THE UNIVERSITY OF
WARWICK

Department of Chemistry

Thesis submitted for the degree of MSc in
Polymer Chemistry

August 2013

Monodisperse Glyco-Clusters. Synthesis and Application as Glycosyl Transferase Inhibitors.

By: **Mary Lilliman**
Supervisor: **Dr Matthew I. Gibson**

Table of Contents

Acknowledgements

I wish to thank Dr Matthew Gibson for allowing me to carry out my research project in the Gibson group. I would also like to thank all members of the Gibson group for their help and support throughout the project.

I would also like to thank all the technical/ support staff in the Chemistry department for their assistance whether it was chasing up chemical orders, providing equipment training or removing stoppers from round bottomed flasks when I had forgotten to grease the joints.

Also, thanks need to go to Science City (Advantage West Midlands) for providing the GPC equipment.

Thank you all.

Abstract

Whilst once hailed as the panacea of modern medicine antibiotics are becoming less and less effective in the treatment of diseases such as tuberculosis due to rising levels of antibiotic resistance. As a result, different drug targets need to be found. One of the most important biosynthetic pathways being targeted is the synthesis of peptidoglycan; the main component of the bacterial cell wall. Two main enzymes are involved in the synthesis of peptidoglycan: glycosyl transferases and transpeptidases. Inhibition of either will lead to incomplete synthesis of the cell wall and therefore cell lysis. Of these two targets glycosyl transferases are seen as the best potential target for antibiotics.

The aim of this project was to investigate the use of polymers based on oligosaccharides as inhibitors of glycosyl transferase enzymes. In order to achieve this aim mono-, di-, tri- and tetra saccharides were methacrylated. As this left them with an activated vinyl group it was possible to carry out Michael addition of cysteine and thiogalactose to the methacrylated sugars.

The methacrylated sugars and the Michael addition products all exhibited low polydispersities, lower than those that would be expected using conventional controlled polymerisation techniques. This makes them more suitable for *in vitro* use, as their properties will be the same from one molecule to another. All reactions were easy to carry out and required minimal work up, making it possible to easily scale up the reactions.

The Michael addition products were then tested for their inhibitory properties against the enzyme galactosyl transferase. Results of this assay were inconclusive but do suggest that the thiogalactose functionalised sugars may competitively inhibit the action of galactosyl transferase enzymes from *E.coli*. Further repeats and quantification of this assay is proposed as an area for further research.

Abbreviations

Boc: Tert-Butyloxycarbonyl
DAP: Diaminopimelic Acid
DMF: Dimethylformamide
ESI: Electrospray Ionisation
ESMS: Electrospray Mass Spectrometry
HCl: Hydrochloric Acid
IR: Infrared Spectroscopy
LC: Liquid Chromatography
$MgSO_4$: Magnesium Sulfate
M_n: Number Average Molecular Weight
M_w: Weight Average Molecular Weight
NAcGlc: N-Acetyl Glucosamine
NaCl: Sodium Chloride
NAcMur: N-Acetyl Muramic Acid
$NaHCO_3$: Sodium Hydrogen Carbonate
NH_4BF_4: Ammonium Tetrafluoroborate
NMR: Nuclear Magnetic Resonance
ONPG: O-Nitrophenol β-Galactoside
TFA: Trifluoroacetic Acid
THF: Tetrahydrofuran
TLC: Thin Layer Chromatography

Amino Acid Abbreviations

Ala: Alanine
Cys: Cysteine
Glu: Glutamic Acid
Lys: Lysine

1. Introduction

1.1. The Burden of Infectious Disease

Whilst Western countries such as the UK and US have sought to eradicate infectious diseases such as tuberculosis for many people in developing countries these diseases are a very real threat. In 2004 in the Democratic Republic of the Congo roughly 57 deaths in every 100,000 were due to tuberculosis compared to less than 1 in every 100,000 in the UK during the same period (World Health Organisation statistics).

Despite the claims in 1969, of the US Surgeon General that it was time to "close the book on infectious diseases" the reality is that infectious diseases are still the biggest cause of death throughout the world.[1] Finding a cure for infectious diseases is therefore essential to make that prediction come true.

1.2. Antibiotics and Resistance

The Oxford English Dictionary gives the origins of the word antibiotic as the Greek biōtikos meaning fit for life, from bios meaning life. Antibiotic therefore literally translates then as 'against life', meaning a compound that interferes with a biological process critical to the survival of bacteria. Of course, an important aspect of antibiotics is that they must be specific for the bacteria, and not cause harm to the eukaryotic host.[2]

Though the discovery of antibiotics is widely attributed to Alexander Fleming, antibiotics have been shown to be in use as far back as 350AD, where skeletons from ancient Sudanese Nubia have been found to contain traces of tetracycline.[3] Whilst most people are familiar with the story of Fleming discovering penicillin by accident in 1928 perhaps what is more surprising is its first real use as an antibiotic was not until World War II when it was used to treat the military.[4] This delay was caused by the difficulties in purification.[3] Following the successful use of penicillin in World War II manufacturing became easier, and antibiotics were soon being hailed as the 'panacea' of medicine; a 'magic bullet' that could cure all.[5,6]

There are many different types of antibiotics available, all of which have different actions.[6–8] However, for each of the different classes of antibiotic that has so far been produced there is at least one mechanism of resistance;[5] some strains of bacteria such as *Mycobacterium tuberculosis* are now resistant to over 100 different antibiotics.[6] The driving

force behind antibiotic resistance is Darwinian selection;[8] the strains of bacteria that are the best suited to their environment survive. It therefore follows that in those places where there are high levels of antibiotic usage, such as developing countries where antibiotics are easily available without prescription; there are also high levels of antibiotic resistance.[5] Ironically we are, as a society, promoting the development of antibiotic resistant strains by our use of antibiotics.[9,10] Only a few years after the introduction of penicillin 50% of all *Staphylococcus aureus* were penicillin resistant.[5]

The rise in levels of antibiotic resistant bacteria, especially those bacteria which are multidrug resistant, is possibly one of the biggest challenges to public healthcare.[5,11,12] Not only does antibiotic resistance lead to an increase in treatment side effects[13] it also prolongs treatment lengths,[7,13] doubles mortality and morbidity and dramatically increases treatment costs.[5,7]

In light of the rising levels of antibiotic resistance alternative targets are being sought[14] to control pathogens such as *Mycobacterium tuberculosis*. These targets include enzymes critical for bacterial cell wall synthesis[12] such as glycosyl transferases.

1.3. Bacterial Cell Walls

The bacterial cell wall is a bag-like structure that controls cell shape and is capable of protecting the cell from changing pressures that could cause it to lyse.[15–17]

Peptidoglycan is the main net like polymer component of bacterial cell walls,[18,19] making up between 5% and 90% of the mass of the cell wall depending on the species.[20] It acts as the main scaffold of the cell, maintaining the cell shape[15,21,22] and providing an anchor for other cell components such as proteins.[15] As the name suggests, peptidoglycan is made up of linear chains of glycan cross linked with short peptide chains.[15,23,24] This forms an elastic network that can protect the cell from lysis by expanding or contracting according to different osmotic pressures.[25,26] The glycan strands are made up from alternating N-acetyl glucosamine (NAcGlc) and N-acetyl muramic acid (NAcMur) residues joined by a β-1,4 linkage.[25,27] The peptide chain is ether linked through its N terminus to the carboxyl group of NAcMur[28,29] and is commonly a pentapeptide of the form L-Ala-ɣ-D-Glu-X-D-Ala-D-Ala where X is either L-Lys or DAP (figure 1.3.1).[29–33]

