic acid chemistry for the synthesis of thiosialosides.

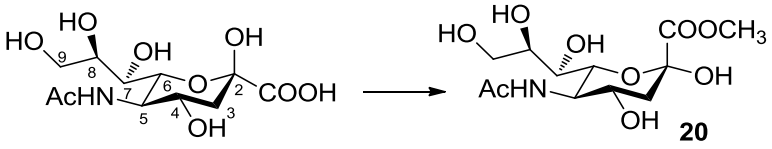
Glycosyl disulfides represent an interesting group of compounds which have received attention due to their potential use as glycosyl donors in synthetic reactions and their potential as probes to increase understanding of biological systems. [127, 128]

We recognised the possibility of synthesising sialic acid-based disulfide compounds from a by-product identified in the synthesis of sialic acid sulfides, when using KSAc in EtOAc – namely acetyl disulfide sialoside (**24**) (Scheme 5.3.1) – a very useful potential building block. [127]

The disulfide by-product was observed when attempting to synthesise akyl and aryl thiosialosides. The synthesis of a second by-product, the acetyl disulfide form stated previously, was identified. This work was summarised in a peer-reviewed publication and appended to this thesis (Appendix, chapter 9).

The key step in the synthesis of thiosialosides, the conversion of 2-chlorosialoside to 2-thioacetate via the use of KSAc, was already known to form also an unsaturated by-product Neu5Ac2en. These by-products were difficult to separate from the main sulphide compound, and HPLC needed to be used, since the disulfide was usually a minor product. [127]

Firstly, a way to encourage synthesis of this particular compound was investigated – i.e. to improve the disulfide:sulfide formation ratio, so that this was the major product and not the sulfide. It was observed that the addition of I_2 to the KSAc in EtOAc medium, drove the reaction to the preferential formation of disulfide. These represent novel conditions for molecules of this type, with considerable potential scope for synthesis of glycosyl disulfide derivatives. An attempt to optimise the conditions for this reaction was therefore proceeded.



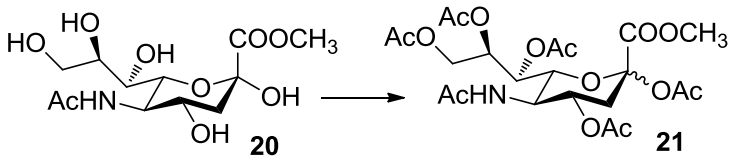
Scheme 5.3.2. – Synthesis of methyl 5-acetamido-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosonate (N-Acetylneuraminic methyl ester, **20**) starting from commercially available sialic acid**.**

Scheme 5.3.1 summarises the principle reactions and compounds made during these disulfide-related studies.

Sialic acid is a sugar that contains an amine (acetate-derivatised, and so effectively protected), five hydroxyl groups and a carboxylic acid. It was therefore necessary to introduce protecting groups to the molecule before being able to derivatise it. [129]

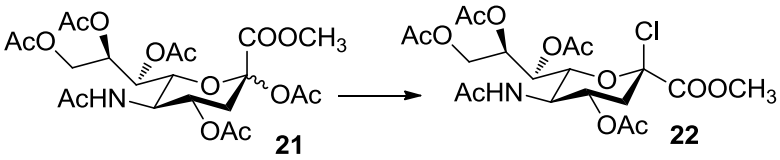
The first protection step led to the synthesis of methyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulo-pyranosonate (*N*-Acetyl-neuraminic methyl ester, **20**) using commercially available sialic acid as starting material (see Scheme 5.3.2). This method utilises MeOH as the solvent, which with the Amberlite resin as catalyst and proton donor, is a commonly used method for the conversion of a carboxylic acid to its ester form. After 48 hrs at RT an almost quantitative yield of compound (**20**) was obtained. The reaction proved to be highly reproducible, even with multi-gram scale-up.

The second protection step consisted of the synthesis of the penta-*O*-acetylated sialic acid methyl ester, 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonate (**21**) starting from previously synthesized methyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (*N*-Acetylneuraminic methyl ester, **20**, see Scheme 5.3.3).



Scheme 5.3.3. – Synthesis of 5-acetamido-2,4,7,8,9-penta-O-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosonate (**21**) starting from methyl 5-acetamido-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosonate (*N*-acetylneuraminic methyl ester, **20**).

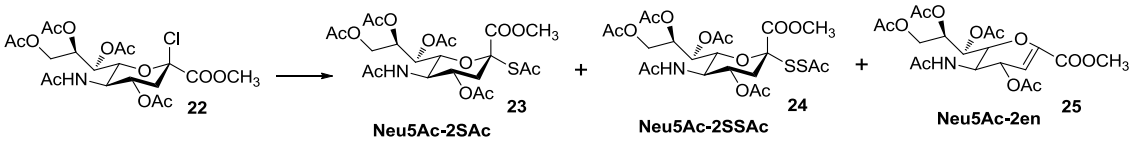
The acetylation process proceeded smoothly. After 24 hrs reaction, and flash column chromatography, the yield was approximately 90%. The compound obtained was actually a mixture of two anomers (i.e. the α and β anomer of compound **21**). These isomers proved impossible to separate by a chromatography column. The β anomer was the major product due to the position of the adjacent ester function.



Scheme 5.3.4. – Synthesis of methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2-chloro-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosonate (**22**) starting from 5-acetamido-2,4,7,8,9-penta-O-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosonate (**21**).

The next step involved chlorination of position C2 to synthesise methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (**22**) starting from, the mixture of anomers at C2 position, 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonate (**21**) (see Scheme 5.3.4).

The chlorination method uses acetyl chloride and a stream of HCl gas a the source of Cl species, first bubbling for 15 min at 0 ºC and then leaving it to stir at RT for a further 48 hrs. The reaction gave successfully compound (**22**) as exclusively β anomer. The reaction was high yielding and reproducible.



Scheme 5.3.5. – Synthesis of 2-(acetylsulfanyl)-5-acetamido-4,7,8,9-tetra-O-acetyl-2,3,5-trideoxy-2-thio-α-D-glycero-D-galacto-2-nonulopyranosonate (Neu5Ac-2SSAc, **24**) starting from methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2-chloro-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosonate (**22**).

Once the chlorinated sialic acid had been synthesised, it was now possible to start considering the synthesis of disulfide sialic acid compounds in some detail. In the first instance, my aim was to synthesise 2-(acetylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyranosonate (Neu5Ac-2SSAc, **24**) from the chlorinated compound (**22**) (see Scheme 5.3.5), while minimising the formation of any by-products (e.g. unsaturated derivative **25**). [127,128] It was determined that the source of KSAc was very important to the outcome of the reaction. [127] The number of equivalents of KSAc used was preferably 6 eq; iodine was initially used at 1.5 eq to allow the formation of the disulfide. At this stage, the precise mechanism of the reaction is not known. The reaction conditions were stirring at RT overnight.

As two batches of KSAc were available, an old one and a newly purchased one (and from different commercial brands), one of the objectives was to assess the influence of the reagent quality (Table 5.3.3 entries 1, 2 and 3 refer to the use of an batch 1 of KSAc reagent which was already opened for some time and entries 4, 5 and 6 to the use of a batch 2 a newly purchased batch) on the outcome of the reaction.

A second objective was to see if the use of a different solvent (other than the normally-used EtOAc). DMF was selected as an alternative (see entry 3 and entry 6 of Table 5.3.4) solvent to be evaluated. The number of equivalents used of KSAc and I_2, 6 eq. and 1.5 eq. accordingly were not changed at this stage. Importance of temperature on the reaction was also explored: since EtOAc was employed either at RT (see entry 1 and 4 of Table 5.3.4) or at 85 ºC (see entry 2 and 5 of Table 5.3.4).

As can be seen in Table 5.3.4, when using the old KSAc batch (entries 1, 2 and 3), the result was the same – synthesis of compound Neu5Ac2en (**25**), independently from the solvent and reaction conditions used. It can therefore be concluded that the reagent was already degraded and for that the main product obtained was the oxidized compound (**25**), methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-glycero-D-talo-non-2-enonate (Neu5Ac2en).

Met.	KSAc batch	Solv.	KSAc	I_2	Conditions		Filt.	Results
					Temp.	Time		
1	ONE (1)	EtOAc	6 eq	1.5 eq	RT	ovn.	yes	Neu5Ac-2en **(25)**
2					85ºC		yes	
3		DMF			Δ 80ºC		no	
4#	**TWO (2)**	**EtOAc**	**6 eq**	**1.5 eq**	**RT**	**ovn.**	**yes**	**Mixture** **Neu5Ac-2SAc (23)** 60% **Neu5Ac-2SSAc (24)** 40%
5					85ºC		yes	Neu5Ac-2en **(25)**
6		DMF			Δ 80ºC		no	Degradation

1) Batch one, LF005421, 25 g Alfa Aesar.
2) Batch two, BCBF7702 Sigma-Aldrich
showed to be the better synthetic method.Adapted from Morais et al (2009) [127]

Table 5.3.4. – Conditions for the synthesis of 2-(acetylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-α-D-glycero-D-galacto-2-nonulopyranosonate (Neu5Ac-2SSAc, **24**) starting from methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosonate (**22**).

The same result was obtained when using the new batch of KSAc, but using EtOAc under reflux conditions (entry 5 Table 5.3.4), which can lead us to conclude that increasing the temperature reduces production of the disulfide compound, in favour of the unsaturated compound (**25**).

Substitution of EtOAc for DMF (entry 6 Table 5.3.4) for better solubilisation of the new batch of KSAc in the reaction medium proved not to be useful, because it only led to complete degradation of the starting

material. It would be useful to try DMF at RT in the future work to see if the problem was the high temperature used, and not the solvent itself.

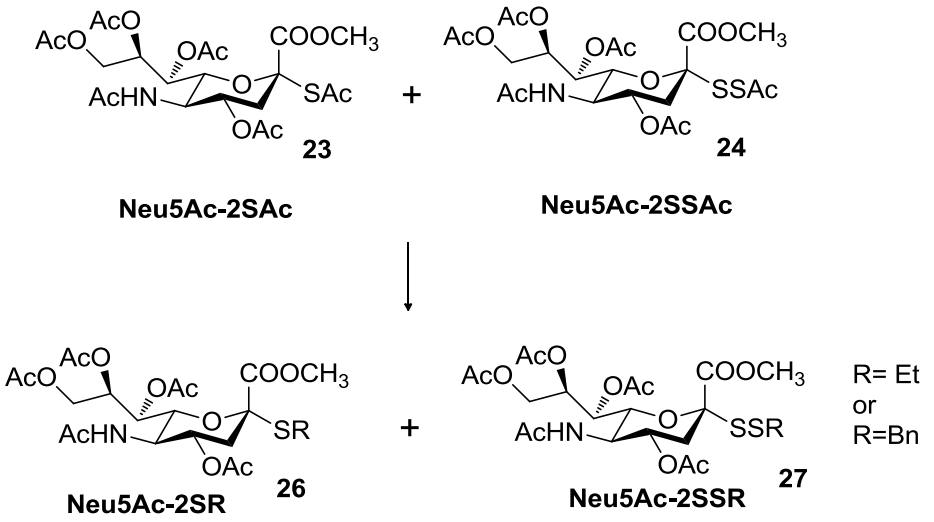
The better method is highlighted in entry 4, Table 5.3.4. Nonetheless it was still a mixture of compounds, the sulfide compound (**23**, Neu5Ac-2SAc) and the disulfide compound (**24**, Neu5Ac-2SSAc) in a ratio of 60% sulfide: 40% disulfide which meant that the desired compound was not the main product.

Met.	KSAc batch	Solv.	KSAc	I_2	Conditions		Filt.	Results
					Temp.	Time		
1[(1)]	**TWO[(2)]**	**EtOAc**	**6 eq**	**1.5 eq**	**RT**	**ovn.**	**yes**	Mixture Neu5Ac-2SAc **(23)** 30% Neu5Ac-2SSAc **(24)** 70%

1) S.m. for this reaction was the mixture 4 (see table 5.3.4).
2) Batch two, BCBF7702 Sigma-Aldrich.

Table 5.3.5. – Conditions for the improvement of mixture 4 (see Table 5.3.4) ratio of 2-(acetylsulfanyl)-5-acetamido-4,7,8,9-tetra-O-acetyl-2,3,5-trideoxy-2-thio-α-D-glycero-D-galacto-2-nonulopyranosonate (Neu5Ac-2SSAc, **24**).

To separate this mixture preparative HPLC was required. However, it was believed that it would be possible to improve the reaction ratio further by reacting the mixture obtained previously (60% sulfide: 40% disulfide) under the same conditions with a further 6 eq of KSAc new batch and 1.5 eq of iodine (see Table 5.3.5). As a result it was possible to improve the reaction ratio, to 30% sulfide: 70% disulfide.



Scheme 5.3.6. – Attempt at the alkylation of mixture 4 (see Table 5.3.4): synthesis of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-S-benzyl-2,3,5-trideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranosonate (Neu5Ac-2SBn, **26**) and 2-(benzylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranosonate (Neu5Ac-2SSBn, **27**).

Having studied the methodology for the synthesis of compound (**24**) and achieving a more satisfactory ratio between sulphide and disulfide, my next objective was to study potential methodology for the alkylation of disulfide sialic acid compounds (see Scheme 5.3.6), starting from the aforementioned acetyl disulfide derivative. Unlike the reaction conditions for the simultaneous de-protection and alkylation of sialosides starting from the thioacetate using diethylamine,[127,130] this reagent was not suitable, resulting in breakdown of the starting material. Triethylamine alone had no effect at all. It was found that initial dissolution in DMF, followed by addition, in a specific order, of triethylamine, alkylating agent and hydrazine acetate, at 50 ºC during 20 min (Table 5.3.6) was sufficient to promote de-protection and alkylation. The chosen alkykating agents were iodoethane (EtI) and benzyl

bromide (BnBr), and the starting material used was the mixture of compounds (**23**) and (**24**) obtained previously (entry 4 Table 5.3.4). A preliminary set of small-scale reactions was investigated, before embarking on preparative HPLC method development in order to separate the mixture, since there was insufficient time to do so before.

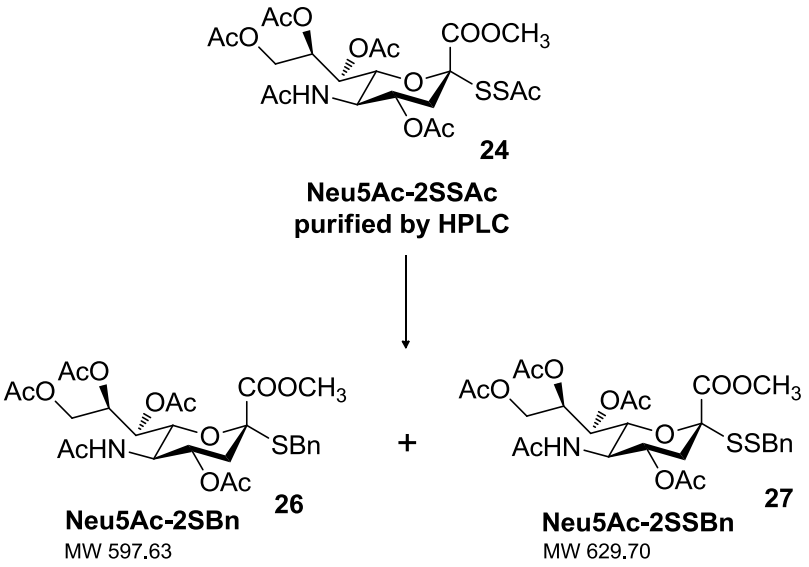
When using EtI (entry 1 Table 5.3.6) complete degradation of the reaction mixture was observed. However, when using BnBr (entry 2 Table 5.3.6) a mixture of compounds (**26**) and (**27**) was observed. These were the expected products, given the nature of the starting material.

Met.	Reagents	Alk. (R)	Conditions		Results
			Temp.	Time	
1(1)	DMF solv. Et_3N cat. (3 eq) Hydrazine acetate (1.1 eq)	EtI (2 eq)	Δ 50 ºC	20 min	Degradation
2(1)	DMF solv. Et_3N cat. (3 eq) Hydrazine acetate (1.1 eq)	BnBr (2 eq)	Δ 50 ºC	20 min	Mixture of Neu5Ac-2SBn (**26**) and Neu5Ac-2SSBn (**27**)

(1) S.m. for this reaction was the mixture 4 (see table 5.3.4) and the reagents were added in a specific order: first the Et_3N, then the alkylating agent and last the hydrazine acetate.

Table 5.3.6. – Conditions for the attempt at the alkylation of mixture 4 (see table 5.3.4).

Following purification of compound (**24**) the method used in entry 2 Table 5.3.6 was re-assessed (see Scheme 5.3.7 and Table 5.3.7).



Scheme 5.3.7. – Attempt at the alkylation of pure 2-(acetylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-α-D-glycero-D-galacto-2-nonulopyranosonate (Neu5Ac-2SSAc, **24**).

Met.	Reagents	Alk. (R)	Conditions		Results
			Temp.	Time	
1(1)	DMF solv. Et_3N cat. (3 eq) Hydrazine acetate (1.1 eq)	BnBr (2 eq)	Δ 50 ºC	20 min	Neu5Ac-2SBn **(26)**

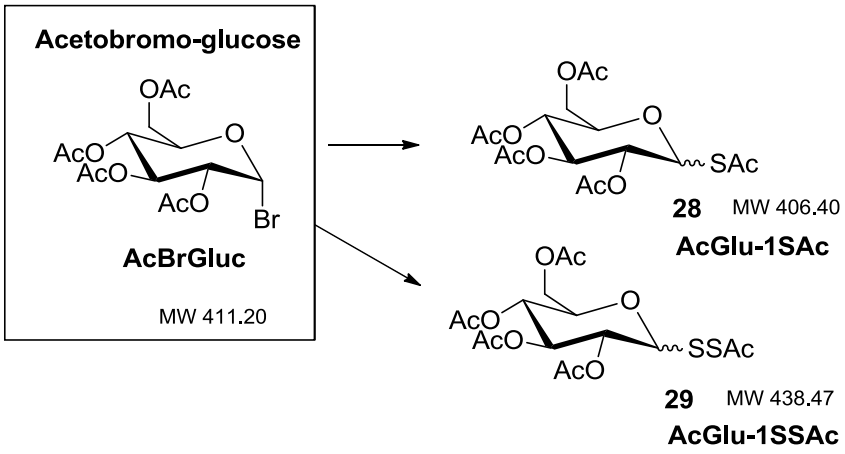
(1) S.m. for this reaction was purified by HPLC and the reagents were added in a specific order: first the Et_3N, then the Alkylating agent and last the hydrazine acetate.