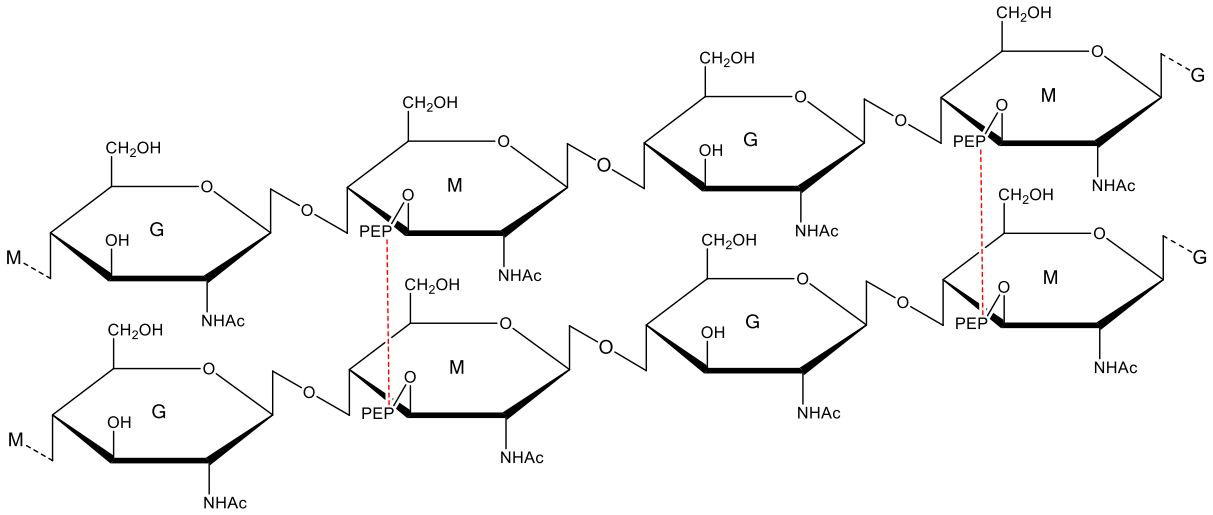


Figure 1.3.1: Structure of peptidoglycan cell wall where G is N-acetyl glucosamine, M is N-acetyl muramic acid; PEP is the pentapeptide and the red lines represent the cross links between the linear chains.[29]

Two main types of enzymes are involved in the polymerisation of peptidoglycan: glycosyl transferases and transpeptidases.[32,34] The linear glycan chains are formed by glycosyl transferases coupling a diphospholipid-linked disaccharide-pentapeptide before cross linking is carried out by transpeptidases.[35] As the peptidoglycan wall is essential for cell viability any inhibition of the biosynthesis of the bacterial cell wall by inhibiting the action of one of these enzymes will leave the bacterial cell vulnerable to lysis, and ultimately lead to cell death.[15,35–37]

1.4. Glycosyl Transferase Enzymes

Peptidoglycan glycosyl transferases are seen as excellent inhibitor targets[30] as they are highly conserved between bacterial species, have no equivalent in mammalian cells[38] so any inhibitors will not affect human cells and are easily accessible from the periplasm.[39,40] These qualities have lead many people to think of glycosyl transferase inhibition as the ‘holy grail’ of drug target research.[39]

Glycosyl transferases catalyze the formation of the glycan chains[32,34] by attachment of a sugar donor, in this case the growing peptidoglycan chain to an aglycone acceptor, the disaccharide intermediate (figure 1.4.1).[41–44] This reaction occurs at no cost to the cell as a phosphoester bond is merely replaced with a glycosidic bond.[29] Depending on the glycosyl transferase the glycosylation reaction can occur with either inversion or retention of the stereochemistry of the donor sugar.[42,45,46]

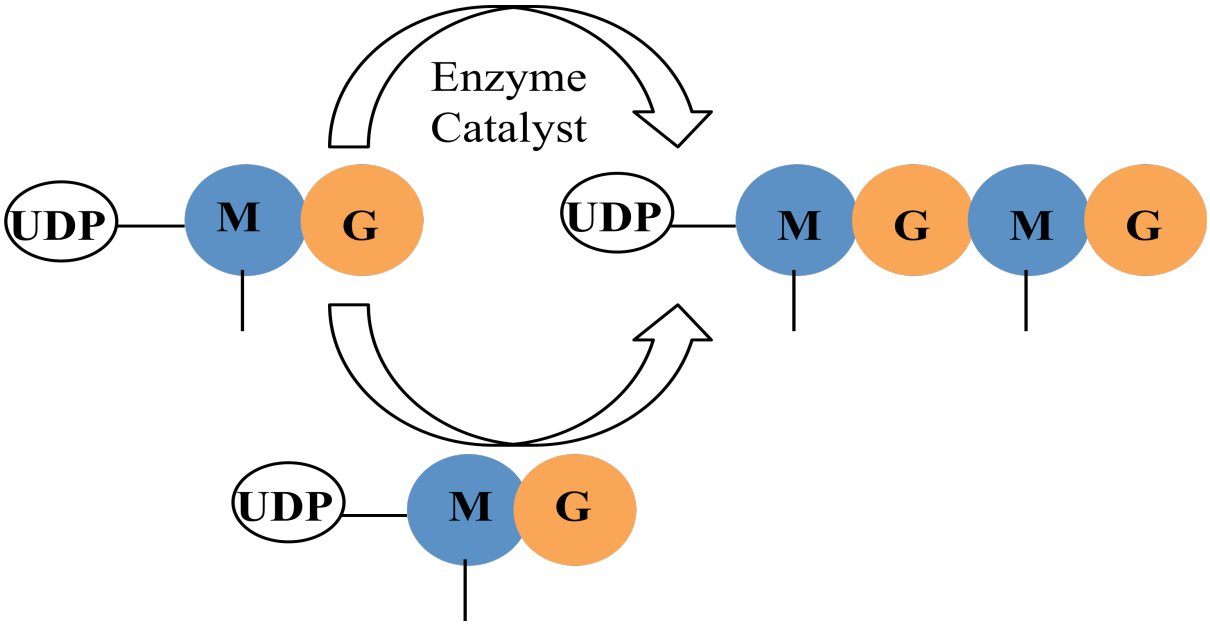


Figure 1.4.1: schematic showing the action of glycosyl transferase enzymes where M is N-acetyl muramic acid, G is N-acetyl glucosamine and UDP is the lipid group anchoring the growing peptidoglycan chain to the cell wall.[23,29]

1.5. Current Inhibition Strategies

The only current antibiotic that directly targets the glycosyl transferase enzymes responsible for peptidoglycan synthesis by binding to them rather than their substrate is the natural product moenomycin A (figure 1.5.1), Moenomycin A is a member of the moenomycin antibiotic family, and can alternatively be called flavomycin or bambermycin.[36,47,48] By binding directly to the enzyme, moenomycin prevents binding of the substrate and therefore polymerisation of the peptidoglycan precursor into peptidoglycan.[49] Whilst moenomycin has many advantageous properties such as a high potency and no reported cases of resistance[36,50] its phosphoglycerate lipid tail means it has poor pharmacokinetics, and therefore has difficulty travelling across cell membranes, as well as having a long serum half life.[51,52] As such, it was only used as a growth promoter in farm animals such as pigs and poultry,[53] but has since been banned by the EU, along with many other antibiotics. This is due to the chance of induced resistance and cross resistance to other antibiotics that could be conferred to bacteria, and which could drastically impact on human health.[47,53]

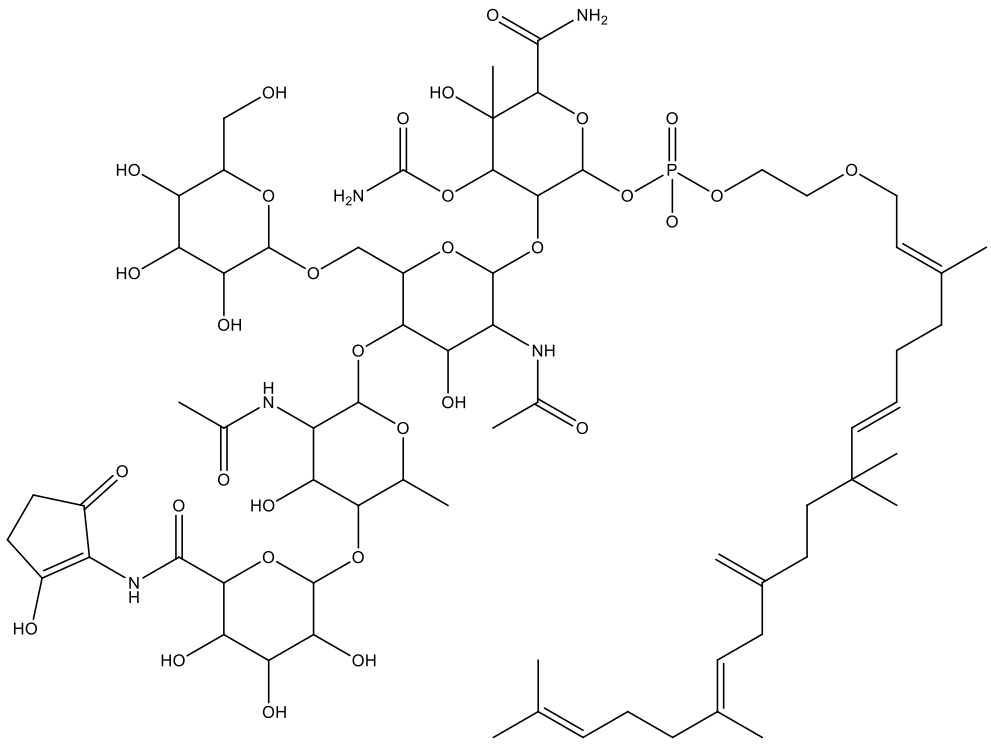


Figure 1.5.1: The structure of the antibiotic moenomycin A.[36]

Therefore, the race is on to find a biocompatible molecule, or family of molecules that have the correct pharmacokinetic properties, and which cause inhibition of the peptidoglycan glycosyl transferase enzymes.

Most attempts to find inhibitors of glycosyl transferases have taken place on eukaryotic galactosyl transferase enzymes and a range of different molecules that successfully inhibit glycosyl transferase enzymes have been found. These molecules all have very different structures, showing there is no idea of what makes a good galactosyl transferase inhibitor, and include molecules as varied as 5-fluorouracil,[54] 4-amino benzyl N-acetyl-β-D-glucosamide,[55] poly(uridine 5'-p-styrenesulfonate)[56] and bis-(p-nitrophenyl) phosphate.[57]

However, some of these only work at high doses, such as 5-fluorouracil which was found to inhibit the galactosyl transferase enzymes in tumour tissue, but only at concentrations of approximately 1mg/ml.[54] They were all tested on a variety of different enzymes, making it difficult to compare their effectiveness, and therefore which ones to potentially base new antibiotics on.

1.6. Glycopolymers

Carbohydrates play a major role in many recognition events.[58] These recognition events are based on the highly specific binding[59] between carbohydrates and the proteins found on the outside of cells, called lectins,[58,59] and are responsible for a number of biological processes such as inflammation, cell-cell communication, fertilisation and signal transmission.[60,61] Binding between an individual carbohydrate and lectin is fairly weak, however concurrent multivalent binding of carbohydrates and lectins increases the binding strength. This phenomenon is referred to as the glyco-cluster effect.[59–62] As polymers are naturally multivalent they are starting to be used as natural carbohydrate mimics,[63] and the term glycopolymer has been used to describe both natural and artificial carbohydrate containing polymers as well as modified sugar based polymers.[58] These polymers can be prepared by both conventional and controlled polymerisation as well as post polymerisation modification,[60] although direct synthesis tends to require additional synthesis and purification steps.[63]

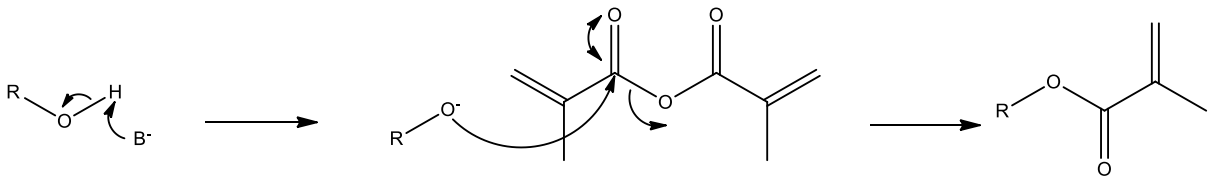
In order for synthetic polymers to be suitable for biomedical applications it is important for them to have a low polydispersity[64] as differences in polymer chain length can affect the solubility and pharmacokinetic properties of the polymer and therefore how effective it is therapeutically.[65] However, even by using controlled polymerisation techniques very low polydispersity glycopolymers are difficult to come by. For example, Suriano *et al.* synthesised a range of triblock copolymers using a ring opening method that ranged in polydispersity from 1.22 to 1.26[66] and Munoz-Bonilla *et al.* used SET-LRP to synthesis a range of block glycopolymers that had polydispersities from 1.15-1.40.[60] Whilst these have polydispersities that are traditionally thought of as low, they are still high compared to those of biological molecules such as proteins.

Therefore, it would be beneficial to be able to produce mono-disperse sugar-based polymers that are biocompatible and biodegradable as well as straightforward to synthesise and purify.

There are a few literature examples of the use of glycopolymers as enzyme inhibitors,[67–70] but only one of these has demonstrated a multivalent effect, similar to that seen with lectins.[70] Therefore it would be of interest to see if it was possible to synthesise a glycosyl transferase inhibitor that demonstrated this multivalent effect.

1.7. Methacrylation of Sugars

There are many examples of the methacrylation of sugars in the literature, dating back as far as 1945.[71] Sugars are an excellent starting material for the development of antibiotics because they are bio available and safe for humans. There are many examples of different reaction conditions and reagents that can be used for the methacrylation of sugars but they all involve a base (scheme 1.7.1).[71–74]

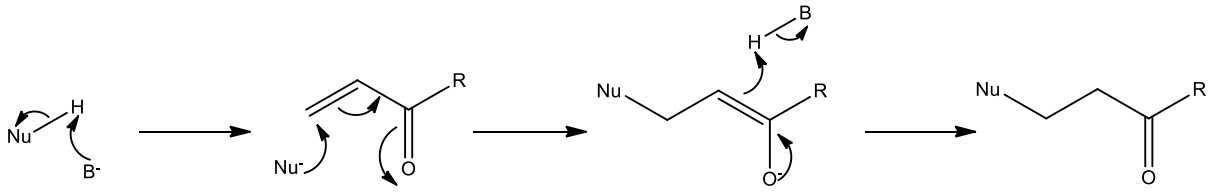


Scheme 1.7.1: General reaction for scheme for sugar methacrylation where B represents a base and R represents the rest of the sugar molecule.