Table 5.3.7. – Conditions for the attempt at the alkylation of pure 2-(acetylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-α-D-glycero-D-galacto-2-nonulopyranosonate (Neu5Ac-2SSAc, **24**).

As can be seen in Table 5.3.7 the result wasn't the one predicted, since compound **26** (Neu5Ac-2SBn, **26**) was exclusively obtained. Further studies are needed to confirm this result. Should it be proved correct, additional investigations are required regarding this methodology to shed some light regarding the mechanism involved and the role that each reagent

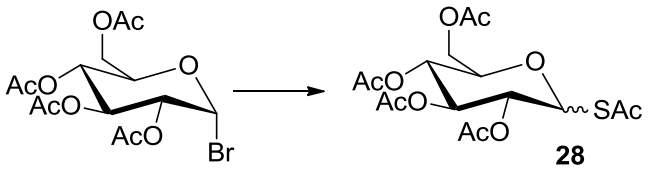
takes in it, so that in the future we could be able to synthesise directly the disulfide compounds and their alkylated forms of sialic acids, but also in the hopes of applying this quite simple methodology to other sugars and aliphatic compounds.

5.4. ACETOBROMO-GLUCOSE METHODOLOGY STUDIES FOR THE SYNTHESIS OF SULFIDE AND DISULFIDE COMPOUNDS



Scheme 5.4.1. – Acetobromo-glucose: tested reactions and synthesized compounds.

As previously mentioned, one of my objectives when developing the methodology for the synthesis of sialic acid-based disulfides, was to investigate whether it would be possible to apply the approach more broadly. To be more specific, whether it would be possible to synthesise disulfides of other compounds (other sugars, aliphatic compounds among others of interest) was studied. In order to examine wider applicability to sugars, glucose was selected as a good starting point, specifically utilising the readily commercially available acetobromo-α-D-glucose (see Scheme 5.4.1).



Scheme 5.4.2. – Synthesis of 1-(acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosate (**28**) starting from commercially available acetobromo-α-D-glucose (AcBrGlu).

The first objective was to apply the methodology previously used for sialic acid sulfide chemistry, for the synthesis of the sulfide 1-(acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosate (**28**, see Scheme 5.4.2) starting from acetobromo-α-D-glucose (AcBrGlu). The method used acetone as the solvent, 6 eq of KSAc, RT overnight. It was predicted that since glucose was less reactive than sialic acid, harsher conditions may be required to achieve introduction of the thioacetyl function in this case.

As before, both the old (entry 1 and 2 Table 5.4.1) and new batches of KSAc (entry 3 and 4 Table 5.4.1) were tested to assess any effect on the outcome of the reactions. As happened previously with sialosides, independently of the reagents and reaction conditions used, the only result was complete degradation. Nonetheless these results did help to determine some key points: the reaction required heating, and when using acetone as solvent the reaction time had to be monitored closely (the optimum conditions proved to be 3-4 hrs).

Met.	KSAc batch	Solv.	KSAc	Conditions		Filt.	Results
				Temp.	Time		
1	ONE[1]	Acetone	6 eq	RT + Reflux 70 ºC	ovn. + 4.30 hrs	yes	Degradation
2		DMF		Δ 80 ºC	ovn.	no	
3	**TWO[2]**	DMF	**6 eq**	Δ 80 ºC	1.20 hrs	no	Mixture AcGluc-1SAc **(28)** α and β anomer
4#		**Acetone**		**Reflux 70 ºC**	**3 hrs**	**yes**	**η ac. (28) = 82%** **α anomer**

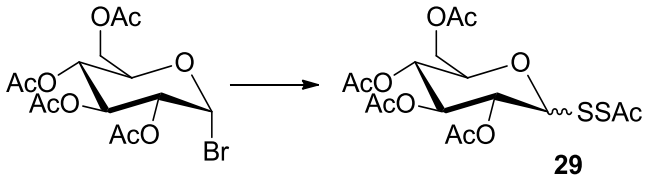
1) Batch one, LF005421, 25 g Alfa Aesar.
2) Batch two, BCBF7702 Sigma-Aldrich.
showed to be the better synthetic method. η ac. = after column yield.
Adapted from Morais et al (2009) [127]

Table 5.4.1. – Conditions for the synthesis of 1-(acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosate (**28**) starting from commercially available acetobromo-α-D-glucose (AcBrGlu).

With these results in hand, the effect of changing the solvent to DMF (entry 3 Table 5.4.1) at 80 ºC from acetone (entry 4 Table 5.4.1) reflux at 70ºC was tested. Using DMF led to more rapid product formation, perhaps partly due to increased solubility of KSAc. More significantly, it was determined that these conditions led to a non stereoselective reaction, giving a mixture of α and β anomers of the desired compound (**28**). When using acetone, after 3 hrs, the α anomer of compound (**28**) was successfully

synthesised exclusively, and with high yield. The methodology using acetone was therefore adopted.

Having successfully synthesised the sulphide (thioacetate) form acetobromo-α-D-glucose, the next goal was to investigate synthesis of the equivalent acetyl disulfide form of the molecule, specifically: 1-(acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranosate (**29**, see Scheme 5.4.3). The reaction conditions established before for the synthesis of compound (**24**) were initially used.



29

Scheme 5.4.3. – Synthesis of 1-(acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranosate (**29**) starting from commercially available acetobromo-α-D-glucose (AcBrGlu).

Again both batches of KSAc were used: batch 1 (see entries 1, 2, 4, 5 and 6 Table 5.4.2) and batch 2 (see entry 3 Table 5.4.2), as previously described for the synthesis of compound (**24**), using 6 eq. of KSAc and 1.5 eq of iodine.

Firstly, batch 2 of KSAc was used, with acetone as solvent (see entry 1 and 2 Table 5.4.2) since these were the conditions determined to be the best for the synthesis of the sulfide compound (**28**) (see Table 5.4.1). We previously noted that for the synthesis of compound (**28**) that heating at raised temperature was required: the reaction did not progress at room temperature. Refluxing in acetone at 70 ºC was selected, in one method for

only 2 hrs (entry 1 Table 5.4.2) and in the other overnight stirring (entry 2 Table 5.4.2). When using the first method a mixture of sulfide compound (**28**) and the desired disulfide compound (**29**) was obtained. TLC analysis indicated the presence of only one compound (**28**), as in the proton and carbon NMR. However, mass spectrometry indicated also the presence of compound (**29**). This result needs confirmation since it can be an artefact of the mass spectrometry process. When the reaction was left overnight the main product was compound (**28**), with degradation occurring.

As synthesis of the disulfide proved unsuccessful, use of DMF as solvent was next tested, using both bacth 1 (entry 3 Table 5.4.2) and batch 2 of KSAc (entry 4 Table 5.4.2) at 80 ºC overnight. As expected from previous studies, DMF did increase the solubility of KSAc salts, but as happened previously (Table 5.4.1 entry 4), the result was a mixture of α and β anomers of compound (**28**). Next, acetonitrile was used, but a similar outcome was observed (entry 5 Table 5.4.2).

On analysing the results obtained, it was possible that it could be that in the first 2 hrs the sulfide compound (**28**) was formed and then afterwards it led to the synthesis of compound (**29**) when iodine was added.

For that reason in entry 6 a reflux in acetonitrile was first attempted, with the acetobromo-glucose starting material and the new batch KSAc for 2 hrs, to allow the formation of SAc. After cooling to RT, iodine was added and the reaction left overnight to allow the formation of disulfide compound (**29**). As result we obtained the same as in entry 1, a mixture sulfide compound (**28**) and the desired disulfide compound (**29**), with the latter still a small minority.

Met.	KSAc batch	Solv.	KSAc	I_2	Conditions		Filt.	Results
					Temp.	Time		
1	TWO (1)	Acetone	6 eq	1.5 eq	Reflux 70 ºC	2 hrs	yes	Mixture AcGluc-1SAc **(28)** **AcGluc-1SSAc (29)**
2						ovn.	yes	Mainly AcGluc-1SAc **(28)** More degradation
3	ONE (2)	DMF	6 eq	1.5 eq	Δ 80 ºC	ovn.	no	Mixture AcGluc-1SAc **(28)** α and β anomer
4	TWO (1)							
5	TWO (1)	MeCN	6 eq	1.5 eq	Reflux 82 ºC	ovn.	no	Mixture AcGluc-1SAc **(28)** α and β anomer
6*					Reflux 82 ºC + RT	2 hrs + ovn.	no	Mixture AcGluc-1SAc **(28)** **AcGluc-1SSAc (29)**

1) Batch two, BCBF7702 Sigma-Aldrich.
2) Batch one, LF005421, 25 g Alfa Aesar.
* reaction 6 was refluxed with KSAc for 2 hrs to form the SAc, and then let to cool to RT to add the iodine and stir ovn. Adapted from Morais et al (2009) [127]

Table 5.4.2. – Conditions for the synthesis of 1-(acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranosate (**29**) starting from commercially available acetobromo-α-D-glucose (AcBrGlu).

Although it proved possible to synthesise the desired sulfide compound using this methodology for the first time, further studies are needed to optimise it.

6. CONCLUSION AND FUTURE WORK

6.1. Conclusion

In this thesis I have described efforts towards the development of chemical methodology to synthesise key building blocks for the synthesis of potential polysialyltransferase inhibitors. The overall strategy, outlining structural modifications for the construction of CMP-Neu5Ac analogues for inhibition of PSA biosynthesis (sialyltransferase inhibitors) that are in the work plan are demonstrated in Scheme 3.1.

The focus of the majority of the work described in this thesis concerned the modification of the core structure of Neu5Ac, through replacement of the sugar sialic acid ring by a carbacycle with similar structure: quinic acid (or quinic acid analogues). This kind of carbomimetic has the potential for potent inhibition of the polysialyltransferase enzymes.

The quinic acid-based chemistry proved to be challenging. Reactions were not easily reproducible because they were very sensitive, often to pH variations of the reaction medium, and therefore it was difficult to optimize the reaction conditions and there is the need for further studies in the near future.

Key reactions proved successful, thereby achieving methodology not previously described for molecules of this type. The Barton-McCombie reaction was successfully optimized, which allowed deoxygenation of the C1 position of quinic acid analogues – a novel approach.

Functionalization of the C1 position is crucial to any future work, as this will be key to conjugation with other moieties (e.g. cytidine). It is also attractive since it is not described in the literature. Success will allow the

synthesis of aliphatic compounds and others (see Scheme 5.1.27). The literature only describes substitution of C3, C4 and C5 hydroxyls; deprotonation of C1 hydroxyl and attachment of others groups, but never complete removal/substitution of the C1 hydroxyl.

Besides carbacycles, replacement of the phosphate linkage between sialic acid and cytidine in the substrate (CMP-sialic acid) was considered. Following identification of a novel acetyl disulfide sialoside derivative, efforts were placed into the potential utility of this compound as a building block for asymmetrical disulfide synthesis. Novel methodology for the synthesis of this acetyl disulfide compound was successfully developed. Reaction conditions were optimised for selectivity and reproducibility.

Consideration was also given to the wider application of this methodology to other compounds: other sugars or even aliphatic compounds. Optimization of the synthesis of disulfide sialic acid compound was accomplished, but unfortunately when adapting it to a glucose sugar (initially acetobromoglucose), limited success was experienced thus far.

6.2. Future Work

The logical starting point would be to attempt to conjugate quinic acid derivatives to the cytidine derivatives synthesised, through suitable linkages. Biological evaluation would follow. Firstly, activity in a cell-free enzyme-based assay to assess inhibition and then secondly functional assays to assess the activity of compounds with regards migration of cells.

Another key area for research is optimisation of the Mukaiyama reaction, as applied to quinic acid. Success here would allow the functionalization of the C1 position in the quinic acid molecule (see schemes 5.1.25, 5.1.26 and 5.1.27), and be highly publishable. It may be necessary to consider alternatives to this approach to achieve the same outcome.

Regarding other manipulations of the CMP-Neu5Ac structure described in scheme 3.1, synthesis of CMP-Neu5Ac with different acyl groups would be sensible, particularly given the reported selectivity for STX inhibiton by Man5But and Man5Pent, in order to create a library of these compounds ready for testing their inhibitory activity towards STX and PST.

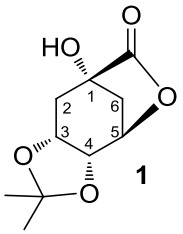
Further possibilities include alteration of the monophosphate linkage, for higher stability by C-, S- (sulfides, disulfides) or N-sialosides. Replacement or modification of the cytidine nucleoside is also planned for the future. Compounds that are transition state mimetics, with delocalized positive charge, are also of interest to future studies.

The synthesis of sialic acid disulfides is a very interesting area of research. Following identification of the novel compound, its utility for synthesis of disulfide compounds became clear. Further optimisation of alkylation conditions would uncover a completely new and easy way to synthesise disulfides. Experiments are already underway in the group.

The ultimate goal would be to arrive at an inhibitor with good activity in the *in vitro* biological assays, to be able to investigate the effects of polysialyltransferase inhibition in vivo.

7. EXPERIMENTAL

7.1. QUINIC ACID-BASED SYNTHESIZED COMPOUNDS



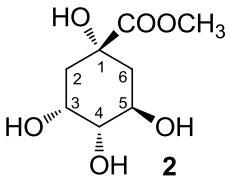
1

7.1.1. 3, 4-*O*-isopropylidene-1,5-quinic lactone (1)

Quinic acid (D(-)-quinic acid 98%, sigma Aldrich, lot 523568-025 MW 192.174 g,20.8 mmol) and of Na_2SO_4 (17.8 g, 6 eq, 62.4 mmol) were suspended in acetone (160 mL). Then concentrated H_2SO_4 (~10 drops) was added as a catalyst. The suspension was stirred at reflux for 24 hrs. After that time TLC (EtOAc /petroleum ether 1:1) showed that no starting material was present. After cooling, the reaction was quenched, , by neutralization to pH 6-7 via the addition of solid $NaHCO_3$. The mixture was filtered and the acetone was evaporated under vacuum. The residue was partitioned between water (50 mL) and DCM (50 mL), the combined organic extracts were dried with Na_2SO_4 and concentrated in vacuum, giving compound **1** as a white solid (4.29 g, 20.03 mmol, 96 %). The analytical data correspond to the reported values.[95, 96, 97]

R*f* (EtOAc): 0.80; R*f* (EtOAc/pet.ether 1:1): 0.5; $[\alpha]^{20}_{D}$ – 34.8 (c 0.175, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 1.33 (s, 3H, CH_3), 1.52 (s, 3H, CH_3), 2.18 (dd, 1H, *J* = 14.7, 2.9 Hz, $H2_{eqt}$), 2.29 - 2.41 (m, 2H, $H6_{eqt}$ and $H2_{ax}$), 2.65 (d, 1H, J = 11.8 Hz, $H6_{ax}$), 4.30 (ddd, 1H, *J* = 6.5, 2.4, 1.4 Hz H4), 4.5 (app. td, 1H, *J* = 7.1, 2.8 Hz, H3) and 4.73 (app. dd, 1H, *J* = 6.1, 2.4 Hz, H5) ppm; ^{13}C NMR

($CDCl_3$): δ 24.29 (CH_3), 26.97 (CH_3), 34.29 (CH_2, C6), 38.30 (CH_2, C2), 71.53 (CH, C4), 72.12 (CH, C3), 75.87 (CH, C5), 78.77 (C1)109.81 ($C_{isopropylidene}$), and 178.71 (C=O) ppm; MS (AP^+) $C_{10}H_{14}O_5$ (214.22) *m/z* (%): 215.0 $[M+H)^+$ (100).

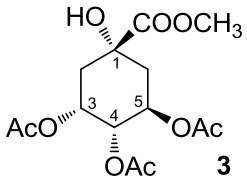


2

7.1.2. Methyl quinate (2)

Quinic acid (D(-)-quinic acid 98%, sigma Aldrich, lot 523568-025 MW 192.17, 2 g, 10.4 mmol) was suspended in MeOH (100 mL). Amberlite ion exchange resin $IR120H^+$ (2.5 g, previously washed with MeOH) was then added to the solution (). The suspension was stirred at reflux for 24 hrs. The reaction was filtered (to remove the resin) and the solvent was evaporated, giving compound **2** as a colourless powder (2.10 g, 10.2 mmol, 98%). The analytical data correspond to the reported values. [36, 98]

R*f* (DCM:MeOH 15%) 0.40; $[\alpha]^{20}_D$ – 31.3 (c 0.28, MeOH); ^{1}H NMR ($CDCl_3$): 1.75-2.13 (m, 4 H, $H2_{ax}$, $H2_{eqt}$, $H6_{ax}$ and $H6_{eqt}$), 2.3 (s, OH5), 2.54 (d, *J* = 9.2 Hz, OH4), 3.33 (td, 1H, *J* = 9.2, 3.4 Hz, H4), 3.44 (d, *J* = 8.6 Hz, OH3), 3.70 (s, 3H, $COOCH_3$), 3.94 - 4.02 (m, 1 H, H3) and 4.07 (app. dd, 1H, *J* = 8.6, 3.2 Hz, H5) ppm; ^{13}C NMR ($CDCl_3$): 38.35 (CH_2, C2), 41.97 (CH_2, C6), 52.93 ($COOCH_3$), 68.21 (CH, C5), 71.47 (CH, C3), 76.54 (C4), 76.82 (CH, C1) and 175.96 (C=O) ppm; MS (AP^+) $C_8H_{14}O_6$ (206.20) *m/z* (%): 207.3 $[M+H]^+$ (100).