1.8. Click Chemistry

The concept of 'click chemistry' was first introduced by Sharpless and co-workers in 2001.[75] Nature exhibits a clear preference for the formation of carbon-heteroatom bonds over carbon-carbon bonds, as demonstrated by nucleic acids, proteins and polysaccharides, all of which are made up of carbon-heteroatom linkages.[76] Click chemistry has now been developed to encompass a few key reactions that link together molecules containing specific chemical groups,[77] all of which have a high thermodynamic driving force, leading to high levels of selectivity.[75] The main idea behind click chemistry is the use of readily available starting products[78] and benign solvents[79] in a reaction that produces good conversions under mild conditions,[80] non toxic by products, and only requires simple, non-chromatographic, purification techniques.[75]

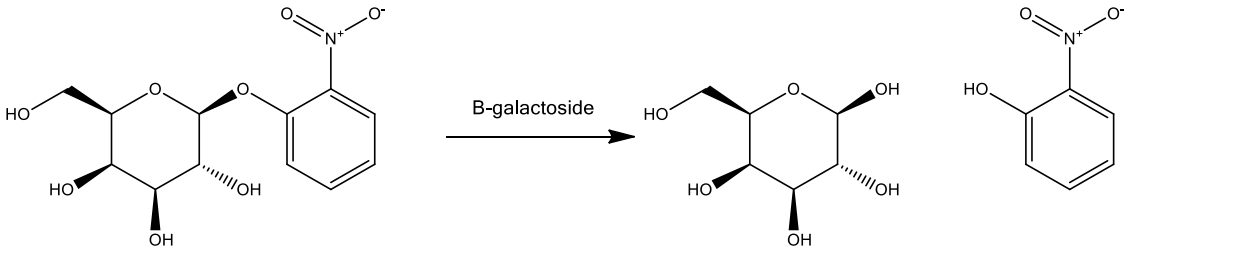
An example of such a reaction is the Michael addition reaction (scheme 1.8.1).[79] Michael addition is a nucleophilic addition of a Michael donor to an electron deficient carbon-carbon double bond, such as that adjacent to an ester group.[80,81] There are a wide variety of catalysts for this reaction, including strong bases, metals, organometallics and Lewis acids,[80] but recent research has focused on the use of tertiary phosphines as nucleophilic catalysts.[82]



Scheme 1.8.1: General reaction scheme of base catalysed Michael Addition where B is base and Nu is a nucleophile such as a thiol.

1.9. Galactosyl Transferase Assay

In order to measure enzyme activity and the effectiveness of different inhibitors enzyme assays are carried out. One of the ways of telling the end point of an assay is if there is a colour change, as is the case in the Miller assay for β-galactosidase activity. β-galactosidase hydrolyzes the colourless substrate o-nitrophenyl-β-D-galactoside to form galactose and o-nitrophenol which is yellow and absorbs light at 420nm (scheme 1.9.1).[83] The activity of the enzyme can then be calculated in Miller units.[84] Recent advances such as the use of 96 well plates and microplate readers[85] have helped increase the reproducibility of results.[86]



Scheme 1.9.1: Miller assay scheme showing starting colourless substrate and yellow product.

1.10. Aims

Synthesise and characterise monodisperse polymer scaffolds based on glucose (monosaccharide), cellobiose (disaccharide), raffinose (trisaccharide) and stachyose (tetrasaccharide).

Use these scaffolds in the synthesis of monodisperse polymers with precise control over the number of carbohydrate groups attached.

Explore the ability of these monodisperse polymers to inhibit a specific galactosyl transferase enzyme from the bacteria *E.coli*.

2. Experimental

2.1. Materials

All materials were used as purchased. Cellobiose was purchased from Acros Organics (Geel, Belgium). Raffinose was purchased from Alfa Aesar (MA, USA). Thiogalactose and stachyose were purchased from Carbosynth (Berkshire, UK). Pyridine was purchased from Fisher Scientific (Loughborough, UK). All other reagents were purchased from Sigma-Aldrich (MO, USA).

2.2. Analytical Methods

NMR: ^{1}H and ^{13}C NMR were recorded on Bruker DPX-400 and DPX-600 spectrometers using deuterated solvents obtained from Sigma-Aldrich. Chemical shifts are reported relative to residual nondeuterated solvent. For simplicity only characteristic peaks have been recorded.

IR: All IR data was recorded on a Bruker Vector 22 GI003097 IR machine. For simplicity only characteristic peaks have been recorded.

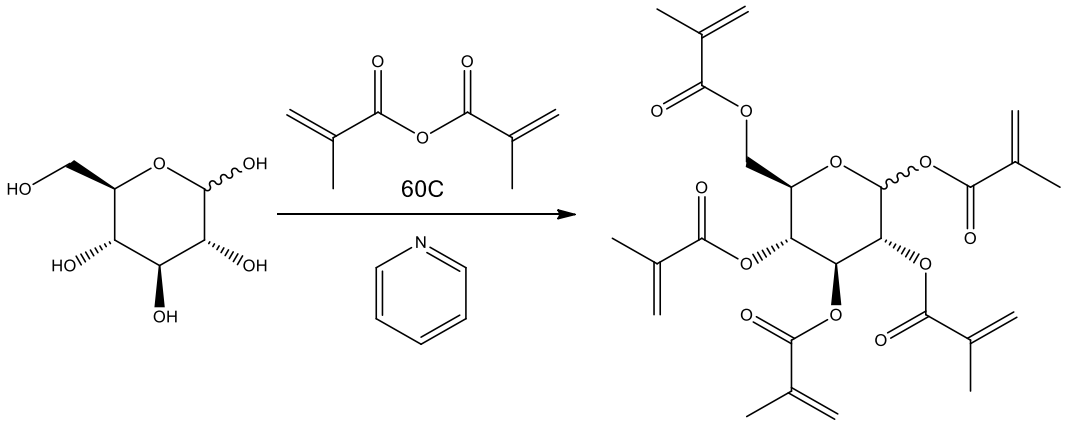
Mass Spec: All Mass Spectra were recorded on an Agilent 6130B ESI-Quad MS using 80% methanol and 20% water as an elution solvent for the LC system. For simplicity only relevant mass spectra peaks have been recorded.

GPC: Gel permeation chromatography (GPC) was used to determine the molecular weights and polydispersities of the synthesized polymers. The DMF GPC system comprised of a Varian 390-LC-Multi detector suite fitted with a differential refractive index (DRI) detector equipped with a guard column (Varian Polymer Laboratories PLGel 5 μm, 50 × 7.5 mm) and two mixed D columns of the same type. The mobile phase was DMF with 5 nM NH_3BF_4 eluent at a flow rate of 1.0 $mL \cdot min^{-1}$, and samples were calibrated against Varian Polymer Laboratories Easi-Vials poly(methylmethacrylate) standards ($162-2.4 \times 10^5$ $g \cdot mol^{-1}$) using Cirrus v3.3.

Assay: The enzyme assay was carried out on 96 well plates (655101) which were purchased from Greiner Bio One (Germany). The plates were read using a BioTek plate reader and Gen5 software.