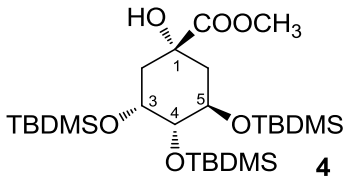
3

7.1.3. Methyl 3,4,5-tri-*O*-acetylquinate (3)

Methyl quinate **(2)** (1.17 g, 5.67 mmol) was dissolved in pyridine (3 mL) and the mixture cooled to 0ºC (ice bath). Then acetic anhydride (1.9 mL, 3.5 eq, 19.8 mmol) was added very slowly dropwise during 30 min with the reaction temperature maintained at 0 ºC. The mixture was allowed to warm up to RT and stir for 3.45 hrs, after which analysis using TLC (DCM/MeOH 15%) showed the total consumption of starting material. The reaction mixture was cooled to 0 ºC and anhydrous MeOH (10 mL) was added and stirred for 1 hr. The mixture was concentrated, extracted with saturated aqueous solution of $NaHCO_3$ and EtOAc, dried ($Na_2SO_{4)}$, filtered and evaporated. Flash column chromatography (EtOAc/pet.ether gradient 1:5 to 1:1) and NMR ($CDCl_3$) enable to identify compound **3** as an amorphous solid (930 mg, 2.8 mmol, 49%). The analytical data correspond to the reported values. [36, 100]

R*f* (EtOAc/PE 1:2) 0.53; $[\alpha]^{20}_D$ – 41.6 (c 0.25, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 1.98 (s, 3H, Ac), 1.99 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.08 - 2.24 (m, 4H, $H2_{ax}$, $H2_{eqt}$, $H6_{ax}$ and $H6_{eqt}$), 3.21 (s, 1H, OH), 3.74 (s, 3 H, COOCH_3), 4.98 (dd,1 H, *J* = 9.5, 3.4 Hz, H4), 5.35 - 5.44 (m, 2 H, H3 and H5) ppm; ^{13}C NMR ($CDCl_3$): δ 20.78 (Ac, *C*H_3), 20.93 (Ac, *C*H_3), 21.11 (Ac, *C*H_3), 35.45 (CH_2, C2), 38.67 (CH_2, C6), 53.25 (COO*C*H_3), 67.04 (CH, C3), 68.71 (CH, C5), 71.63 (CH, C4), 73.66 (C1), 168.88 (Ac, C=O), 170.20 (Ac, C=O),

170.32 (Ac, C=O), 174.51 (*C*OOCH$_3$) ppm; MS (AP$^+$) $C_{14}H_{20}O_9$ (332.30) *m/z* (%): 333.0 $[M+H]^+$ (100).



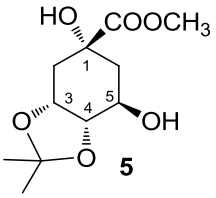
4

7.1.4. 3,4,5-tri(*tert*-butyldimethylsilyloxy)-1-hydroxy cyclohexane-1-carboxylate (4)

Methyl quinate **(2)** (100 mg, 0.48 mmol) was dissolved in anhydrous DMF (5 mL) and imidazole (500 mg, 15 eq, 7.35 mmol) and TBDMSCl (740 mg, 10 eq, 4.8 mmol) were added. The reaction was then stirred at 80 ºC for 72 hrs. The solvent was evaporated and the crude mixture washed with water (100 mL) and extracted with DCM (100 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated. TLC (DCM/MeOH 5%) showed several impurities and therefore column chromatography had to be conducted (DCM/MeOH gradient DCM to 5% MeOH). NMR ($CDCl_3$) of the one of the obtained pure fractions corresponded to compound **4** as a colourless solid (247 mg, 0.45 mmol, 93%). The analytical data correspond to the reported values. [101]

R*f* (DCM/MeOH 5%) 0.8 ; $[\alpha]^{20}_D$ + 4.78 (c 0.23, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ - 0.01 (2s, 6H, SiC*H*$_3$), 0.04 (2s, 6H, SiC*H*$_3$), 0.08 (2s, 6H, SiC*H*$_3$), 0.82 (1s, 9H, SiC(C*H*$_3$)$_3$), 0.86 (1s, 9H, SiC(C*H*$_3$)$_3$), 0.87 (1s, 9H, SiC(C*H*$_3$)$_3$), 1.89 (dd, 1H, *J* = 12.4, 10.2 Hz, H6$_{ax}$), 1.97 - 2.10 (m, 3H, H2$_{ax}$, H2$_{eqt}$ and H6$_{eqt}$), 3.53 (m, 1H, H4), 3.68 (s, 3H, COOC*H*$_3$), 3.86 (m, 1H, H5) and 4.24

(m, 1H, H3) ppm; ^{13}C NMR ($CDCl_3$): δ - 4.98 (C, Si*C*H_3), - 4.86 (C, Si*C*H_3), - 4.81 (C, Si*C*H_3), - 4.70 (C, Si*C*H_3), - 4.45 (C, Si*C*H_3), - 4.17 (C, Si*C*H_3), 18.09 (C, Si*C*$(CH_3)_3$), 18.17 (C, Si*C*$(CH_3)_3$), 18.20 (C, Si*C*$(CH_3)_3$), 25.81(3C, SiC(*C*$H_3)_3$), 25.96 (3C, SiC(*C*$H_3)_3$), 26.03 (3C, SiC(*C*$H_3)_3$), 36.62 (CH_2, C2), 39.75 (CH_2, C6), 52.21 (COO*C*H_3), 68.26 (CH, C3), 70.92 (CH, C5), 74.27 (CH, C4), 77.21 (C1) and 174.46 (C7) ppm; MS (AP^+) $C_{26}H_{56}O_6Si_3$ (548.98) *m/z (%)*: 549.4 $[M+H]^+$ (100).



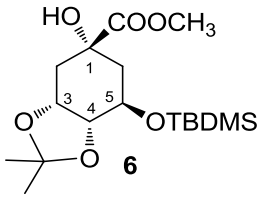
5

7.1.5. Methyl 3,4-*O*-isopropylidene quinate (5)

To a solution of quinide acetal **(1)** (500 mg, 2.3 mmol) in anhydrous MeOH (17 mL) was added molecular sieves and to this mixture was added slowly dropwise NaOMe solution (6 mL 0.5 M in MeOH, 1.3 eq, 3 mmol) at 0 ºC during 30 min, under a N_2 atmosphere. Then the mixture was stirred to warm up to RT for 22 hrs. Analysis using TLC (EtOAc) showed the absence of starting material (R*f* 0.8) and so the reaction was neutralized with Amberlite resin IR-120 H^+ only until pH 7 (smallest amount possible used). The reaction was filtered and evaporated. NMR (CD_3OD) showed the desired compound **5** as colourless oil (358 mg, 1.5 mmol, 62%). The analytical data correspond to the reported values. [38, 86, 97, 102, 103]

R*f* (EtOAc): 0.4; $[\alpha]^{20}_D$ – 1.21 (c 0.165, CH_2Cl_2); ^{1}H NMR (CD_3OD): δ 1.23 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.70 (dd, 1H, *J* = 13.4, 11.2 Hz, $H6_{ax}$),

1.84 (app. ddd, 1H, *J* = 13.4, 4.2, 1.8 Hz, $H6_{eqt}$), 1.88 - 1.97 (m, 1H, $H2_{ax}$), 2.14 (dd, 1H, *J* = 15.1, 5.4, $H2_{eqt}$), 3.64 (s, 3H, COOCH_3), 3.80 (m, 1H, H4), 3.96(ddd, 1H, *J* = 11.3, 7.2, 4.3 Hz, H5) and 4.33 (app. dd, 1H, *J* = 9.8, 5.4 Hz, H3) ppm; ^{13}C NMR (CD_3OD): δ 24.19 (CH_3), 26.76 (CH_3), 35.23 (CH_2, C2), 38.06 (CH_2, C6), 53.30 (COOCH_3), 67.42 (CH, C3), 73.19 (CH, C5), 73.66 (C1), 78.94 (CH, C4),109.67 ($C_{isopropylidene}$), and 175.67 (C=O) ppm; MS (AP^-) $C_{11}H_{18}O_6$ (246.26) *m/z* (%): 269 $[M+Na]^+$ (100) and 247 $[M+H]^+$ (45).



6

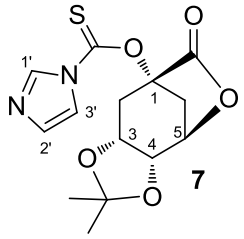
7.1.6. 5-(*tert*-butyldimethylsilyloxy)-3,4-O-isopropylidene quinate (6)

Methyl 3,4-O-isopropylidene quinate **(5)** (47 mg, 0.19 mmol) was dissolved in DMF (1 mL) and then imidazole (38.6 mg, 3 eq, 0.57 mmol) and TBDMSCl (57 mg, 2 eq, 0.38 mmol) were added.

The resulting mixture was then stirred at 60 ºC for 3.5 hrs. The reaction was then quenchedthrough DMF evaporation under vacuum and the residue was washed with water (100 mL) and extracted with DCM (100 mL). The organic layers were dried ($Na_2SO_{4)}$, filtered and evaporated. Column chromatography was conducted to remove impurities (EtOAc/petroleum ether 1:9 to 1:1) and NMR ($CDCl_3$) of the one of the obtained fractions showed compound **6** as a colourless oil (30 mg, 0.083 mmol, 44%).

R*f* (EtOAc/PE 1:2) 0.52; $[\alpha]^{20}_D$ – 54.12 (c 0.09,CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ -0.01 (1, 3H, SiCH_3), 0.02 (1s, 3H, SiCH_3), 0.81 (s, 9H,

SiC(CH_3)$_3$),1.28 (s, 3H, CH_3),1.45 (s, 3H, CH_3), 1.77 (dd, 1H, *J* = 13.7, 10.5 Hz, H6$_{ax}$), 1.90 (ddd, 1H, *J* = 13.7, 4.6, 2.0 Hz, H6$_{eqt}$), 2.10 - 2.17 (m, 1H, H2$_{ax}$), 2.20 (dd, 1H, *J* = 15.7, 4.3 Hz, H2$_{eqt}$), 3.35 (s, 1H, OH), 3.71 (s, 3H, COOCH_3), 3.86 (t, 1H, *J* = 6.1 Hz, H4), 4.03 (ddd, 1H, *J* = 10.8, 6.5, 4.7 Hz, H5) and 4.37 – 4.41 (m, 1H, H3) ppm; ^{13}C NMR ($CDCl_3$): δ - 4.81(C, SiCH_3), - 4.53 (C, SiCH_3), 18.01 (SiC(CH_3)$_3$), 25.58 (3C, SiC(CH_3)$_3$), 25.81 (CH_3), 28.24 (CH_3), 34.29 (CH_2, C2), 40.58 (CH_2, C6), 52.84 (COOCH_3), 69.18 (CH, C5), 73.84 (CH, C3), 74.14 (C1), 80.29 (CH, C4),108.86 (C$_{isopropylidene}$), and 174.96 (C=O) ppm; MS (AP$^+$) $C_{17}H_{32}O_6Si$ (360.52) *m/z* (%): 361.2 $[M+H]^+$ (100).



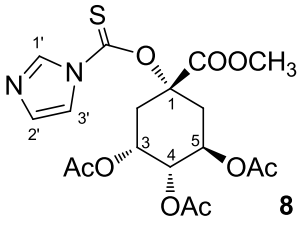
7

7.1.7. 1-*O*-(thiocarbonyldiimidazolyl)-3,4-*O*-isopropylidene-1,5-quinic lactone (7)

Quinide acetal **(1)** (1 g, 4.7 mmol) was dissolved in anhydrous DCE (25 mL) and DMAP (230 mg, 0.4 eq, 1.88 mmol) was added. Then thiocarbonyldiimidazole (1.7 g, 2 eq, 9.4 mmol) was added and the mixture stirred at reflux under N_2 atmosphere for 24 hrs. The solvent was evaporated and the residue washed with a saturated aqueous solution of NH_4Cl and extracted with DCM. The organic phase was dried (Na_2SO_4), filtered and evaporated. Column chromatography (EtOAc/pet.ether gradient 1:1 top 2:1)

and NMR ($CDCl_3$) allow isolation of compound **7** as a colourless sugar-like (1.3 g, 4 mmol, 86%).

R*f* (EtOAc) 0.65; $[\alpha]^{20}_D$ – 2.3 (c 0.13, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 1.38 (s, 3H, CH_3), 1.57 (s, 3H, CH_3), 2.66 (ddd, 1H, *J* = 14.3, 7.6, 2.4 Hz, $H6_{eqt}$), 2.76 (d, 1H, *J* = 14.3, 3.0 Hz, $H6_{ax}$), 2.92 (d, 1H, *J* = 11.6 Hz, $H2_{ax}$), 3.59 (dddd, 1H, *J* = 11.5, 6.5, 2.2, 1.3 Hz, $H2_{eqt}$), 4.41 (ddd, 1H, *J* = 6.5, 2.3, 1.1 Hz, H4), 4.63 (td, 1H, *J* = 7.2, 3.0 Hz, H3), 4.92 (dd, 1H, *J* = 6.5, 2.4 Hz, H5), 7.06 (d, 1H, *J* = 0.8 Hz, $H2'_{imidazole}$), 7.63 (app. t, 1H, *J* = 1.4 Hz, $H3'_{imidazole}$) and 8.32 (s, 1H, $H1'_{imidazole}$) ppm; ^{13}C NMR ($CDCl_3$): δ 24.27 (CH_3), 26.91 (CH_3), 29.79 (CH_2, C6), 35.63 (CH_2, C2), 71.03 (CH, C5), 72.35 (CH, C4), 75.73 (CH, C3), 82.30 (C1), 110.15 ($C_{isopropylidene}$), 117.99 ($C2'_{imidazole}$), 131.09 ($C3'_{imidazole}$), 136.74 ($C1'_{imidazole}$), 171.3 (C=O) and 179.22 (C=S) ppm; MS (AP^+) $C_{14}H_{16}N_2O_5S$ (324.35) m/z (%): 325.1 $[M+H]^+$ (100).



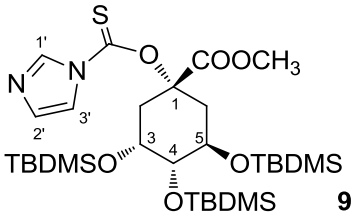
8

7.1.8. 1-*O*-(thiocarbonyldiimidazolyl)-3,4,5-tri-*O*-acetylquinate (8)

Methyl 3,4,5-tri-*O*-acetylquinate **(3)** (87 mg, 0.26 mmol) was dissolved in anhydrous DCE (5 mL), and then DMAP (46 mg, 1.5 eq, 0.39 mmol) and thiocarbonyldiimidazole (133 mg, 3 eq, 0.78 mmol), batch two lot BCBF5117V Sigma-Aldrich) were added. The mixture stirred at reflux for 48 hrs. The reaction was evaporated, washed with saturated aqueous solution of NH_4Cl (50 mL) and extracted with DCM (50 mL). The organic phase was

dried ($Na_2SO_{4)}$, filtered and evaporated. Column chromatography (EtOAc/pet.ether gradient 1:5 to 2:1) and NMR ($CDCl_3$) gave compound **8** as a yellow oil (16 mg, 0.036 mmol, 14% yield, the low yield was expected due to the large amount of unreacted starting material showed by TLC analysis during the monitoring of the reaction).

R*f* (EtOAc/PE 2:1) 0.41; $[\alpha]^{20}_D$ – 24 (c 0.075,CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 1.62 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 2.14 (m, 1H, $H2_{eqt}$), 2.50 (dd, 1H, *J* = 16.6, 3.3 Hz, $H6_{ax}$), 2.88 (m, 1H, $H2_{ax}$), 3.38 (dt, 1H, *J* = 16.6, 3.3 Hz , $H6_{eqt}$), 3.75 (s, 3H, $COOCH_3$), 5.07 (dd, 1H, *J* = 10.4, 3.6 Hz H4), 5.42 (tt, 1H, *J* = 11.3, 5.6 Hz, H3), 5.61 (q, 1H, *J* = 3.4 Hz, H5), 7.06 (d, 1H, *J* = 0.8 Hz, $H2'_{imidazole}$), 7.72 (app. t , 1H, *J* = 1.4 Hz, $H3'_{imidazole}$) and 8.45 (s, 1H, $H1'_{imidazole}$) ppm; MS (AP^+) $C_{18}H_{22}N_2O_9S$ (442.44) *m/z (%)*: 443.1 $[M+H]^+$ (100).



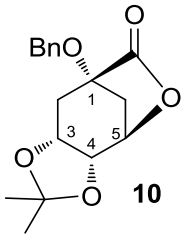
9

7.1.9. 1-*O*-(thiocarbonyldiimidazolyl)-3,4,5-tri-*O*-(*tert*-butyldimethylsilyloxy)cyclohexane-1-carboxylate (9)

3,4,5-tri(*tert*-butyldimethylsilyloxy)-1-hydroxy cyclohexane-1-carboxylate **(4)** (80 mg, 0.146 mmol) was dissolved in DCE (4 mL), and then DMAP (13.5 mg, 0.8 eq, 0.11 mmol) and thiocarbonyldiimidazole (102 mg, 4 eq, 0.57 mmol) were added. The mixture stirred at reflux for 48 hrs. The

reaction was quenched with saturated aqueous solution of NH_4Cl (50 mL) and extracted with DCM (50 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated. Column chromatography (EtOAc/pet.ether gradient pet.ether only to 1:4) and NMR ($CDCl_3$) gave one fraction that corresponded to the unreacted starting material **(4)** (white solid, 34.5 mg, 0.063 mmol, 36% recovered, R*f* 0.6 in 80% PE in EtOAc solvent) and an UV-reactive fraction as compound **9** a colourless oil (30 mg, 0.046 mmol, 31% yield).