2.3. Methacrylation of Sugars

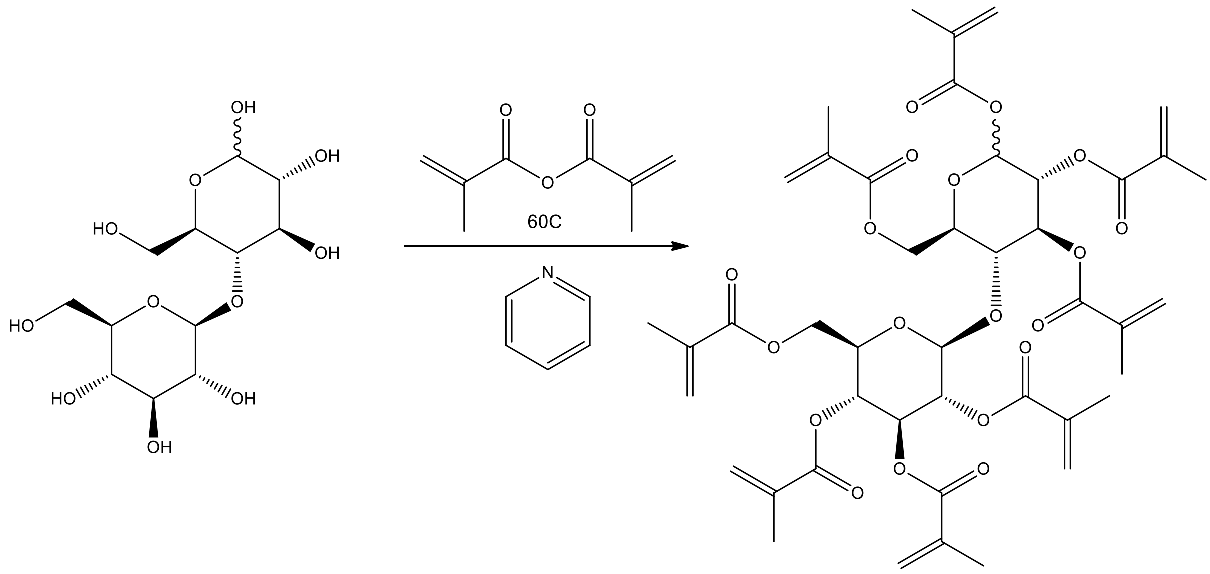
Methacrylation of Glucose



Scheme 2.3.1: Methacrylation of glucose

Glucose (5.0500g, 27.75mmol, 1eq), methacrylic anhydride (21.5252g, 0.14mol, 5.1eq) and pyridine (125ml) were stirred at room temperature overnight before being heated to 60°C for 3 hours. Upon cooling, dichloromethane (50ml) was added and the product washed with 0.1M HCl (40x50ml), water (2x50ml), brine (50ml) and saturated $NaHCO_3$ (2x50ml) before being dried with $MgSO_4$. The solvent was then removed under vacuum at 40°C to yield a clear sticky product which was purified from THF and water (2.9101g, 20% yield). ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm): 6.45 (d, *J*=3.8Hz, 0.5H, β anomer), 6.27-6.03 (m, 5H, C=H_1H_2), 5.87 (d, J=8.0Hz, 0.5H, α anomer), 5.76-5.44 (m, 6H, 5 C=H_1H_2, 1 C$H_{glucose}$), 5.39-5.09 (m, 2H, C$H_{glucose}$), 4.44-4.15 (m, 3H, C$H_{glucose}$, C$H_{2\ glucose}$), 2.01-1.84 (m, 15H, CH_3); ^{13}C NMR ($CDCl_3$, 600MHz, PENDANT) δ (ppm) 166.9-164.9 (*C*=O), 135.7-134.8 (R_2C=CH_2), 128.2-126.4 (R_2C=CH_2), 92.3-92.1 (β anomer), 89.8-89.7 (α anomer), 76.1-68.2 (C$H_{glucose}$), 62.8-62.0 (C$H_{2\ glucose}$), 18.2-18.0 (CH_3); IR υ (cm^{-1}) 1721 (s, C=O ester), 1637 (m, C=C), 1453 (m, CH_2/CH3 bend), 1317 (m, CH_3 bend), 1292 (m, alkyl C-H), 1140 (s, C-O stretch); ESMS (positive mode) m/z 475.1 $[M(4mer)+Na]^+$ $C_{22}H_{28}O_{10}$, m/z 543.2 $[M(5mer)+Na]^+$ $C_{26}H_{32}O_{11}$; GPC (DMF) M_n 432, M_w 442, M_w/M_n 1.02.

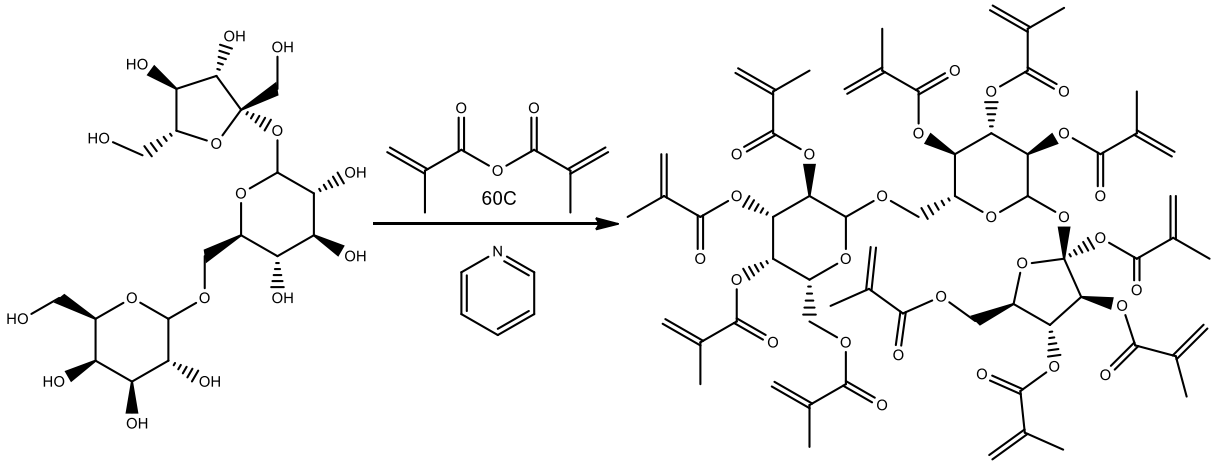
Methacrylation of Cellobiose



Scheme 2.3.2: Methacrylation of Cellobiose

Cellobiose (1.0001g, 2.92mmol, 1eq), methacrylic anhydride (3.60065g, 0.14mol, 5.1eq) and pyridine (25ml) were stirred at room temperature for 48 hours before being heated to 60°C for 24 hours. Upon cooling, dichloromethane (50ml) was added and the product washed with 0.1M HCl (20x50ml), water (2x50ml), brine (50ml) and saturated $NaHCO_3$ (2x50ml) before being dried with $MgSO_4$. The solvent was then removed under vacuum at 40°C to yield a clear sticky product which was purified from THF and water (0.8765g, 34% yield). ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 6.23-6.04 (m, 8H C=C$H_1$$H_2$), 5.75-5.54 (m, 9H, C=$CH_1$$H_2$, C$H_{cellobiose}$); 5.25-4.96 (m, 3H, C$H_{cellobiose}$), 4.70-3.85 (m, 10H, C$H_{cellobiose}$, C$H_{2\ cellobiose}$), 2.01-1.81 (m, 24H, C$H_3$); ^{13}C NMR ($CDCl_3$, 600MHz, PENDANT) δ (ppm) 167.2-165.1 (*C*=O), 135.8-134.7 (R_2*C*=CH_2), 127.9-126.2 (R_2C=*C*H_2), 101.8 (*C*$H_{cellobiose}$), 92.2 (anomer), 82.2-81.8 (anomer), 75.2-64.9 (*C*$H_{cellobiose}$), 63.2-62.1 (*C*$H_{2\ cellobiose}$), 18.4-18.0 (*C*H_3); IR υ (cm^{-1}) 1721 (s, C=O ester), 1637 (m, C=C), 1453 (m, CH_2/CH3 bend), 1317 (m, CH_3 bend), 1293 (m, alkyl C-H), 1140 (s, C-O stretch); ESMS (positive mode) m/z 637.2 $[M(4mer)+Na]^+$ $C_{28}H_{38}O_{15}$, m/z 705.2 $[M(5mer)+Na]^+$ $C_{32}H_{42}O_{16}$, m/z 773.2 $[M(6mer)+Na]^+$ $C_{36}H_{46}O_{17}$, m/z 841.3 $[M(7mer)+Na]^+$ $C_{40}H_{50}O_{18}$; GPC (DMF) M_n 1173, M_w 1292, M_w/M_n 1.10.