R*f* (80% PE in EtOAc) 0.5; $[\alpha]^{20}_{D}$ + 16.25 (c 0.16,CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 0.04 (1s, 3H, SiCH_3), 0.05 (1s, 3H, SiCH_3), 0.08 (1s, 3H, SiCH_3), 0.11 (1s, 3H, SiCH_3), 0.12 (1s, 3H, SiCH_3), 0.13 (1s, 3H, SiCH_3), 0.87 (1s, 9H, SiC(CH_3)$_3$), 0.90 (1s, 9H, SiC(CH_3)$_3$), 0.92 (1s, 9H, SiC(CH_3)$_3$), 1.94 - 2.07 (ddd, 3H, *J* = 39.8, 14.8, 5.1 Hz, H2$_{ax}$, H6$_{eqt}$ and H6$_{ax}$), 2.74 (dd, 1H, *J* = 13.4, 3.0 Hz, H2$_{eqt}$), 3.66 (m, 1H, H4), 3.76 (s, 3H, COOCH_3), 4.12 - 4.18 (m, 1H, H5), 4.40 - 4.47 (m, 1H, H3), 7.06 (d, 1H, *J* = 0.8 Hz, H2'$_{imidazole}$), 7.36 (app. t , 1H, *J* = 1.4 Hz, H3'$_{imidazole}$) and 8.08 (s, 1H, H1'$_{imidazole}$); MS (AP$^+$) $C_{30}H_{58}N_2O_6SSi_3$ (659.11) *m/z (%)*: 660.3 $[M+H]^+$ (100).



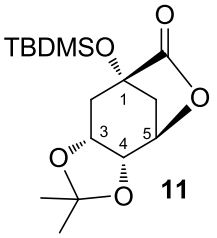
10

7.1.10. 1-*O*-benzyl-3,4-*O*-isopropylidene-1,5-quinic lactone (10)

Sodium hydride (22.4 mg, 2 eq, 0.934 mmol, 60% suspension in oil) was suspended in DMF (2 mL) at 0 ºC under N_2 atmosphere. Then a solution of quinide acetal **(1)** (100 mg, 0.47 mmol) in DMF (1mL) and benzyl bromide (66 µL, 1.2 eq, 0.56 mmol) was added dropwise during 30 min to the NaH solution in DMF at 0 ºC with stirring. The mixture was left stirring to warm up to RT for 19 hrs, after which TLC analysis (EtOAc/petroleum ether 1:1) showed only a small portion of unreacted starting material and an UV reactive compound. The reaction was then quenched slowly throught the use of a saturated aqueous solution of NH_4Cl (2 mL) at 0 ºC. The mixture was thenextracted with DCM (5 mL), and the organic phases were dried (Na_2SO_4), filtered and evaporated to give a white crude. Column chromatography (EtOAc/petroleum ether 1:2 to EtOAc only) and NMR ($CDCl_3$) gave compound **10** as a colourless solid (73mg, 0.24 mmol, 51%). The analytical data correspond to reported values. [118]

R*f* (EtOAc/pet.ether 1:1) 0.8; $[\alpha]^{20}_D$ + 8 (c 0.05, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 1.34 (s, 3H, CH_3), 1.52 (s, 3H, CH_3), 2.26 (dd, 1H, *J* = 8.0, 3.4 Hz, $H2_{eqt}$), 2.46 – 2.56 (m, 3H, $H2_{ax}$ and H6), 4.32 (m, 1H, H4), 4.55 (dd, 1H, *J* = 7.0, 2.7 Hz, H3), 4.58 - 4.67 (A, B of AB, 2H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.70 (dd, 1H, *J* = 6.0, 2.4 Hz, H5), 7.29 – 7.40 (m, 5H, C_6H_5) ppm; ^{13}C NMR ($CDCl_3$): δ

24.3, 27.0 (CH_3), 30.7 (C6), 36.1 (C2), 67.0 (CH_2Ph), 71.4 (C3), 72.4 (C4), 75.0 (C5), 76.9 (C1), 109.6 ($C_{isopropylidene}$), 127.8, 127.9, 128.4, 137.4 (6C, C_6H_5), 175.8 (C=O) ppm; MS (ES^+) $C_{17}H_{20}O_5$ (304.34) m/z (%): 305.1 $[M+H]^+$ (100).



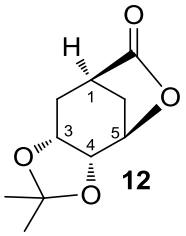
11

7.1.11. 1-(*tert*-butyldimethylsilyloxy)-3,4-*O*-isopropylidene-1,5-quinic lactone (11)

Quinide acetal **(1)** (100 mg, 0.47 mmol) was dissolved in anhydrous DCM (2 mL) and pyridine (75 µL, 2 eq, 0.94 mmol). Then TBDMSCl (212 mg, 3 eq, 1.41 mmol) was added at 0 ºC. The mixture stirred for 30 min at 0 ºC and then for 20 hrs at RT. After that time TLC analysis (EtOAc) showed total consumption of starting material and so the reaction was quenched with DCM (50 mL) and water (50 mL). The aqueous layer was separated and acidified with an aqueous solution of HCl 10% and then extracted twice with DCM (50 mL). The organic phase was then dried (Na_2SO_4), filtered and evaporated. NMR ($CDCl_3$) gave compound **11** as colourless crystals (88 mg, 0.27 mmol, 57%). The analytical data of the crude correspond to reported values. [119]

R*f* (EtOAc/pet. ether 1:2) 0.8; $[\alpha]^{20}_D$ + 1.9 (c 1.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.01(s, 3H, SiCH_3), 0.03 (s, 3H, SiCH_3), 0.82 (s, 9H, SiC(CH_3)$_3$), 1.23 (s, 3H, CH_3), 1.42 (s, 3H, CH_3), 2.08 (dd, 1H, *J* = 11.6, 3.1 Hz, $H6_{eqt}$), 2.23 – 2.30 (m, 2H, $H2_{eqt}$ and $H6_{ax}$), 2.55 (d, 1H, *J* = 11.7 Hz, $H2_{ax}$), 4.19

(ddd, 1H, *J* = 6.5, 3.5, 1.4 Hz, H4), 4.37 – 4.42 (m, 1H, H3), 4.62 (dd, 1H, *J* = 6.1, 2.5 Hz, H5) ppm; ^{13}C NMR ($CDCl_3$): δ - 3.1 (C, $SiCH_3$), - 3.2 (C, $SiCH_3$), 17.82 ($SiC(CH_3)_3$), 24.42 (C, $SiC(CH_3)_3$), 25.59 (C, $SiC(CH_3)_3$), 27.24 (C, $SiC(CH_3)_3$), 35.57 (C6), 39.36 (C2), 71.67 (C3), 72.20 (C4), 73.1 (C1), 75.1 (C5), 109.64 ($C_{isopropylidene}$), 176.9 (C=O) ppm; MS(ES^+) $C_{16}H_{28}O_5Si$ (328.48) m/z (%): 329.8 $[M+H]^+$ (100).

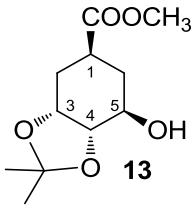


12

7.1.12. 3,4-*O*-isopropylidene-1,5-lactone cyclohexane (12)

1-*O*-(thiocarbonyldiimidazolyl)-3,4-*O*-isopropylidene-1,5-quinic lactone **(7)** (80 mg, 0.25 mmol) was dissolved in anhydrous toluene (5 mL) and AIBN was added as a catalyst (8 mg, 0.2 eq, 0.05 mmol). The mixture was purged under a N_2 atmosphere for 5 min. Then TTMSS was added (150 µL, 2 eq, 0.5 mmol) and the mixture was stirred at 90 ºC under N_2 atmosphere for 20 hrs. After that time, TLC analysis (EtOAc/pet.ether 1:3) showed total consumption of the starting material. The reaction mixture was then quenched with saturated aqueous solution of NH_4Cl (50 mL) and extracted with DCM (50 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated. Column chromatography (EtOAc/pet.ether gradient 1:4 to 2:3) and NMR ($CDCl_3$) gave compound **12** as colourless crystals (47 mg, 0.24 mmol, 96%). Nonetheless the reaction presented very inconsistent yields varying from 56 to 96%.

R*f* (EtOAc/pet.ether 1:3) 0.45; $[\alpha]^{20}_{D}$ – 42.9 (c 0.07, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 1.19 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.95 (ddd, 1H, *J* = 15.1, 6.4, 3.2 Hz, $H2_{eqt}$), 2.05 - 2.12 (m, 1H, $H2_{ax}$), 2.18 (ddt, 1H, *J* = 15.1, 7.6, 1.8 Hz, $H6_{eqt}$), 2.28 (d, 1H, *J* = 12.2 Hz, $H6_{ax}$), 2.48 (dd, 1H, *J* = 8.3, 3.5 Hz, H1), 4.12 - 4.16 (m, 1H, H4), 4.25 - 4.30 (m, 1H, H3) and 4.57 (dd, 1H, *J* = 5.7, 2.5 Hz, H5) ppm; ^{13}C NMR ($CDCl_3$): δ 24.41 (CH_3), 27.13 (CH_3), 27.97 (CH_2, C6), 29.96 (CH_2, C2), 35.37 (CH, C1), 70.48 (CH, C3), 72.76 (CH, C4), 77.41 (CH, C5), 109.35 ($C_{isopropylidene}$), 178.90 (C=O) ppm; MS(AP^+) $C_{10}H_{14}O_4$ (198.22) m/z (%): 199.7 $[M+H]^+$ (100).

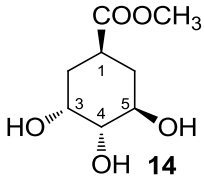


13

7.1.13. 3,4-O-isopropylidene-cyclohexane-1-carboxylate (13)

3,4-*O*-isopropylidene-1,5-lactone cyclohexane **(12)** (126 mg, 0.64 mmol) in anhydrous MeOH (2mL) was added slowly dropwise to a NaOMe solution (1.7 mL, 0.5 M in MeOH, 1.3 eq, 0.83 mmol) at 0 ºC during 30 min, under a N_2 atmosphere. Then the reaction mixture was left stirring to warm up to RT for 3 hrs. TLC analysis (EtOAc/PE 2:1) gave a compound with R*f* 0.5 and total consumption of starting material (R*f* 0.8) and for that reason the reaction mixture was neutralized with Amberlite resin IR until pH 3-4, filtered and evaporated. Column chromatography (EtOAc/pet.ether gradient PE only to 2:1) andNMR ($CDCl_3$) gave compound **13** as a colourless solid (25 mg, 0.109 mmol, 17% yield).

R*f* (EtOAc/PE 2:1) 0.5; $[\alpha]^{20}_{D}$ – 116.3 (c 0.08, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 1.30 (s, 3H, CH_3), 1.47 (s, 3H, CH_3), 1.83 (dd, 1H, *J* = 13.7, 10.6 Hz, $H6_{ax}$), 2.00 (dd, 1H, *J* = 13.6, 4.1 Hz, $H6_{eqt}$), 2.19 (d, H, *J* = 3.7 Hz, H2), 2.68 (tt, 1H, H1), 3.76 (s, 3H, COOCH_3), 3.90 - 3.94 (m, 1H, H4), 4.07 (ddd, 1H, *J* = 10.6, 7.5, 4.0 Hz, H5) and 4.41 (dt, 1H, *J* = 5.7, 3.7 Hz, H3) ppm; ^{13}C NMR ($CDCl_3$): δ 25.65 (CH_3), 28.17 (CH_3), 34.69 (CH_2, C2), 38.88 (CH_2, jC6), 53.23 (COO*C*H_3), 68.27 (CH, C1), 73.47 (CH, C3), 73.84 (CH, C5), 79.84 (CH, C4), 109.36 ($C_{isopropylidene}$), and 175.7 (C=O) ppm; MS (AP^+) $C_{11}H_{18}O_5$ (230.26) *m/z* (%): 231.2 $[M+H]^+$ (100).



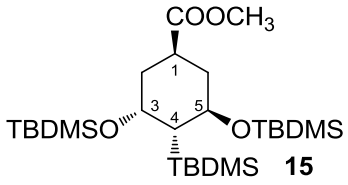
14

7.1.14. 3,4,5-tri-*O*-hydroxy-cyclohexane-1-carboxylate (14)

3,4-*O*-isopropylidene-1,5-lactone cyclohexane **(12)** (100 mg, 0.51 mmol) in anhydrous MeOH (2mL) was added slowly dropwise to a NaOMe solution (1.4 mL, 0.5 M in MeOH, 1.3 eq, 0.66 mmol) at 0 ºC during 30 min, under a N_2 atmosphere. The mixture was then left stirring to warm up to RT for 3 hrs. TLC analysis (EtOAc/PE 2:1) showed a the presence of a compound with R*f* 0.49 and total consumption of the starting material. Therefore the reaction mixture was neutralized with an excess amount of Amberlite resin IR until acidic pH. After neutralization TLC analysis showed that the compound had now a lower R*f* of 0.11, as it was expected if the formation of the desired compound was achieved. The reaction was then

filtered and evaporated. Column chromatography (EtOAc/PE gradient 1:1 to EtOAc only) and NMR (CD_3OD) gave compound **14** as a colourless solid (66 mg, 0.35 mmol, 68%).

R*f* (EtOAc/MeOH 5%) 0.37; $[\alpha]^{20}_D$ – 52 (c 0.05, MeOH); ^{1}H NMR ($CDCl_3$): δ 1.35 (dd, 1H, *J* = 24.1, 12.5 Hz, $H6_{ax}$), 1.48 - 1.57 (m, 1H, $H2_{eqt}$), 1.92 - 1.96 (m, 1H, $H2_{ax}$), 2.02 - 2.09 (m, 1H, $H6_{eq}$), 2.75 (tt, 1H, *J* = 12.5, 3.6 Hz, H1), 3.19 (dd, 1H, *J* = 9.2, 3.0 Hz, H4), 3.59 (s, 3H, COOCH_3), 3.63 - 3.71 (m, 1H, H5) and 3.97 (dd, 1H, *J* = 6.0, 3.0 Hz, H3) ppm; ^{13}C NMR ($CDCl_3$): δ 33.35 (CH_2, C6), 34.78 (CH_2, C2), 35.8 (CH, C1), 50.8 (COOCH_3), 68.6 (CH, C3), 68.9 (CH, C5), 75.79 (CH, C4) and 175.6 (C=O) ppm; MS (AP^+) $C_8H_{14}O_5$ (190.19) *m/z* (%): 191.2 $[M+H]^+$ (100).



15

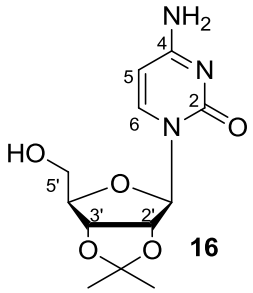
7.1.15. 3,4,5-tri-*O*-(*tert*-butyldimethylsilyloxy) cyclohexane-1-carboxylate (15)

3,4,5-tri-*O*-hydroxy-cyclohexane-1-carboxylate **(14)** (172 mg, 0.91 mmol) was dissolved in pyridine (4 mL) and then imidazole (558 mg, 9 eq, 8.2 mmol) and TBDMSCl (823 mg, 6 eq, 5.5 mmol) were added. The reaction mixture stirred at 80 ºC for 24 hrs, and after that time was quenched throughtpyridine evaporation under vacuum and then the residue was washed with a saturated aqueous solution of NH_4Cl (100 mL) and extracted

with DCM (100 mL). The organic layers were then dried (Na_2SO_4), filtered and evaporated. Column chromatography (EtOAc/PE gradient PE only to 5% EtOAc) and NMR ($CDCl_3$) gave compound **15** as a colourless syrup (380 mg, 0.71 mmol, 78%).

R*f* (EtOAc 5% in PE) 0.7; ^{1}H NMR ($CDCl_3$): δ - 0.01(2s, 6H, SiC*H*$_3$), 0.03 (2s, 6H, SiC*H*$_3$), 0.07 (2s, 6H, SiC*H*$_3$), 0.84 (s, 9H, SiC(C*H*$_3$)$_3$), 0.86 (s, 9H, SiC(C*H*$_3$)$_3$), 0.88 (s, 9H, SiC(C*H*$_3$)$_3$), 1.78 - 2.02 (m, 4H, H6 and H2), 2.61 (tt, 1H, *J* = 12.5, 3.6 Hz, H1), 3.51 - 3.55 (m, 1H, H4), 3.68 (s, 3H,COOC*H*$_3$), 3.77 - 3.80 (m, 1H, H3) and 4.15 - 4.17 (m, 1H, H5) ppm; ^{13}C NMR ($CDCl_3$): δ - 4.96 (C, SiC*H*$_3$), - 4.87 (C, SiC*H*$_3$), - 4.81 (C, SiC*H*$_3$), - 4.59 (C, SiC*H*$_3$), - 4.51(C, SiC*H*$_3$), - 4.32 (C, SiC*H*$_3$), 18.08 (C, Si*C*(CH$_3$)$_3$), 18.18 (C, Si*C*(CH$_3$)$_3$), 18.22 (C, Si*C*(CH$_3$)$_3$), 25.85 (3C, SiC(*C*H$_3$)$_3$), 25.94 (3C, SiC(*C*H$_3$)$_3$), 26.07 (3C, SiC(*C*H$_3$)$_3$), 28.28 (CH_2, C2), 29.63 (CH_2, C6), 51.38 (COO*C*H$_3$), 67.99 (CH, C3), 71. 83 (CH, C1), 71.92 (CH, C5), 75.10 (CH, C4) and 175.54 (C=O) ppm; MS (AP$^+$) $C_{26}H_{56}O_5Si_3$ (532.98) *m/z* (%): 533.2 $[M+H]^+$ (100).

7.2. CYTIDINE SYNTHESIZED COMPOUNDS



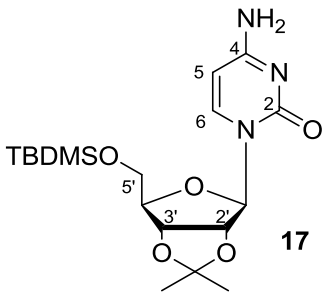
16

7.2.1. 2',3'-*O*-isopropylidene cytidine (16)

To a suspension of cytidine (alfa aesar lot 10132023, MW 243.22) (2.09 g, 8.6 mmol) in dry acetone (300 mL) was added *p*-toluene sulfonic acid monohydrate (15.17 g, 79.7 mmol, 9.3 eq) and the mixture was stirred at reflux for 2 hrs. Then solid $NaHCO_3$ was added until the yellow solution was white in colour (pH7, deprotonation of NH_3^+). The solid was then filtered under vacuum, giving a yellow oil crude residue. Column chromatography (DCM/MeOH 10%) and NMR ($CDCl_3$) gave compound **16** as a colourless solid compound (2.2 g, 7.8 mmol, 95 %). The analytical data correspond to reported values.