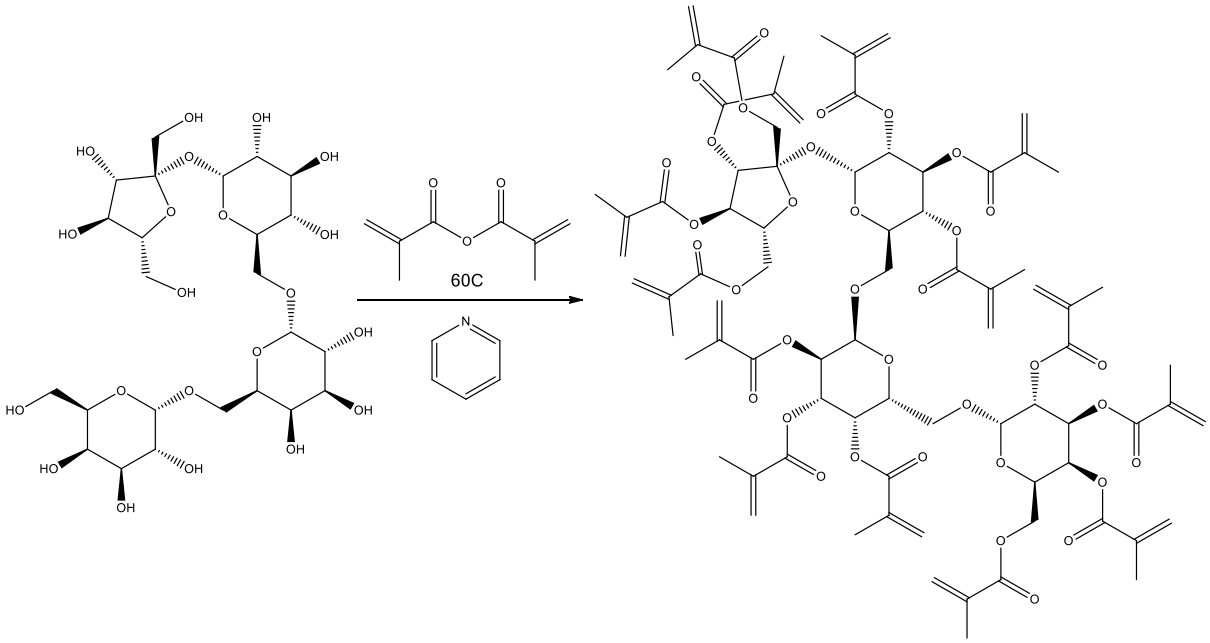
Methacrylation of Raffinose



Scheme 2.3.3: Methacrylation of Raffinose

Raffinose pentahydrate (1.0048g, 1.68mmol, 1eq), methacrylic anhydride (3.1020g, 0.14mol, 5.1eq) and pyridine (25ml) were stirred at room temperature for 48 hours before being heated to 60°C for 30 hours. Upon cooling, dichloromethane (50ml) was added and the product washed with 0.1M HCl (20x50ml), water (2x50ml), brine (50ml) and saturated $NaHCO_3$ (2x50ml) before being dried with $MgSO_4$. The solvent was then removed under vacuum at 40°C to yield a clear sticky product which was then purified from THF and water (1.1838g, 56% yield). ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 6.28-6.09 (m, 11H, C=CH_1H_2), 5.74-5.53 (m, 13H, 11 C=CH_1H_2, 2 C$H_{raffinose}$), 5.50-4.82 (m, 3H, C$H_{raffinose}$), 4.58-3.78 (m, 16H, C$H_{raffinose}$, C$H_{2\ raffinose}$), 2.02-1.88 (m, 33H, CH_3); ^{13}C NMR ($CDCl_3$, 700MHz, PENDANT) δ (ppm) 167.6-165.7 (*C*=O), 136.0-134.9 (R_2C=CH_2), 128.5-126.1 (R_2C=CH_2), 99.5-98.9, 97.0-96.5, 93.3-92.7, 90.6-89.5 (*C*H$_{raffinose}$), 80.1-67.1 (*C*H$_{raffinose}$), 66.3-62.9 (*C*H$_{2\ raffinose}$), 18.4-18.0 (*C*H$_3$); IR υ (cm^{-1}) 1717 (s, C=O ester), 1636 (m, C=C), 1453 (m, CH_2/CH3 bend), 1319 (m, CH_3 bend), 1295 (m, alkyl C-H), 1147 (s, C-O stretch); GPC (DMF) M_n 1634, M_w 1668, M_w/M_n 1.02.

Methacrylation of Stachyose

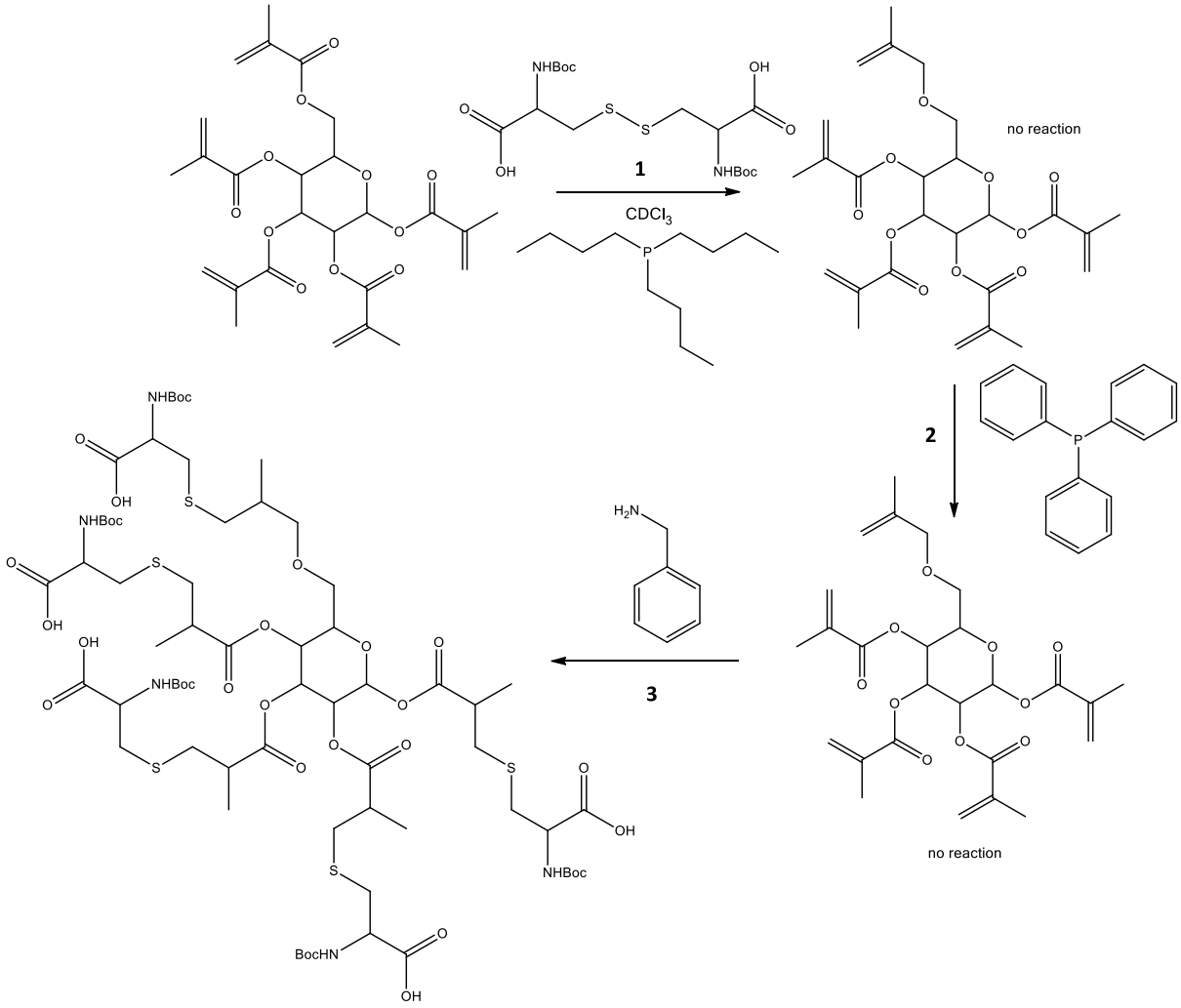


Scheme 2.3.4: Methacrylation of Stachyose

Stachyose (0.5092g, 0.76mmol, 1eq), methacrylic anhydride (1.7304g, 11.22mmol, 14.8eq) and pyridine (18ml) were stirred at room temperature for 5 hours before being heated to 60°C for 45 hours. Upon cooling, dichloromethane (50ml) was added and the product washed with 0.1M HCl (20x50ml), water (2x50ml), brine (50ml) and saturated $NaHCO_3$ (2x50ml) before being dried with $MgSO_4$. The solvent was then removed under vacuum at 40°C to yield orange crystals which were then purified from THF and water (0.1556g, 13% yield). ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 6.28-6.05 (m, 14H, C=CH_1H_2), 5.73-5.53 (m, 18H, 14 C=CH_1H_2, 4 C$H_{stachyose}$), 5.24-5.01 (m, 3H, C$H_{stachyose}$), 4.51-4.08 (m, 21H, C$H_{stachyose}$, C$H_{2\ stachyose}$), 2.01-1.81 (m, 42H, CH_3); ^{13}C NMR ($CDCl_3$, 600MHz, PENDANT) δ (ppm) 167.3-166.2 (*C*=O), 135.7-135.0 (R_2C=CH_2), 127.3-126.3 (R_2C=CH_2), 96.4-96.2, 90.6, 88.9, 88.3, 87.9, 86.9, 85.9, 82.4 (*C*H$_{stachyose}$), 73.8-68.1 (*C*H$_{stachyose}$), 68.0-64.9 (*C*H$_{2\ stachyose}$), 18.5-18.0 (*C*H$_3$); IR υ (cm^{-1}) 1715 (s, C=O ester), 1636 (m, C=C), 1454 (m, CH_2/CH3 bend), 1318 (m, CH_3 bend), 1295 (m, alkyl C-H), 1146 (s, C-O stretch); GPC (DMF) M_n 2312, M_w 2467, M_w/M_n 1.07.

2.4. Michael Addition of Boc-Cysteine

Michael Addition in NMR Tube



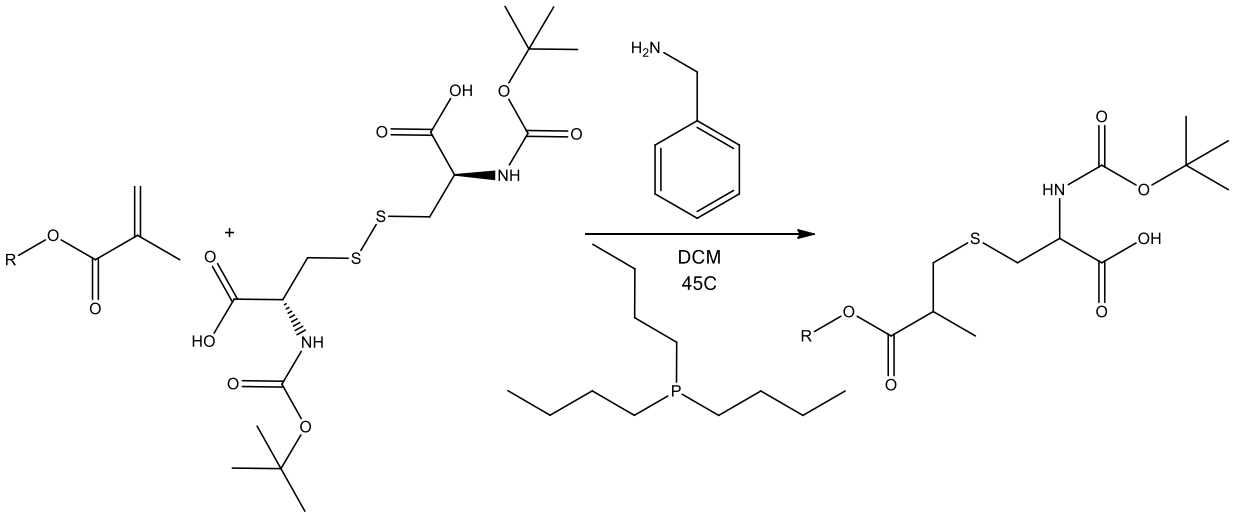
Scheme 2.4.1: Michael Addition of boc-cysteine to methacrylated glucose showing the different reagents used and whether or not the reaction was successful.

Step 1: Methacrylated glucose (3.22mg, 6.19μmol, 1eq), $(\text{boc-cysteine})_2$ (4.37mg, 9.92μmol, 1.6eq), tributyl phosphine (drop) and deuterated chloroform were placed in an NMR tube and the reaction monitored by NMR.

Step 2: Triphenyl phosphine (catalytic amount) was added to the NMR tube and the reaction again monitored by NMR.

Step 3: Benzyl amine (drop) was added to the NMR tube and the reaction was again monitored by NMR.

Michael Addition of Boc-Cysteine to Methacrylated Glucose



Scheme 2.4.2: General reaction scheme for the Michael addition of boc-cysteine to methacrylated sugars, where R is the rest of the sugar molecule.

Methacrylated glucose (0.0521g, 96µmol, 1eq), boc-cysteine (0.1143g, 250µmol, 2.6eq), tributyl phosphine (drop, catalytic) and benzylamine (drop, catalytic) were dissolved in DCM (3ml) and stirred at 45°C for 72 hours. DCM (10ml) was then added and the product washed with water (2x20ml) and brine (20ml) before the solvent was removed under vacuum. The product (Gluco-Cys5) was then purified in THF and water. IR υ (cm^{-1}) 2960 (m, O-H), 2927 (m, C-H stretch), 1720 (s, C=O ester), 1637 (m, C=C), 1454 (m, CH_2/CH3 bend), 1318 (m, CH_3 bend), 1160 (s, C-O stretch), 751 (m, C-S); GPC (DMF) M_n 946, M_w 1190, M_w/M_n 1.26.

The product was then dissolved in DCM, and TFA (4 drops) added to remove the boc protecting group. The product was then dialysed and the water removed by freeze drying to yield a sticky orange solid (0.0072g, 7 %yield)

Michael Addition of Boc-Cysteine to Methacrylated Cellobiose

Methacrylated cellobiose (0.0495g, 56µmol, 1eq), boc-cysteine (0.1022g, 230µmol, 4.1eq), tributyl phosphine (drop, catalytic) and benzylamine (drop, catalytic) were dissolved in DCM (3ml) and stirred at 45°C for 72 hours. DCM (10ml) was then added and the product washed with water (2x20ml) and brine (20ml) before the solvent was removed under vacuum. The product (Cello-Cys8) was then purified in THF and water. GPC (DMF) M_n 2126, M_w 2153, M_w/M_n 1.01.

The product was then dissolved in DCM, and TFA (4 drops) added to remove the boc protecting group. The product was then dialysed and the water removed by freeze drying to yield a sticky orange solid (0.0015g, 1% yield).

Michael Addition of Boc-Cysteine to Methacrylated Raffinose

Methacrylated raffinose (0.0492g, 40μmol, 1eq), boc-cysteine (0.1004g, 226μmol, 5.6eq), tributyl phosphine (drop, catalytic) and benzylamine (drop, catalytic) were dissolved in DCM (3ml) and stirred at 45°C for 80 hours. DCM (10ml) was then added, and the product washed with water (2x20ml) and brine (20ml) before the solvent was removed under vacuum. The product (Raffi-Cys11) was then purified from THF and water. GPC (DMF) M_n 2001, M_w 2028, M_w/M_n 1.01.

The product was then dissolved in DCM, and TFA (4 drops) added to remove the boc protecting group. The product was then dialysed and the water removed by freeze drying to yield a sticky orange solid (0.0053g, 5% yield).