R*f* (DCM/MeOH 10%) 0.24. ^{1}H NMR ($CDCl_3$): δ 1.32 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 3.77 (dd, 1H, *J* = 11.9, 4.7 Hz, H5'), 3.87 (dd, 1H, *J* = 11.9, 3.6 Hz, H5'), 4.26 (m, 1H, H4'), 4.73 (dd, 1H, *J* = 6.4, 3.6 Hz, H3'), 5.05 (d, 1H, *J* = 2.5 Hz, H2'), 5.55 (d, 1H, *J* = 7.4 Hz, H1'), 5.88 (d, 1H, *J* = 7.4 Hz, H5), 7.45 (d, 1H, *J* = 7.4 Hz, H6) ppm. ^{13}C NMR ($CDCl_3$): δ 25.23 (CH_3), 27.17 (CH_3), 61.97 (C5'), 80.50 (C3'), 84.28 (C2'), 87.32 (C4'), 95.58 (C1'), 96.45 (C5, C=C), 113.75 ($C_{isopropylidene}$), 143.77 (C6, C=C), 156.36 (C2, C=O), 166.33 (C4) ppm; MS (AP^+) $C_{12}H_{17}N_3O_5$ (283.28) m/z (%) 284.2

$[M+H]^+$ (100); HRMS (ESI) (*m/z*) $[M+H]^+$ calculated for $C_{12}H_{18}N_3O_5$ found 284.1241.



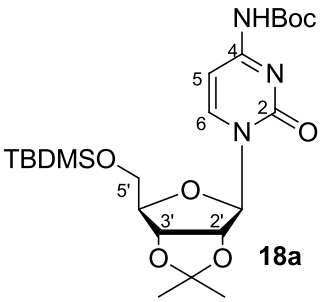
17

7.2.2. 2´, 3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (17)

Propargyl cytidine **(16)** (2.13 g, 7.5 mmol) was dissolved in anhydrous DMF (8 mL). Imidazole (2.55 g, 5 eq, 37.5 mmol) and TBDMSCl (2.48 g, 2.2 eq, 16.5 mmol) were added to the reaction mixture and stirred for 1 hr at RT under a N_2 atmosphere. TLC analysis (DCM/MeOH 10%) showed total consumption of the starting material. DMF solvent was then removed under vacuum and the crude residue was dissolved in DCM (100 mL) and extracted with water (100 mL) to separate the excess of imidazole and TBDMSCl. The organic extracts were then dried (Na_2SO_4), then filtered and evaporated. NMR ($CDCl_3$) gave compound **17** as a yellow oil (2.9 g, 7.3 mmol, 97%). The analytical data correspond to reported values.

R*f* (DCM/MeOH 10%) 0.37; ^{1}H NMR ($CDCl_3$): δ 0.00 (s, 6H, SiCH_3), 0.82 (s, 9H, SiC(CH_3)$_3$), 1.27 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 3.72 (dd, 1H, *J* = 11.4, 3.9 Hz, H5'), 3.84 (dd, H, *J* = 11.4, 3.9 Hz, H5'), 4.20 (dd, 1H, *J* = 6.5 Hz, 3.3 H4'), 4.65 – 4.71 (m, 2H, H2' and H3'), 5.66 (d, 1H, *J* = 7.4 Hz, H5), 5.82 (d, 1H, *J* = 1.5 Hz, H1'), 7.57 (d, 1H, *J* = 7.4 Hz, H6) ppm; ^{13}C NMR

($CDCl_3$): δ - 5.45 (2C, Si*C*H_3), 18.12 (Si*C*$(CH_3)_3$), 25.38 (CH_3), 25.87 (3C, SiC(CH_3)$_3$), 27.23 (CH_3), 63.28 (C5'), 80.19 (C2'), 85.87 (C3'), 87.27 (C4'), 93.33 (C1'), 94.42 (C5, C=C), 113.25 ($C_{isopropylidene}$), 141.57 (C6, C=C), 155.00 (C2, C=O), 165.75 (C4) ppm; MS (AP^+) $C_{18}H_{31}N_3O_5Si$ (397.54) m/z (%) 398.2 $[M+H]^+$ (100). HRMS (ESI) (*m/z*) $[M+H]^+$ calcd for $C_{18}H_{32}N_3O_5Si$ found 398.2103.



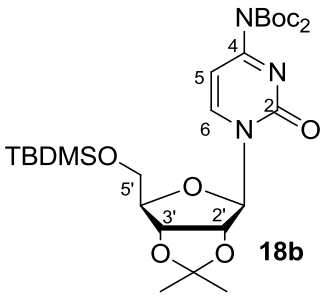
Cyt-5'TBDMS-Boc1

7.2.3. *N-tert*-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (18a)

Silylated cytidine **(17)** (394 mg, 0.99 mmol) was dissolved in anhydrous THF (5 mL) and DMAP (115 mg, 0.95 eq, 0.94 mmol) and di-*tert*-butyl dicarbonate (Boc_2O, 373 mg, 1.7 eq, 1.7 mmol) were added. The reaction mixture was stirred at RT overnight. TLC analysis (EtOAc/pet.ether 1:1) showed total consumption of starting material and so the reaction was quenched with MeOH (5 mL) and stirred for 1 hr. The solvents were then evaporated and flash column chromatography was preformed (EtOAc/pet.ether gradient 1:2 to 1:1). NMR ($CDCl_3$) gave compound **18a** as a

colourless sugar-like (207 mg, 0.42 mmol, 35%). The analytical data correspond to reported values.

R*f* (EtOAc/pet.ether 1:1) 0.4; ^{1}H NMR ($CDCl_3$): δ 0.00 (d, 6H, *J* 1.6 Hz, $Si(CH_3)_2$), 0.80 (s, 9H, $SiC(CH_3)_3$), 1.27 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.43 (s, 9H, $NCOOC(CH_3)_3$), 3.73 (dd, 1H, *J* = 11.5, 3.5 Hz, H5'), 3.88 (dd, 1H, *J* = 11.5, 2.6 Hz, H5'), 4.30 (dd, 1H, *J* = 5.8, 2.8 Hz, H4'), 4.68 (dd, 1H, *J* = 6.2, 2.3 Hz, H3'), 4.68 - 4.71 (m, 1H, H2'), 5.88 (d, 1H, *J* = 1.5 Hz, H1'), 7.07 (d, 1H, *J* = 7.5 Hz, H5), 7.98 (d, 1H, *J* = 7.5 Hz, H6) ppm; ^{13}C NMR ($CDCl_3$): δ -5.50 (2C, $Si(CH_3)_2$), 25.27 (CH_3), 25.81 ($SiC(CH_3)_3$), 25.92 (3C, $SiC(CH_3)_3$), 27.17 (CH_3), 27.97 (3C, $NCOOC(CH_3)_3$), 63.25 (C5'), 82.50 (C3'), 84.87 (3C, $NCOOC(CH_3)_3$), 86.05 (C2'), 87.91 (C4'), 94.02 (C1'), 94.32 (C5, C=C), 113.61 ($C_{isopropylidene}$), 144.67 (C6, C=C), 151.21 ($NCOOC(CH_3)_3$), 154.83 (C2, C=O), 162.76 (C4) ppm. MS (ES^+) $C_{23}H_{39}N_3O_7Si$ (497.66) m/z (%) 498.1 $[M+H]^+$ (100); HRMS (ESI) (*m/z*) $[M+H]^+$ calculated for $C_{23}H_{40}O_7N_3Si$ 498.2630, found 498.2625.



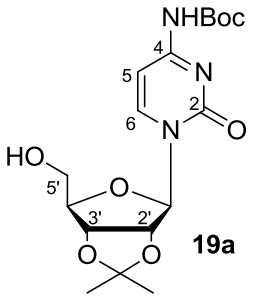
Cyt-5'TBDMS-Boc2

7.2.4. *N,N*-Di-*tert*-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine (18b)

Silylated cytidine **(17)** (394 mg, 0.99 mmol) was dissolved in anhydrous THF (5 mL) and DMAP (115 mg, 0.95 eq, 0.94 mmol) and di-*tert*-butyl dicarbonate (Boc_2O, 373 mg, 1.7 eq, 1.7 mmol) were added. The reaction mixture stirred at RT overnight. TLC analysis (EtOAc/pet.ether 1:1) showed total consumption of the starting material and so the reaction was quenched with MeOH (5 mL) and stirred for 1 hr. The solvents were then evaporated and flash column chromatography was preformed (EtOAc/pet.ether gradient 1:2 to 1:1). NMR ($CDCl_3$) gave compound **18b** as a colourless sugar-like (261 mg, 0.44 mmol, 44%). The analytical data correspond to reported values.

R*f* (EtOAc/pet.ether 1:1) 0.70; ^{1}H NMR ($CDCl_3$): δ 0.00 (d, 6H, *J* = 1.0 Hz, SiC*H*$_3$), 0.81 (3s, 9H, SiC(C*H*$_3$)$_3$), 1.27 (s, 3H, CH_3), 1.48 (s, 18H, NCOOC(C*H*$_3$)$_3$), 1.50 (s, 3H, CH_3), 3.73 (dd, 1H, *J* = 11.5, 3.8 Hz, H5'), 3.86 (dd, 1H, *J* = 11.5, 2.7 Hz, H5'), 4.28 (dd, 1H, *J* = 6.4, 3.3 Hz, H4'), 4.66 (dd, 1H, *J* = 6.1, 3.3 Hz, H3'), 4.71 (dd, 1H, *J* = 6.1, 1.9 Hz, H2'), 5.84 (d, 1H, *J* = 1.8 Hz, H1'), 6.93 (d, 1H, *J* = 7.6 Hz, H5), 7.92 (d, 1H, *J* = 7.6 Hz, H6) ppm; ^{13}C NMR ($CDCl_3$): δ - 5.43 (2C, Si*C*H$_3$), 18.34 (Si*C*(CH$_3$)$_3$), 25.32 (CH_3),

25.92 (3C, SiC(CH_3)$_3$), 27.24 (CH_3), 27.97 (6C, NCOOC(CH_3)$_3$), 63.33 (C5'), 80.19 (C3'), 84.87 (2C, NCOOC(CH_3)$_3$), 86.04 (C2'), 88.04 (C4'), 94.23 (C1'), 95.67 (C5, C=C), 113.71 ($C_{isopropylidene}$), 144.30 (C6, C=C), 149.54 (2C, NCOOC(CH_3)$_3$), 154.09 (C2, C=O), 162.51 (C4) ppm. MS (ES^+) $C_{28}H_{47}N_3O_9Si$ (597.77) m/z (%) 598.2 $[M+H]^+$ (100).



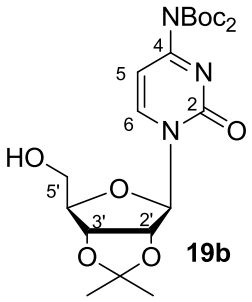
Cyt-5'OH-Boc1

7.2.5. *N-tert*-butyldicarbonate 2´,3´-*O*-isopropylidene cytidine (19a)

N-tert-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine **(18a)** and *N,N*-Di-*tert*-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine **(18b)** mixture (468 mg, 0.78 mmol) were dissolved in anhydrous THF (3 mL) and then TBAF (0.94 mL, 1.2 eq, 1.0 M in THF) was added dropwise. The reaction mixture was then stirred at RT for 2 hrs, after which the solvents were removed under vacuum. TLC analysis (EtOAc/pet.ether 1:1) showed several impurities, 2 of them UV-reactive. Flash column chromatography was preformed (EtOAc/pet.ether gradient 1:2 to EtOAc only) andNMR ($CDCl_3$) gave compound **19a** as a white solid (368 mg, 0.96 mmol, 54%). The analytical

data correspond to reported values. The pure compounds were given to Bradley Springett for his experiments.

R*f* (EtOAc) 0.4; ^{1}H NMR (CD_3OD): δ 1.27 (s, 3H, CH_3), 1.44 (s, 9H, NCOOC(CH_3)$_3$), 1.48 (s, 3H, CH_3), 3.65 (d, 1H, *J* = 4.6, 12.0 Hz, H5'), 3.74 (dd, 1H, *J* = 3.5, 12.0 Hz, H5'), 4.23 (dd, 1H, *J* = 5.6, 3.1 Hz, H4'), 4.75 (dd, 1H, *J* = 6.5, 3.4 Hz, H3'), 4.82 (dd, 1H, *J* = 6.5, 2.7 Hz, H2'), 5.80 (d, 1H, *J* = 2.7 Hz, H1'), 7.19 (d, 1H, *J* = 7.5 Hz, H5), 8.13 (d, 1H, *J* = 7.5 Hz, H6) ppm; ^{13}C NMR (CD_3OD): δ 25.49 (CH_3), 27.50 (CH_3), 28.33 (3C, NCOOC(CH_3)$_3$), 63.08 (C5'), 82.29 (C3'), 83.15 (NCOO*C*(CH_3)$_3$), 86.84 (C2'), 89.50 (C4'), 96.21 (C1'), 96.50 (C5, C=C), 114.85 ($C_{isopropylidene}$), 147.16 (C6, C=C), 153.43 (N*C*OOC(CH_3)$_3$), 157.72 (C2, C=O), 165.44 (C4) ppm; MS (AP^+) $C_{17}H_{25}N_3O_7$ (383.40) m/z (%) 384.1 $[M+H]^+$ (100); HRMS (ESI) (*m/z*) $[M+H]^+$ calculated for $C_{17}H_{25}N_3O_7$ 384.1765, found 384.1769.



Cyt-5'OH-Boc2

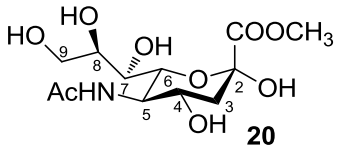
7.2.6. *N,N*-Di-*tert-butyldicarbonate* 2´,3´-*O*-isopropylidene cytidine (19b)

N-tert-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine **(18a)** and *N,N*-Di-*tert*-butyldicarbonate 2´,3´-*O*-isopropylidene-5´-*tert*-butyldimethylsilyloxy cytidine **(18b)** mixture (468 mg, 0.78 mmol) were dissolved in anhydrous THF (3 mL) and then TBAF (0.94 mL, 1.2 eq, 1.0 M in THF) was added dropwise. The reaction mixture stirred at RT for 2 hrs, after which the solvents were removed under vacuum. TLC analysis (EtOAc/pet.ether 1:1) showed several impurities, 2 of them UV-reactive, reason by which flash column chromatography was preformed (EtOAc/pet.ether gradient 1:2 to EtOAc only). NMR ($CDCl_3$) gave compound **19b** as a white solid (756 mg, 1.87 mmol, 43%).The analytical data correspond to reported values. The pure compounds were given to Bradley Springett for his experiments

R*f* (EtOAc) 0.6; ^{1}H NMR (CD_3OD): δ 1.27 (s, 3H, CH_3), 1.44 (s, 18H, $NCOOC(CH_3)_3$), 1.48 (s, 3H, CH_3), 3.65 (d, 1H, *J* = 4.6, 12.0 Hz, H5'), 3.74 (dd, 1H, *J* = 3.5, 12.0 Hz, H5'), 4.23 (dd, 1H, *J* = 5.6, 3.1 Hz, H4'), 4.75 (dd, 1H, *J* = 6.5, 3.4 Hz, H3'), 4.82 (dd, 1H, *J* = 6.5, 2.7 Hz, H2'), 5.80 (d, 1H, *J* = 2.7 Hz, H1'), 7.19 (d, 1H, *J* = 7.5 Hz, H5), 8.13 (d, 1H, *J* = 7.5 Hz, H6) ppm;

^{13}C NMR (CD_3OD): δ 25.49 (CH_3), 27.50 (CH_3), 28.33 (6C, NCOOC(CH_3)$_3$), 63.08 (C5'), 82.29 (C3'), 83.15 (2C, NCOO*C*(CH_3)$_3$), 86.84 (C2'), 89.50 (C4'), 96.21 (C1'), 96.50 (C5), 114.85 ($C_{isopropylidene}$), 147.16 (C6), 153.43 (2C, N*C*OOC(CH_3)$_3$), 157.72 (C2), 165.44 (C4) ppm; MS (AP^+) $C_{22}H_{33}N_3O_9$ (483.51) m/z (%) 484.1 $[M+H]^+$ (100).

7.3. SIALIC ACID SYNTHESIZED COMPOUNDS

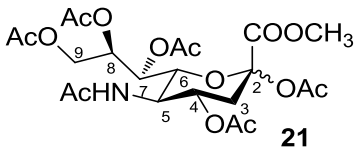


20

7.3.1. Methyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (*N*-Acetylneuraminic methyl ester) (20)

Starting material *N*-Acetylneuraminic acid (5 g, 16.18 mmol, MW 309 g/mol, lot CCDx42-71 Dextra Lab.) was dispersed in anhydrous MeOH (180 mL), then Amberlyte resin IR-120 H^+ (8.5 g) was added. The reaction mixture stirred at RT for 48 hrs. Then it was filtered and evaporated. NMR (CD_3OD) gave compound **20** as a yellow sugar (4.98 g, 15.4 mmol, 95% yield). The analytical data correspond to reported values.

$[\alpha]^{20}_D$ – 24 (c 0.15,MeOH); ^{1}H NMR (CD_3OD): δ 1.86 (s, 3H, NAc), 1.92 (dd, 1H, *J* = - 12 Hz, $H3_{ax}$), 2.28 (dd, 1H, *J* = 4.5 Hz, $H3_{eq}$), 3.53 (dd, 1H, *J* = 10 Hz, H5), 3.65 (dd, 1H, *J* = 5, - 11.5 Hz, H9a), 3.74 (m, 1H, *J* = 2, 5 Hz, H8), 3.82 (s, 3H, COOCH_3),3.84 (m, 2H, *J* = 1 Hz, H7 and *J* = 2, - 11.5 Hz, H9b), 3.96 (dd, 1H, *J* = 1, 10 Hz, H6), 4.1 (m,1H, *J* = 10 Hz, H4); ^{13}C NMR (CD_3OD): δ 21.04 (NHCOCH_3), 39.14 (CH_2, C3), 51.59 (COOCH_3), 52.81 (CH, C7), 63.19 (CH_2, C9), 66.30 (CH, C4), 68.61 (CH, C5), 70.08 (CH, C8), 70.48 (CH, C6), 94.97 (C2), 170.19 (NH*C*OCH_3) and 173.65 (*C*OOCH_3) ppm; MS (AP^-) $C_{12}H_{21}NO_9$ (323.30) m/z (%) 324 $[M+H]^+$ (100).



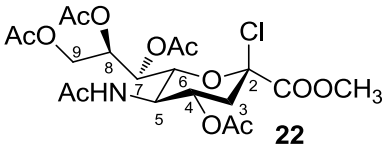
21

7.3.2. Methyl 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonate (21)

N-Acetylneuraminic methyl ester **(20)** (4.36 g, 13.5 mmol) was suspended in anhydrous pyridine (45 mL) and acetic anhydride (45 mL) under a N_2 atmosphere at 0 ºC. The reaction mixture was stirred and allowed to warm up to RT and then stirred for 24 hrs more. TLC analysis (EtOAc/MeOH 20:1) showed total consumption of the starting material and therefore the reaction was slowly quenched with cold MeOH (100 mL) (0ºC, since it's very exotermic reaction) and stirred for 1 hr. Afterwards the solvents were evaporated and the residue was dissolved in DCM (100 mL) and extracted with saturated aqueous solution of $NaHCO_3$ (100 mL, very fizzy reaction). Then the organic phases were washed with water (100 mL), dried ($MgSO_4$), filtered and evaporated. Flash column chromatography (EtOAc/MeOH gradient EtOAc only to EtOAc/MeOH 20:1) and NMR ($CDCl_3$) gave compound **21** as a white solid (5.95 g, 11.2 mmol, 83%).

R*f* (EtOAc/MeOH 20:1) 0.53 and 0.47; ^{1}H NMR ($CDCl_3$): δ 1.86 (s, 3H, NAc), 2.00 (2s, 6H, 2OAc), 2.03 (s, 3H, OAc), 2.11 (2s, 6H, 2OAc), 2.03 - 2.10 (m, 1H, $H3_{ax}$), 2.52 (dd,1H, J = 13.4, 4.9 Hz, $H3_{eq}$), 3.76 (s, 3H, COOCH_3) plus mixture of peaks from both anomers but with predominance of the β anomer; ^{13}C NMR ($CDCl_3$): δ 20.75 (CH_3, AcO), 20.78 (CH_3, AcO), 20.81 (CH_3, AcO), 20.87 (CH_3, AcO), 20.93 (CH_3, AcO), 23.20 (NHCOCH_3), 35.90 (CH_2, C3), 49.28 (CH, C7), 53.23 (COOCH_3), 62.19 (CH_2, C9), 67.78

(CH, C4), 68.30 (CH, C5), 71.35 (CH, C8), 72.95 (CH, C6), 97.40 (C2), 166.34 (NH*C*OCH$_3$), 168.25 (C=O, AcO), 170.08 (C=O, AcO), 170.28 (C=O, AcO), 170.64 (C=O, AcO), 170.83 (C=O, AcO) and 171.03 (*C*OOCH$_3$) ppm; MS (ES$^+$) $C_{22}H_{31}NO_{14}$ (533.48) m/z (%) 534 $[M+H]^+$ (100).



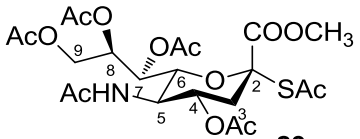
22

7.3.3. Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (22)

Methyl 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonate **(21)** (5.95 g, 11.2 mmol) was dissolved in acetyl chloride (110 mL). Dry hydrogen chloride was then bubbled through the solution for 15 min at 0 ºC. The solution was tightly closed and the mixture stirred and left to warm up to RT for 48 hrs. The excess of HCl in the mixture was removed with the use of a stream of N_2 gas for some minutes, and the excess of acetyl chloride was removed with the use of the vacuum. The crude residue was then co-evaporated with ether to give compound **22** as a colourless sugar-like (5.6 g, 10.98 mmol, 99%, β anomer) as confirmed by NMR ($CDCl_3$).

R*f* (EtOAc/MeOH 20:1) 0.59; ^{1}H NMR ($CDCl_3$): δ 1.90 (s, 3H, NAc), 2.04 (2s, 6H, 2OAc), 2.06 (s, 3H, OAc), 2.09 (s, 3H,OAc), 2.27 (dd, 1H, *J* = 13.9, 9.2 Hz, H3$_{ax}$), 2.77 (dd, 1H, *J* = 13.9, 4.8 Hz, H3$_{eq}$), 3.86 (s, 3H, COOC*H*$_3$), 4.04 (dd, 1H, *J* = 10.8, 5.4 Hz, H9a), 4.20 (dd, 1H, *J* = 10.8, 2.4 Hz, H9b), 4.33 (m, 1H, H5), 4.40 (m, 1H, H6), 5.17 - 5.18 (m, 1H, H4), 5.39

(dd, 1H, *J* = 6.9, 2.4 Hz, H8), 5.46 (dd, 1H, *J* = 6.7, 2.4 Hz, H7); ^{13}C NMR ($CDCl_3$): δ 20.76 (CH_3, AcO), 20.81 (CH_3, AcO), 20.85 (CH_3, AcO), 20.95 (CH_3, AcO), 23.13 ($NHCOCH_3$), 40.62 (CH_2, C3), 48.69 (CH, C5), 53.82 ($COOCH_3$), 62.07 (CH_2, C9), 66.84 (CH, C4), 68.77 (CH, C7), 69.97 (CH, C8), 73.87 (CH, C6), 96.59 (C2), 165.64 ($NHCOCH_3$), 169.73 (C=O, AcO), 169.98 (C=O, AcO), 170.53 (C=O, AcO), 170.70 (C=O, AcO) and 171.02 ($COOCH_3$) ppm; MS (ES^+) $C_{20}H_{28}ClNO_{12}$ (509.89) m/z (%) 510 $[M+H]^+$ (100).



23

7.3.4. Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-S-acetyl-2,3,5-trideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyranosonate (Neu5Ac-2SAc, 23)

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate **(22)** (5.6 g, 9.92 mmol) was dissolved in EtOAc (50 mL), then potassium thioacetate (7.6 g, 6 eq, 66.2 mmol, batch two BCBF7702 Sigma-Aldrich) and iodine (4.2 g, 1.5 eq, 16.6 mmol) were added. The reaction mixture was stirred at RT overnight. The insoluble salts were then filtered and the reaction mixture was washed with saturated aqueous solution of sodium thiosulphate (50 mL). The organic phases were then dried (Na_2SO_4), filtered and evaporated. Flash column chromatography (EtOAc/PE gradient 1:1 to EtOAc only) and NMR ($CDCl_3$)

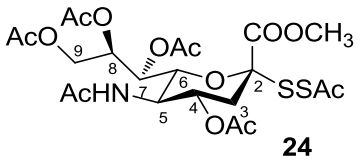
gave a mixture of compound **23** and compound **24** as a orange sugar-like crude, in a SAc:SSAc ratio of 60% to 40%.

To improve the SAc:SSAc ratio of the mixture **(23** and **24)**, 500 mg (0.98 mmol) were dissolved in EtOAc (5 mL), then potassium thioacetate (672 mg, 6 eq, 5.88 mmol, batch two BCBF7702 Sigma-Aldrich) and iodine (373 mg, 1.5 eq, 1.47 mmol) were added. The reaction mixture stirred at RT overnight. Then the insoluble salts were filtered and the mixture was washed with saturated aqueous solution of sodium thiosulphate (50 mL). The organic phases were then dried (Na_2SO_4), filtered and evaporated. Flash column chromatography (EtOAc/PE 2:1 to EtOAc only) and NMR ($CDCl_3$) gave an improved ratio of SAc 30% and SSAc 70%.

Characterization of compound **23**:

R*f* (EtOAc/PE 9:1) 0.41; ^{1}H NMR ($CDCl_3$): δ 1.88 (s, 3H, NHAc), 2.00 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.13 (s, 3H, OAc), 2.09 (dd, 1H, *J* = 12.9, 9.2 Hz, $H3_{ax}$), 2.27 (s, 3H, SAc), 2.60 (dd,1H, *J* = 12.9, 4.6 Hz, $H3_{eqt}$), 3.78 (s, 3H, COOCH_3), 4.02 (dd,1H, *J* = 12.4, 5.8 Hz, H9a), 4.08 (m, 1H, H5), 4.37 (dd, 1H, *J* = 12.4, 2.4 Hz, H9b), 4.65 (dd, 1H, *J* = 10.8, 2.2 Hz, H6), 4.87 - 4.91 (m, 1H, H4), 5.16 (d, 1H, *J* = 5.1 Hz, NH), 5.20 - 5.22 (m, 1H, H8), 5.34 (dd, 1H, *J* = 6.7, 2.2 Hz, H7) ppm; ^{13}C NMR ($CDCl_3$): δ 20.05 (CH_3, AcO), 20.56 (CH_3, AcO), 20.86 (CH_3, AcO), 20.90 (CH_3, AcO), 23.25 (NHCOCH_3), 30.26 (SAc, CH_3), 37.45 (CH_2, C3), 49.28 (CH, C7), 53.60 (COOCH_3), 62.41 (CH_2, C9), 67.75 (CH, C4), 68.91 (CH, C5), 70.25 (CH, C8), 75.18 (CH, C6), 84.54 (C2), 167.37 (NHCOCH$_3$), 170.19 (C=O, AcO), 170.23 (C=O, AcO), 170.55 (C=O, AcO), 170.88 (C=O, AcO), 171.97

(*C*OOCH$_3$) and 191.54 (S*C*OCH$_3$) ppm; MS (AP$^-$) $C_{22}H_{31}NO_{13}S$ (549.55) m/z (%) 572.1 $[M+Na]^+$ (100).



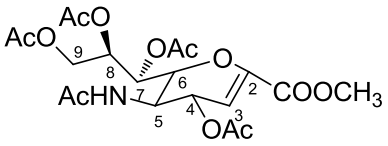
24

7.3.5. Methyl 2-(acetylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-α-D-glycero-D-galacto-2-nonulopyranosonate (Neu5Ac-2SSAc, 24)

Data according to previous work from the group.

R*f* (EtOAc/PE 9:1) 0.41; $[\alpha]^{20}_D$ + 155 ± 1.0 (c 0.85, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 1.88 (s, 3H, NHAc), 2.02 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.07 (dd, 1H, *J* = 12.2, 9.4 Hz, $H3_{ax}$), 2.46 (s, 3H, SSAc), 2.81 (dd,1H, *J* = 12.2, 4.4 Hz, $H3_{eqt}$), 3.77 (s, 3H, COOC*H*$_3$), 3.86 (d,1H, *J* = 9.4 Hz, H6), 4.00 - 4.05 (m, 1H, H5), 4.12 (dd, 1H, *J* = 12.1, 4.7 Hz, H9a), 4.34 (d, 1H, *J* = 12.1 Hz, H9b), 4.83 - 4.88 (m, 1H, H4), 5.21 (d, 1H, *J* = 10.8 Hz, NH), 5.29 - 5.33 (m, 2H, H7 and H8) ppm; ^{13}C NMR ($CDCl_3$): δ 20.87 (2CH_3, AcO), 20.90 (CH_3, AcO), 21.24 (CH_3, AcO), 23.20 (NHCO*C*H$_3$), 28.81 (SSAc, CH_3), 37.07 (CH_2, C3), 49.45 (CH, C7), 53.50 (COO*C*H$_3$), 62.01 (CH_2, C9), 67.23 (CH, C4), 69.04 (CH, C5), 69.57 (CH, C8), 74.44 (CH, C6), 84.72 (C2), 166.85 (NH*C*OCH$_3$), 170.00 (C=O, AcO), 170.18 (C=O, AcO), 170.80 (C=O, AcO), 170.89 (C=O, AcO), 171.68 (*C*OOCH$_3$) and 192.51 (SS*C*OCH$_3$) ppm; MS (AP$^-$) $C_{22}H_{31}NO_{13}S_2$ (581.61)

m/z (%) 582.2 $[M+H]^+$ (30), 604.2 $[M+Na]^+$ (100); HRMS (ES^+) found 599.1575, calculated for $C_{22}H_{31}NO_{13}S_2$ $[M+NH_4]^+$.



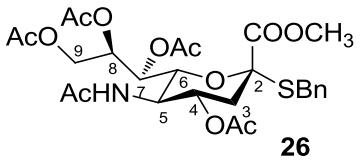
25

7.3.6. Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-glycero-D-talo-non-2-enonate (Neu5Ac2en, 25)

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate **(22)** (5 g, 9.92 mmol) was dissolved in of solvent (50 mL), then potassium thioacetate (6.8 g, 6 eq, 59.5 mmol, batch one LF005421, Alfa Aesar) and iodine (3.8 g, 1.5 eq, 14.9 mmol) were added. Using as conditions: EtOAc RT overnight or EtOAc reflux overnight or DMF heating 80ºC overnight. The insoluble salts were then filtered and the mixture was washed with saturated aqueous solution of sodium thiosulphate (50 mL). The organic phases were dried (Na_2SO_4), filtered and evaporated. Flash column chromatography (EtOAc/MeOH gradient EtOAc only to 5% MeOH) and NMR ($CDCl_3$) gave compound **25** as a yellow sugar-like. Using the same method but batch two of potassium thioacetate under EtOAc reflux overnight, the obtained compound was also **25** as a red sugar-like.

^{1}H NMR ($CDCl_3$): δ 1.90 (s, 3H, NHAc), 2.01 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.09 (s, 3H, OAc), 3.77 (s, 3H, COOCH_3), 4.17 (dd, 1H, *J* = 12.3, 6.9 Hz, H9a), 4.35 - 4.38 (m, 2H, H4 and H6), 4.57 (dd, 1H, *J* =

12.3, 3.4 Hz, H9b), 5.33 - 5.34 (m, 1H, NH), 5.34 - 5.42 (m, 2H, H5 and H7), 5.58 (m, 1H, H8) and 5.98 (d, 1H, *J* = 2.7 Hz, H3) ppm; MS (AP^+) $C_{20}H_{27}NO_{12}$ (473.43) m/z 474 $[M+H]^+$, 496 $[M+Na]^+$.



26

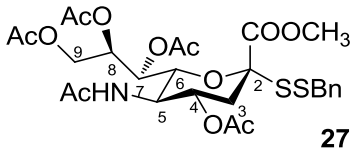
7.3.7. Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-S-benzyl-2,3,5-trideoxy-2-thio-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (Neu5Ac-2SBn, 26)

Mixture of compounds **23** and **24**, 100 mg was dissolved in DMF (2 mL). Then Et_3N (72 µL, 3 eq, 0.516 mmol) was added dropwise, afterwards benzylbromide (40 µL, 2 eq, 0.344 mmol) was added, and then finally hydrazine acetate (17,4 mg, 1.1 eq, 0.189 mmol). The reaction mixture was then stirred at 50 ºC for 20 min. Then DMF was evaporated under vacuum, and the crude residue washed with saturated aqueous solution of NH_4Cl (50 mL) and extracted with DCM (50 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated. Flash column chromatography (EtOAc/PE 9:1) and NMR ($CDCl_3$) gave a mixture of compounds **26** and **27** as a yellow sugar-like, which was confirmed by mass spectrometry. Data according to previous work from the group not yet published.

When purified compound **24** was used as starting material, for the same reaction conditions stated above, NMR ($CDCl_3$) gave compound **26**, which was confirmed by mass spectometry. MS (ES^+) $C_{27}H_{35}NO_{12}S$ (597.7) m/z (%) 620.1 $[M+Na]^+$(100).

Characterization of compound **26**:

R*f* (EtOAc/PE 9:1) 0.52;^{1}H NMR ($CDCl_3$): δ 1.93 (s, 3H, NHAc), 1.98 (dd, 1H, *J* = 11.8, 12.7 Hz, $H3_{ax}$), 2.02 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.14 (s, 3H, OAc), 2.18 (s, 3H, OAc), 2.70 (dd,1H, *J* = 12.6, 4.7 Hz, $H3_{eqt}$), 3.54 (s, 3H, COOCH_3), 3.80 (d, 1H, *J* = 13.6 Hz, SCH_2Bn), 3.89 (dd, 1H, *J* = 9.6, 2.4 Hz, H6), 3.91 (d, 1H, *J* = 13.6 Hz, SCH_2Bn), 4.04 (m, 1H, H5), 4.11 (dd, 1H, *J* = 12.5, 5.1 Hz, H9a), 4.31 (dd, 1H, *J* = 12.5, 2.5 Hz, H9b), 4.85 (m, 1H, H4), 5.28 (d, 1H, *J* = 2.4 Hz, NH), 5.32 – 5.37 (m, 1H, H7), 5.43 - 5.46 (m, 1H, H8), 6.95 - 7.28 (m, 5H, Bn) ppm; ^{13}C NMR ($CDCl_3$): δ 20.79 (CH_3, AcO), 20.91 (CH_3, AcO), 20.96 (CH_3, AcO), 21.25 (CH_3, AcO), 23.32 (NHCOCH_3), 37.43 (CH_2, C3), 42.91 (SCH_2Bn), 49.44 (CH, C5), 53.31 (COOCH_3), 61.29 (CH_2, C9), 68.71 (CH, C4), 69.42 (CH, C7), 70.92 (CH, C8), 75.19 (CH, C6), 89.43 (C2), 127.75 (1C, Bn), 128.76 (2C, Bn), 129.89 (2C, Bn), 134.18 (1C, Bn), 168.10 (NHCOCH_3), 170.05 (C=O, AcO), 170.21 (C=O, AcO), 170.32 (C=O, AcO), 170.73 (C=O, AcO) and 170.07 (COOCH_3) ppm; MS (AP^+) $C_{27}H_{35}NO_{12}S$ (597.63) m/z (%) 598.3 $[M+H]^+$ (100).



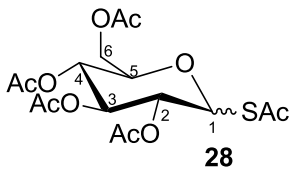
27

7.3.8. Methyl 2-(benzylsulfanyl)-5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (Neu5Ac-2SSBn, 27)

Data according to previous work from the group.

R*f* (EtOAc/PE 9:1) 0.52; $[\alpha]^{20}_{D}$ - 29 ± 1.0 (c 0.53, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.88 (s, 3H, NHAc), 1.94 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.17 (s, 3H, OAc), 2.23 (dd, 1H, *J* = 12.6, 12.5 Hz, $H3_{ax}$), 2.69 (dd, 1H, *J* = 12.6, 4.7 Hz, $H3_{eqt}$), 3.81 (s, 3H, COOCH_3), 4.00 - 4.14 (m, 5H, H9b, H5, NH and SCH_2), 4.36 (dd, 1H, *J* = 12.5, 2.7 Hz, H9a), 4.89 (app. td, 1H, *J* = 11.7, 4.7 Hz, H4), 5.26 (d, 1H, *J* = 10.1 Hz, H6), 5.34 (dd, 1H, *J* = 10.1, 2.2 Hz, H7), 5.34 - 5.39 (m, 1H, H8), 7.24 - 7.33 (m, 5H, Bn) ppm; ^{13}C NMR ($CDCl_3$): δ 20.79 (CH_3, AcO), 20.91 (CH_3, AcO), 20.96 (CH_3, AcO), 21.25 (CH_3, AcO), 23.32 (NHCOCH_3), 37.54 (CH_2, C3), 44.71 (SSCH_2Bn), 49.44 (CH, C5), 53.25 (COOCH_3), 62.23 (CH_2, C9), 67.47 (CH, C4), 69.43 (CH, C7), 69.79 (CH, C8), 75.01 (CH, C6), 89.47 (C2), 127.77 (1C, Bn), 128.66 (2C, Bn), 129.69 (2C, Bn), 136.41 (1C, Bn), 168.00 (NHCOCH$_3$), 170.05 (C=O, AcO), 170.21 (C=O, AcO), 170.32 (C=O, AcO), 170.73 (C=O, AcO) and 171.09 (COOCH$_3$) ppm; MS (ES^+) $C_{27}H_{35}NO_{12}S_2$ (629.70) m/z (%) 630.3 $[M+H]^+$ (100); HRMS (ES^+) found 630.16, calculated for $C_{27}H_{35}NO_{12}S_2$ $[M+H]^+$.

7.4. ACETOBROMO-GLUCOSE SULFIDE AND DISSULFIDE SYNTHESIZED COMPOUNDS



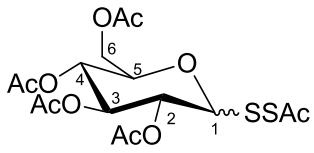
28

7.4.1. 1-(Acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosate (AcGluc-1SAc, 28)

Starting material acetobromo-α-D-glucose (AcBrGlu, lot 077k5304, 10 g, Sigma-Aldrich) (100 mg, 0.24 mmol) was dissolved in anhydrous acetone (5 mL). Then KSAc (165 mg, 6 eq, 1.44 mmol, BCBF7702 Sigma-Aldrich) was added and the mixture stirred at reflux for 3 hrs. TLC analysis (EtOAc/PE 1:2) showed that there was still unreacted starting material but nonetheless the reaction was filtered (to remove the insoluble KSAc salts) and then concentrated under vacuum. Flash column chromatography (EtOAc/PE gradient 1:5 to 2:5) NMR ($CDCl_3$) gave compound **28,** α isomer, as a yellow oil (81 mg, 0.2 mmol, 82%).

R*f* (EtOAc/PE 1:2) 0.3; $[\alpha]^{20}_D$ + 13.33 (c 0.24,CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 2.00 (s, 3H, AcO), 2.02 (2s, 6H, 2AcO) 2.07 (s, 3H, AcO), 2.38 (s, 3H, SAc), 3.83 (ddd, 1H, *J* = 10.1, 4.4, 2.1 Hz, H5), 4.09 (dd, 1H, *J* = 12.6, 2.6 Hz, H6a), 4.26 (dd,1H, *J* = 12.6, 4.4 Hz, H6b), 5.09 - 5.14 (m, 2H, H3 and H4) and 5.23 - 5.29 (m, 2H, H2 and H1) ppm; ^{13}C NMR ($CDCl_3$): δ 20.60 (CH_3, AcO), 20.61 (CH_3, AcO), 20.63 (CH_3, AcO), 20.75 (CH_3, AcO), 30.88 (SAc, CH_3), 61.65 (CH_2, C6), 67.82 (CH, C4), 69.93 (CH, C3), 73.94 (CH, C2), 76.35 (CH, C5), 80.18 (CH, C1), 169.38 (C=O, AcO), 169.42 (C=O,

AcO), 170.08 (C=O, AcO), 170.68 (C=O, AcO) and 192.08 (S*C*OCH_3); MS (AP^+) $C_{16}H_{22}O_{10}S$ (406.4) m/z (%) 407 $[M+H]^+$ (100).



29

7.4.2. 1-(Acetylsulfanyl)-2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranosate (AcGluc-1SSAc, 29)

Starting material acetobromo-α-D-glucose (AcBrGlu, lot 077k5304, 10 g, Sigma-Aldrich) (100 mg, 0.24 mmol) was dissolved in anhydrous acetone (5 mL). Then KSAc (165 mg, 6 eq, 1.44 mmol, BCBF7702 Sigma-Aldrich) and iodine (91 mg, 1.5 eq, 0.36 mmol) were added and the mixture stirred at reflux for 2 hrs. TLC analysis (EtOAc/PE 1:2) showed unreacted starting material but nontheless the reaction was filtered and evaporated. The crude residue was then washed with saturated aqueous solution of sodium thiosulphate (50 mL) and extracted with EtOAc (50 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated. Flash column chromatography (EtOAc/PE gradient 1:5 to 1:1) and NMR ($CDCl_3$) gave a mixture of compounds **28** and **29** as yellow oil**.**

When starting material acetobromo-α-D-glucose, (100 mg, 0.24 mmol) were dissolved in anhydrous acetonitrile (5 mL) and then KSAc (165 mg, 6 eq, 1.44 mmol, BCBF7702 Sigma-Aldrich) was added and the mixture stirred at reflux for 2 hrs (after 2 hrs there wasn't unreacted starting material). Then the reaction mixture was cooled down to RT and iodine (91 mg, 1.5 eq, 0.36 mmol) was added and the reaction stirred at RT overnight. The reaction was

filtrated, concentrated under vacuum and the residue was washed with saturated aqueous solution sodium thiosulphate (50 mL) and extracted with DCM (50 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated. Flash column chromatography (EtOAc/PE gradient 90% PE only to 1:1) and NMR ($CDCl_3$) gave again a mixture of compounds **28** and **29**, in a ratio of 39% SSAc: 61% SAc.

Characterization of compound **29**:

R*f* (EtOAc/PE 1:2) 0.3; ^{1}H NMR ($CDCl_3$): δ 2.00 (s, 3H, AcO), 2.02 (2s, 6H, 2AcO) 2.07 (s, 3H, AcO), 2.44 (s, 3H, SSAc), 3.98 (ddd, 1H, *J* = 10.1, 4.1, 2.3 Hz, H5), 4.07 (dd, 1H, *J* = 7.1, 2.3 Hz, H6a), 4.31 (dd,1H, *J* = 7.1, 4.0 Hz, H6b), 5.09 - 5.14 (m, 2H, H3 and H4), 5.21 – 5.25 (m, 1H, H2) and 6.24 (d, 1H, *J* = 5.2 Hz, H1) ppm; ^{13}C NMR ($CDCl_3$): δ 20.60 (CH_3, AcO), 20.61 (CH_3, AcO), 20.63 (CH_3, AcO), 20.75 (CH_3, AcO), 30.92 (SSAc, CH_3), 61.65 (CH_2, C6), 67.82 (CH, C4), 69.93 (CH, C3), 73.94 (CH, C2), 76.35 (CH, C5), 81.23 (CH, C1), 169.38 (C=O, AcO), 169.42 (C=O, AcO), 170.08 (C=O, AcO), 170.68 (C=O, AcO) and 192.13 (SS*C*OCH_3); MS (AP^+) $C_{16}H_{22}O_{10}S_2$ (438.4) m/z (%) 439 $[M+H]^+$ (10), 407.1 $[M-S]^-$ (40).

8. REFERENCES

(1) Cooper GM, Hausman RE. (2004). The Cell: A molecular approach. 3rd Ed. Boston University ASM Press. 632-668.

(2) Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. (2002). Molecular Cell Biology. 4th ed. W. H. Freeman and Company. 1054-1082.

(3) Hanahan D, Weinberg RA. (2000). The Hallmarks of Cancer. *Cell.* **100**, 57-70.

(4) Katzung BG.(2004). Basic and Clinial Pharmacology. 9th ed. McGraw Hill. 898-929

(5) Hanahan D, Weinberg RA. (2011). Hallmarks of Cancer: The Next Generation. *Cell*.**144**, 646-674.

(6) Palmer, T. D., Ashby, W. J., Lewis, J. D., Zijlstra, A. (2011). Targeting tumour cell motility to prevent metastasis. *Adv. Drug Delivery Rev.***63**, 568-81.

(7) Ferlay J, Shin HR, Bray F,Forman D, Mathers C, Parkin DM. (2010). GLOBOCAN 2008 vl.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No.10. Lyon, France: International Agency for Research on Cancer. Available from: http://globocan.iarc.fr

(8) Amoureux, M. et al. (2010). Polysialic acid neural cell adhesion molecule (PSA-NCAM) is an adverse prognosis factor in glioblastoma, and regulates olig2 expression in glioma cell lines.BMC Cancer. 10 (91). doi:10.1186/1471-2407-10-91

(9) Wells, A., Grahovac, J., Wheeler, S., Ma,B., Lauffenburger, D. (2013).Targeting tumor cell motility as a strategy against invasion and metastasis. *Trends Pharmacol Sci*. **34** (5), 283-9.

(10) Cancer Research UK Stats Report. (2010). Childhood Cancer. Available from: http://info.cancerresearchuk.org/cancerstats/childhoodcancer/

(11) Jimbo, T., Nakayama, J., Akahana, K., Fukuda, M.(2001). Effect of polysialic acid on tumour xenografts implanted into nude mice. *Int. J. Cancer*. **94**(2), 192-9.

(12) Suzuki, M. et al. (2005). Polysialic acid facilitates tumor invasion by glioma cells. *Glycobiology*. **15**(9), 887-94.

(13) Hildebrandt, H. et al. (1998). Polysialic acid on neural cell adhesion molecule correlates with expression of polysialyltransferases and promotes neuroblastoma cell growth. *Cancer Res*. **58**(4), 779-84.

(14) Gluer, S., Scholp, C., von Schweinitz, D., Gerardy-Schahn, R. (1998). Polysialylated neural cell adhesion molecule in childhood rhabdomyosarcoma. *Pediatr. Res*. **43**(1), 145-7.

(15) Eggers, K. et al. (2011). Polysialic acis controls NCAM signals at cell-cell contacts to regulate focal adhesion independent from FGF receptor activity. *J. Cell Sci*. **124**(19), 3279-91.

(16) Gluer, S., Zense, M., Radtke, E., von Schweinitz, D. (1998). Polysialylated neural cell adhesion molecule in childhood ganglioneuroma and neuroblastoma of different histological grade and clinical stage. *Langenbecks Arch Surg*. **383**(5), 340-4.

(17) Tanaka, F. et al. (2000). Expression of polysialic acid and STX, a human polysialyltransferase, is correlated with tumor progression in non small cell lung cancer. *Cancer Res*. **60**(11), 3072-80.

(18) Daniel, L. et al. (2001). A nude mice model of human rhabdomyosarcoma lung metastases for evaluating the role of polysialic acids in metastatic process. *Oncogene.* **20**, 997-1004.

(19) Valentiner, U., Muhlenhoff, M., Lehmann, U., Hildebrandt, H., Schummacher, U. (2011). Expression of neural cell adhesion molecule and polysialic acid in human neuroblastoma cell lines. *Int. J. Oncol*. **39**(2), 417-24.

(20) Seifert, A., Glanz, D., Glaubitz, N., Horstkorte, R., Bork, K. (2012). Polysialylation of the Neural Cell Adhesion Molecule: Interfering with polysialylation and migration in neuroblastoma cells. *Arch. Biochem. Biophys.* **524**(1), 56-63.

(21) Schreiber, S. C. et al. (2008). Polysialylated NCAM represses E-Cadherin-mediated cell-cell adhesion in pancreatic tumour cells. *Gastroenterology.* **134**,*1555-66.*

(22) Daniel, L. et al. (2000). Polysialylated-neural cell adhesion molecule expression in rat pituitary transplantable tumours (spontaneous mammotropic transplantable tumour in Wistar-Furth rats) is related to growth rate and malignancy. *Cancer Res.* **60**, 80-85

(23) Komminoth, P. et al. (1991). Polysialic acid of neural cell adhesion molecule distinguishes small cell lung carcinoma from carcinoides. *Am. J. Pathol*. **139**(2), 297-304.

(24) Roth, J. et al. (1988). Presence of long chain form of polysialic acid of the neural cell adhesion molecule in Wilm's tumour: identification of a cell adhesion molecule as an oncodevelopmental antigen and implication of tumour histogenesis. *Am. J. Pathol*. **133**(2), 227-40.

(25) Fernández-Briera, I., García-Parceiro E., Cuevas E., Gil-Martín. (2010). Effect of Human Colorectal Carcinogenesis on the Neural Cell Adhesion Molecule Expression and Polysialylation. *Oncology*. **78**, 196–204.

(26) Seidenfaden, R., Krauter, A., Schertzinger, F., Gerardy-Schahn, R., Hildebrandt, H. (2003). Polysialic acid directs tumour cell growth by controlling heterophilic neural cell adhesion molecule interactions. *Mol. Cell. Biol*. **23**(16), 5908-18.

(27) Gascon, E., Vutskits, L., Kiss, J. Z. (2007). Polysialic acid-neural cell adhesion molecule in brain plasticity: from synapses to integration of new neurons. *Brain Res. Rev*. **56**, 101-18.

(28) Angata, K., Fukuda, M. (2003). Polysialyltransferases: major players in polysialic acid synthesis on the neural cell adhesion molecule.*Biochimie*.**85**, 195-206.

(29) Rutishauser, U., et al. (1976). Mechanisms of adhesion among cells from the neural tissues of the chick embryo. *Proc.Natl.Acad.Sci*. U.S.A. **73**, 577-581.

(30) Close, B. E. et al. (2003). The minimal structural domains required for neural cell adhesion molecule polysialylation by PST/St8SiaIV and STX/ST8SiaII. *J. Biol*. *Chem*. **278**(33), 30796-805.

(31) Fuster, M. M., Esko, J.D. (2005). The sweet and sour of cancer: glycans as novel therapeutic targets. Nat. Rev. *Cancer*. **5**(7), 526-42.

(32) Varki, A. (1999) in Essentials of Glycobiology (eds Varki, A. et al.). Cold Spring Harbor Laboratory Press, New York. 537-549.

(33) Feizi, T. (1985).Carbohydrate antigens in human cancer. *Cancer Surv*. **4**, 245-269.

(34) Bianco, A., Brufani, M., Manna, F., Melchioni, C. (2001). Synthesis of a carbocyclic sialic acid analogue for the inhibition of influenza virus neuraminidase. *Carbohydrate Research*.**332**, 23-31.

(35) Wilson, J. C., Kiefrel, M. J., Itzstein, M. V. (2005). In Handbook of carbohydrate engineering. Sialic acid and sialyl mimetics: useful chemical probes of sialic acid-recognizing proteins. Taylor & Francis Group, LLC, Chapter 25, 687-748.

(36) Mohan, S. et al. (2010). Carbacycles related to oseltamivir as influenza virus group-1-specific neuraminidase inhibitors. Binding to N1 enzymes in the context of virus-like particles. *J. Med. Chem.***53**,7377-91

(37) Angata, K., Suzuki, M., Fukuda, M. (2002). ST8SiaII and ST8SiaIV polysialyltransferases exhibit marked differences in utilizing various acceptors containing oligosialic acid and short polysialic acid: the basis for cooperative polysialylation by two enzymes. *J. Biol. Chem*. **277**(39), 36808-17.

(38) Galuska, S. P. et al. (2008). Enzyme-dependent variations in the polysialylation of the Neural Cell Adhesion Molecule (NCAM) *in vivo*. *J. Biol.Chem*. **283** (1),17-28

(39) Bork, K., Reutter, W., Gerardy-Schahn, R., Horstkorte, R. (2005). The intracellular concentration of sialic acid regulates the polysialylation of the neural cell adhesion molecule. *FEBS Lett.* **579**, 5079-83.

(40) Roth, J. et al. (1988). Presence of long chain form of polysialic acid of the neural cell adhesion molecule in Wilm's tumour: identification of a cell adhesion molecule as an oncodevelopmental antigen and implication of tumour histogenesis. *Am. J. Pathol*. **133**(2), 227-40.

(41) Finne, J.(1982). Occurrence of unique polysialosyl carbohydrate units in glycoproteins of developing brain. J. Biol. Chem. 257,11966–11970.

(42) Finne, J., Finne U., Deagostini-Bazin H., Goridis C. (1983). Occurrence of alpha 2-8 linked polysialosyl units in a neural cell adhesion molecule.*Biochem. Biophys. Res. Commun*. **112,** 482–487.

(43) Muhlenhoff, M., Eckhardt, M., Gerardy-Schahn, R. (1998). Polysialic acid: three-dimensional structure, biosynthesis and function. *Curr. Opin. Struct. Biol.***8**, 558-64.

(44) Horstkorte, R. et al. (2004). Selective inhibition of polysialyltransferase ST8SiaII by unnatural sialic acids. *Exp. Cell Res*. **298**, 268-74.

(45) Eckhardt, M. et al. (2000). Mice deficient in the polysialyltransferase ST8SiaIV/PST-1 allow discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity. *J. Neurosci.* **20**(14), 5234-44.

(46) Angata, K., Suzuki, M., Fukuda, M. (1998). Differential and cooperative polysialylation of the neural cell adhesion molecule by two polysialyltransferases, PST and STX. *J. Biol. Chem*. **273**(43), 28524-32.

(47) Rutishauser, U., et al. (1982). Binding properties of a cell adhesion molecule from neural tissue. *Proc.Natl.Acad.Sci*. U.S.A. **79**, 685-689.

(48) Yang, P., Yin, X., Rutishauser, U. (1992). Intercellular space is affected by the polysialic acid content of NCAM. *J. Cell. Biol*. **116**, 1487-96.

(49) Yang, P., Major, D., Rutishauser, U. (1994). Role of charge and hydration in effects of polysialic acid on molecular interactions and between cell membranes. *J. Biol*. *Chem*. **269**(37), 23039-44.

(50) Janas, Teresa; Janas, Tadeusz. (2011). Membrane oligo- and polysialic acids. *Biochim. Biophys. Acta*. doi:10.1016/j.bbamem.2011.08.036.

(51) Storms, S. D., Rutishauser, U. (1998). A role for polysialic acid in neural cell adhesion molecule heterophilic binding to proteoglycans. *J. Biol. Chem*. **273**(42), 27124-29.

(52) Rey-Gallardo, A. et al. (2011). Polysialic acid is required for neuropilin-2a/b-mediated control of CCL21-driven chemotaxis of

mature dendritic cells and for their migration in vivo. *Glycobiology*. **21**(5),655-62.

(53) Heimburg-Molinaro, J. et al. (2011). Cancer vacines and carbohydrate epitopes. *Vaccine*. doi:10.1016/j.vaccine.2011.09.009.

(54) Maness, P. F., Schachner, M. (2007). Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. *Nat. Neurosci.* **10**(1), 19-26.

(55) Krug, L. M. et al. (2004). Vaccination of small cell lung cancer patients with polysialic acid or N-propionylated polysialic acid conjugated to keyhole limpet hemocyanin. *Clin. Cancer Res*. **10**, 916-23.

(56) Liu, T., Guo, Z., Yang, Q., Sad, S., Jennings, H. J. (2000). Biochemical engineering of surface α2-8 polysialic acid for immunotargeting tumour cells. *J. Biol*. *Chem*. **275**(42), 32832-36.

(57) Barbeau, D. Liang,J.J., Robitalille, Y., Quirion, R., Srivastava, L. K. (1995). Decreased expression of the embryonic form of the neural cell adhesion molecule in schizophrenic brains. *Proc. Natl. Acad. Sci*. 2785–2789.

(58) Mikkonen, M., Soininen, H., Alafuzof, I., Miettinen, R. (2001) Hippocampal plasticity in Alzheimer's disease. *Rev. Neurosci*. **12** 311–325.

(59) Fewou, S. N., Ramakrishnan, H., Bussow, H., Gieselmann, V., Eckhardt, M. (2007). Down-regulation of polysialic acid is required for efficient myelin formation. *J. Biol*. *Chem*. **282**(22), 16700-11.

(60) El Maarouf, A., Petridis, A. K., Rutishauser, U. (2006). Use of polysialic acid in repair of the central nervous system. *Proc. Natl. Acad. Sci.* **103**(45), 16989-94.

(61) Mehanna, A. et al. (2010). Polysialic acid glycomimetic promotes functional recovery and plasticity after spinal cord injury in mice.*Mol. Ther*. **18**(1), 34-43.

(62) Nakayama, J., Fukuda, M. (1996). A human polysialyltransferase directs *in vitro* synthesis of polysialic acid. *J. Biol*. *Chem*. **271**(4), 1829-32.

(63) Angata, K. et al. (2000). Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptor by three distinct α-2,8-sialyltransferases, ST8SiaIV(PST), ST8SiaII(STX) and ST8SiaIII. *J. Biol. Chem*. **775**(24), 18594-601.

(64) Ong, E. et al. (1998). Developmental regulation of polysialic acid in mouse directed by two polysialyltransferases, PST and STX. *Glycobiology*. **8**(4), 415-24.

(65) Nakayama, J., Angata, K., Ong, E., Katsuyama, T., Fukuda, M. (1998). Polysialic acid, a unique glycan that is developmentally regulated by two polysialyltransferases, PST and STX, in the central nervous system: from biosynthesis to function. *Pathol. Int*. **48**(9), 665-77.

(66) Angata,K., Nakayama,J., Fredette,B., Chong,K., Ranscht,B. and Fukuda,M. (1997) Human STX polysialyltransferase forms the embryonic form of the neural cell adhesion molecule. Tissue specific expression, neurite outgrowth and chromosomal

localization in comparison with another polysialyltransferase PST. *J Biol.Chem*. **272**, 7182–7190.

(67) Kitagawa,H. and Paulson,J.C. (1994) Differential expression of five sialyltransferase genes in human tissues. *J. Biol. Chem*. **269**, 17872–17878.

(68) Angata, K., Chan, D., Thibault, J., Fukuda, M. (2004). Molecular Dissection of the ST8Sia IV Polysialyltransferase: distinct domains are required for neural cell adhesion molecule recognition and polysialylation. *J. Biol*. *Chem*. **279**(24), 25883-90.

(69) Charter, N. W., Mahal, L. K., Jr. Koshland, D. E., Bertozzi, C. R. (2000). Biosynthetic incorporation of unnatural sialic acids into polysialic acid on neural cells. *Glycobiology*. **10**(10), 1049-56.

(70) Charter, N. W., Mahal, L. K., Jr. Koshland, D. E., Bertozzi, C. R. (2002). Differential effects of unnatural sialic acids on the polysialylation of the neural cell adhesion molecule and neuronal behavior. *J. Biol*. *Chem*. **277**(11), 9255-61.

(71) Franceschini, I. et al. (2001). Polysialyltransferase ST8SiaII (STX) polysialylates all the major isoforms of NCAM and facilitates neurite outgrowth. *Glicobiology.* **11**(3), 231-39.

(72) Close, B. E. et al. (2001). The polysialyltransferase ST8SiaII/STX: post translational processing and role of auto polysialylation in the polysialylation of neural cell adhesion molecule. *Glycobiology*. **11**(11), 997-1008.

(73) Muhlenhoff, M., Manegold, A., Windfuhr, M., Gotza, B., Gerardy-Schahn, R. (2001). The impact of *N*-Glycosylation on the functions of Polysialyltransferases. *J. Biol*. *Chem*. **276**(36), 34066-73.

(74) Nakano, T. A., Steirer, L. M., Moe, G. R. (2011). The expression profile of De-*N*-acetyl polysialic acid (NeuPSA) in normal and disease human tissue. *J. Biol*. *Chem*. **286**(46), 40343-53.

(75) Izet M. Kapetanovic (2011). Drug discovery and development-present and future.InTech, Croatia.

(76) Xiu-Ping Chen, Guan-Hua Du. (2007). Target validation: A door to drug discovery. *Drug Discov Ther*. **1**(1):23-29.

(77) Compain, P., Martin, O. R. (2001). Carbohydrate mimetics based-glycosyl transferase inhibitors. *Bioorg.Med.Chem*. **9**, 3077-92.

(78) Schaub, C., Muller, B., Schmidt, R. R. (1998). New sialyl transferase inhibitors based on CMP-quinic acid: development of new sialyltransferase assay. *Glycoconj.* **15**,345-54.

(79) Wang, X., Zhang, L., Ye, X. (2003). Recent development in design of sialyltransferase inhibitors. *Medicinal Research Reviews*. **23**(1), 32-47.

(80) Horenstein, BA., Bruner, M. (1996). Acid-catalysed solvolysis of CMP-N-acteyl neuraminate:evidence for a sialyl cation with finite lifetime. *J. Am.Chem.Soc*. **118**, 10371-79.

(81) Wilson, J. C., Kiefrel, M. J., Itzstein, M. V. (2005). In Handbook of carbohydrate engineering. Sialic acid and sialyl mimetics: useful chemical probes of sialic acid-recognizing proteins. Taylor & Francis Group, LLC, Chapter 25, 687-748.

(82) Hashimoto et al.(1993). Characterization of inhibitory activities and binding mode of synthetic 6'-modified methyl N-acetyl-β-lactosaminide toward rat liver CMP-D-Neu5Ac:D-galactoside-(2-6)-α-D-sialyltransferase. Carbohydrate Res. **247**, 179-193.

(83) Amann, F., Schaub, C., Muller, B., Schmidt, R. R. (1998). New potent sialyltransferase inhibitors-synthesis of donor and of transition-state analogues of sialyl donor CMP-Neu5Ac. *Chem. Eur.J*. **4**(6), 1106-15.

(84) Schaub, C., Muller, B., Schmidt, R. R. (2000). Sialyltransferase inhibitors based on CMP-quinic acid. *Eur.J.Org.Chem*. 1745-58

(85) Schmidt, R. R. (1998). New approaches to glycoconjugate synthesis. *Pure&Appl.Chem*. **70**(2), 397-402.

(86) Girard, C. et al. (2005). Sialyl Lewis^x analogs based on a quinic acid scaffold as the fucose mimic. *Bioorg.Med.Chem.Lett*. **15**, 3224-28.

(87) Grandjean, C. et al. (2001). Novel hyperbranched glycomimetics recognized by human mannose receptor: quinic and shikimic acid derivatives as mannose bioisosteres. *Chembiochem*. **2**, 747-57.

(88) Metaferia, B.B. et al. (2006). Synthetic macrolides that inhibit breast cancer cell migration *in vitro*. *J. Am.Chem. Soc*. **129**, 2434-35.

(89) Wu, Z. et al. (2011). Universal phosphatase-coupled glycosyltransferase assay. *Glicobiology*. **21**(6),727-33.

(90) Inoue, S. & Inoue, Y. (2001).Developmental profile of neural cell adhesion molecule glycoforms with a varying degree of polymerization of polysialic acid chains. *J Biol Chem.* **276**, 31863-70.

(91) Flores-Parra, A., Gutierrez-Avella, D. M., Contreras, R., Huu, F. (1989). ^{13}C and ^{1}H-RMN investigations of quinic acid derivatives:complete spectral assigment and elucidation of preferred conformations. *Magn. Reson. Chem.* **27**,544-55.

(92) Greene, W. T., Wuts, P. G. M. (1999) In Protective groups in organic synthesis, 3rd ed. Greene, W. T., Wuts, P. G. M., Eds. Wiley, New York.

(93) Herrera, L. et al. (2003). Synthesis of anellated carbasugars from -(-) quinic acid. *Carbohydrate Research***. 338**, 293–98.

(94) Flores-Parra, A., Gutierrez-Avella, D. M., Contreras, R., Huu, F. (1989). ^{13}C and ^{1}H-RMN investigations of quinic acid derivatives:complete spectral assigment and elucidation of preferred conformations. *Magn. Reson. Chem.* **27**,544-55.

(95) Barco, A. et al. (1997). D-(-)-Quinic acid: a chiron store for natural product synthesis. *Tetrahedron: Asymmetry.* **8**(21), 3515-45.

(96) Baptistella, L. H. B., Cerchiaro, G. (2004). Studies for the transformation of carbacycles into carbohydrates: approach toward the synthesis of higher sugar derivatives. *Carbohydrate Research*.**339**, 665-71.

(97) Sanchez-Abella, L., Fernandez, S., Armesto, N., Ferrero, M., Gotor, V. (2006). Novel and efficient syntheses of (-)-methyl 4-*epi*-shikimate and 4,5-epoxy-quinic and -shikimic acid derivatives as key precursors to prepare new analogues. *J. Org. Chem.* **71**(14), 5396-99.

(98) Frank, M., Miethchen, R. (1998). Non-conventional epimerization and functionalisation of quinic acid and shikimic acid methyl esters. *Carbohydrate Research*.**313**, 49-53.

(99) Chatgilialoglu, C.; Ferreri, C.; Gimisis, T. (1998). In The chemistry of organic silicon compounds; Rappoport, Z., Apeloig, Y., Eds. The Chemistry of Functional Groups Series; Patai, S., Series Ed. Wiley-Interscience: New York, Vol. 2, 1–3, 1539–1579.

(100) Kondo, T., Kato, Y., Yoshida, K. (2005). Essential structure of co-pigment for blue sepal-color development of hydrangea. *Tetrahedron.***46**, 6645-49.

(101) Sanchez-Abella, L., Fernandez, S., Verstuyf, A., Verlinden, L., Gotor, V., Ferrero, M. (2009). Synthesis, conformational analysis, and biological evaluation of 19-nor-vitamin D_3 analogues with A-ring modifications. *J. Med. Chem.* **52**(19), 6158-62

(102) Kok, S. H. L., Lee, C. C., Shing, T. K. M. (2001). A new synthesis of Valienamine. *J. Org. Chem.* **66**(21), 7184-90.

(103) Wang, Z., Miller, S. M., Anderson, O. P., Shi, Y. (1999). A class of *C*2 and pseudo *C*2 symmetric ketone catalysts for asymmetric epoxidation. Conformational effect on catalysis. *J. Org. Chem.***64**(17), 6443-58.

(104) Hanessian, S., Pan, J., Carnell, A., Bouchard, H., Lesage, L.(1997). Total synthesis of (-)-Reserpine using the chiron approach. *J. Org. Chem.* **62**(3), 465-73.

(105)Zeng, K, et al. (2009). Synthesis and biological evaluation of quinic acid derivatives as anti-inflammatory agents. *Bioorg.Med.Chem.Lett.***19**, 5458-60.

(106)Barton, D. H. R., McCombie, S. W. (1975). A new method for the deoxygenation of secondary alcohols. *J.Chem.Soc*. **16**, 1574-85.

(107)Crich, D., Puintero, L. (1989). Radical chemistry associated with thiocarbonyl group. *Chem. Rev*. **89**, 1413-32.

(108)Spiegel, D. et al. (2005). Deoxygenation of alcohols employing water as hydrogen atom source. *J. Am. Chem. Soc*. **127**(36), 12513-515.

(109)Barton, D. H. R., Ok, J. D., Jaszberenyi, J. C. (1990). On the mechanism of deoxygenation of secondary alcohols by tin hydride reduction of methyl xanthates and other thiocarbonyl derivatives. *Tetrahedron Lett.* **31**(28), 3991-94.

(110)Barton, D. H. R., Jaszberenyi, J. C. (1989). Improved methods for the radical deoxygenation of secondary alcohols. *Tetrahedron Lett.* **30**(20), 2619-22.

(111)Hartwig, W. (1983).Modern methods for the radical deoxygenation of alcohols. *Tetrahedron.* **39**(16), 2609-45.

(112)Derek H.R. Barton, Wolfang Hartwig, Robyn S. Hay Motherwell, William B. Motherwell, Andreas Stange. (1982). Radical deoxygenation of tertiary alcohols.*Tetrahedron Lett.* **23**(19), 2019-22.

(113)Clive, D.L.J., Wang, J. (2001). A tin hydride designed to facilitate removal of tin species from products of stannane-mediated radical reactions. *J. Org. Chem.* **67**, 1192-1198.

(114)Bowman, W. R., Krintel, S. L., Schilling, M. B. (2004). Tributylgermanium hydride as a replacement for tributyltin hydride in radical reactions*. Org. Biomol. Chem*. **2**, 585-92.

(115)Postigo, A., Kopsov, S., Ferreri, C., Chatgilialoglu, C. (2007). Radical reactions in aqueous medium using TTMSS. *Org.Lett*. **9** (25), 5159-62.

(116)Boxer, M. B., Yamamoto, H. (2006). Tris(trimethylsilyl)silyl-Governed Aldehyde Cross-Aldol Cascade Reaction. *J. Am. Chem. Soc*. **128**(1), 48-49.

(117)Crich, D. et al. (1995).The origin of the beta-oxygen effect in the Barton deoxygenation reaction. *J. Am. Chem. Soc.* **117**(34), 8757-68.

(118)Schwardt, O. et al (2010). Design, synthesis, biological evaluation, and modeling of a non-carbohydrate antagonista of the myelin-associated glycoprotein. *Bioorg. Med. Chem*. **18**, 7239–51

(119)Prazeres, V. F. V., Castedo, L., Gonzalez-Bello, C. (2008). Stereoselective synthesis of novel cyclic γ-amino acids and triazole derivatives. *Eur. J. Org. Chem.* 3991–4003

(120)Mukaiyama, T., Banno, K., Narasaka, K. (1973). New aldol type reaction. *Chem. Lett.* 1011.

(121)Mukaiyama, T., Banno, K., Narasaka, K. (1975). *Chem. Lett.* 745.

(122)Inamoto, Y., Nishimoto, Y., Yasuda, M., Baba, A. (2012). Direct use of esters in the Mukaiyama Aldol Reaction: a powerful and convenient alternative to aldehydes. *Org. Lett*. **14**(4), 1168-71.

(123)Mukaiyama, T. (1982). The Directed Aldol Reaction, *Organic Reactions*. **28**, 203.

(124)Mukaiyama, T. (2004). Explorations into new reaction chemistry. *Angew Chem Int.* **43**(42), 5590-614. Carreira, E. (1999).

(125)Mukaiyama Aldol Reaction, In Comprehensive Asymmetric Catalysis, Jacobsen, E. N., Pfaltz, A., Yamamoto, H. Editors; Springer Verlag: Heidelberg. Vol III, 998-1059.

(126)Mukaiyama, T., Banno, K., Narasaka, K. (1974). New Cross-Aldol reactions- reactions of silyl enol ethers with carbonyl compounds activated by titanium tetrachloride. *J. Am. Chem. Soc.* **96**(24), 7503-09.

(127)Morais, G. R., Oliveira, I. F., Humphrey, A. J., Falconer, R. A. (2009). Identification of an acetyl disulfide derivative in the synthesis of thiosialosides. *Carbohydrate Research.* doi:10.1016/j.carres.2009.10.017

(128)Morais, G. R., Oliveira, R. S., Falconer, R. A. (2009). Selective synthesis of Neu52en and its oxazoline derivative using $BF_3.Et_2O$. *Tetrahedron Lett.***50**, 1642-44.

(129)Haverkamp, J. et al. (1982). High-resolution ^{1}H-RMN spectroscopy of free and glycosidically linked *O*-acetylated sialic acids. *Eur.J.Biochem.***122**, 305-11.

(130) S. Bennett et al. (1994). A simple method for the preparation of thioglycosides of N-Acetylneuraminic acid. Carbohydrate research, **259**(2), 293-99.

9. APPENDIX

Ribero Morais, G., Oliveira, I.P.F., Humphrey, A.J. and Falconer, R.A. (2009). Identification of an acetyl disulfide derivative in the synthesis of thiosialosides. Carbohydrate Research. Vol. 345, No. 1, pp. 160-162.
DOI: http://dx.doi.org/10.1016/j.carres.2009.10.017