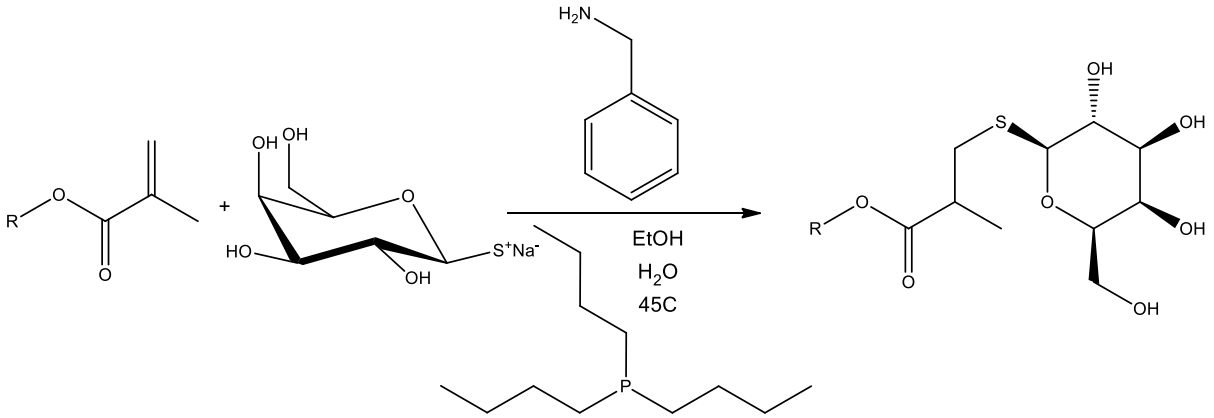
Michael Addition of Boc-Cysteine to Methacrylated Stachyose

Methacrylated stachyose (0.0511g, 31μmol, 1eq), boc-cysteine (0.0996g, 219μmol, 7.1eq), tributyl phosphine (drop, catalytic) and benzylamine (drop, catalytic) were dissolved in DCM (3ml) and stirred at 45°C for 80 hours. DCM (10ml) was then added, and the product washed with water (2x20ml) and brine (20ml) before the solvent was removed under vacuum. The product (Stach-Cys14) was then purified from THF and water. GPC (DMF) M_n 2467, M_w 2575, M_w/M_n 1.04.

The product was then dissolved in DCM, and TFA (4 drops) added to remove the boc protecting group. The product was then dialysed and the water removed by freeze drying to yield a sticky orange solid (0.0021g, 2% yield).

2.5. Michael Addition of Thiogalactose

Michael Addition of Thiogalactose to Methacrylated Glucose



Scheme 2.5.1: General reaction scheme for the Michael addition of thiogalactose to methacrylated sugars where R is the remainder of the sugar molecule.

Methacrylated glucose (14.1mg, 26.9μmol, 5.1eq), β-D-thiogalactose sodium salt (30.0mg, 137μmol, 1eq), benzylamine (drop, catalytic), tributyl phosphine (drop, catalytic) were dissolved in a mixture of ethanol and water (1:1, 5ml) before being stirred at 45°C for 72 hours. The ethanol was then removed under vacuum, and the remaining solution dialysed before the water was removed by freeze drying to yield a yellow sticky solid (Gluco-Gal5, 0.0125g, 31% yield). GPC (DMF) M_n 1639, M_w 1691, M_w/M_n 1.03.

Michael Addition of Thiogalactose to Methacrylated Cellobiose

Methacrylated cellobiose (0.0301g, 33.8μmol, 1eq), β-D-thiogalactose sodium salt (0.0599g, 274μmol, 8.1eq), benzylamine (drop, catalytic), tributyl phosphine (drop, catalytic) were dissolved in a mixture of ethanol and water (1:1, 5ml) before being stirred at 45°C for 72 hours. The ethanol was then removed under vacuum, and the remaining solution dialysed before the water was removed by freeze drying to yield a yellow sticky solid (Cello-Gal8, 0.0017 mg, 2 % yield). GPC (DMF) M_n 1938, M_w 1952, M_w/Mn 1.01.

Michael Addition of Thiogalactose to Methacrylated Raffinose

Methacrylated raffinose (0.0317g, 24.7μmol, 1eq), β-D-thiogalactose sodium salt (0.0600g, 274μmol, 11.1eq), benzylamine (drop, catalytic), tributyl phosphine (drop, catalytic) were dissolved in a mixture of ethanol and water (1:1, 5ml) before being stirred at 45°C for 80 hours. The ethanol was then removed under vacuum, and the remaining solution

dialysed before the water was removed by freeze drying to yield a yellow sticky solid (Raffi-Gal11, 0.0108g, 14% yield). GPC (DMF) M_n 3424, M_w 3446, M_w/M_n 1.01.

Michael Addition of Thiogalactose to Methacrylated Stachyose

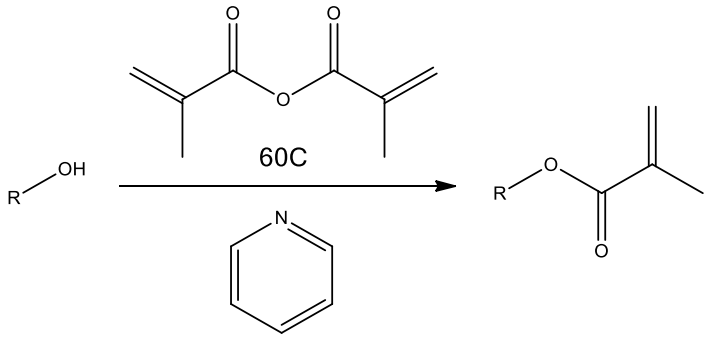
Methacrylated stachyose (0.0316g, 19.1μmol, 1eq), β-D-thiogalactose sodium salt (0.0600g, 274μmol, 14.1eq), benzylamine (drop, catalytic), tributyl phosphine (drop, catalytic) were dissolved in a mixture of ethanol and water (1:1, 5ml) before being stirred at 45°C for 80 hours. The ethanol was then removed under vacuum, and the remaining solution dialysed before the water was removed by freeze drying to yield a yellow sticky solid (Stach-Gal14, 0.1083g, 130% yield, probably due to not all water being removed during freeze drying). GPC (DMF) M_n 3475, M_w 3521, M_w/M_n 1.01.

2.6. Galactosyl Transferase Assay

Tris(hydroxymethyl)methylamine (0.1211g, 1mM) was made up to pH 7 with glacial acetic acid before NaCl (0.0584g, 0.1mM) and 2-mercaptoethanol (0.7813g, 1mM) were added and the solution made up to 100ml with distilled water. Solutions of β-galactosidase (*E.coli*), ONPG, and the thiogalactose functionalised sugars were made up in the buffer solution. Concentrations used were as follows: β-galactosidase 1mg/ml, ONPG 3.3-16.6mM, Gluco-Gal5 0.85mM-3.3mM, Cello-Gal8 86.6μM-346.2μM, Raffi-Gal11 0.42mM-1.69mM, Stach-Gal14 0.6mM-2.5mM. The assay was then carried out in a 96 well plate. The absorbance at 405nm was measured every 5 minutes for 2 hours. Standard curves were prepared with 3.3-16.6mM of ONPG as well with the thiogalactose functionalised sugars. All reactions were carried out at room temperature.

3. Results and Discussion

3.1. Methacrylation of Sugars



Scheme 3.1.1: General reaction scheme for methacrylation of sugars, where R is the remainder of the sugar molecule.

The first step of the project was to methacrylate a range of different sized sugars using the method shown (scheme 3.1.1). The first method attempted for the methacrylation step was that used by Mukhopadhyay *et al.*[73] which uses iodine as a catalyst. However, examination of ^{1}H NMRs of the product showed a lack of vinyl peaks suggesting that the reaction was unsuccessful, or at least didn't yield the expected product, possibly due to addition of the iodine across the double bond. Therefore the method used by Treadway and Yanovsky[71] (scheme 3.1.1) was used for all further methacrylations. This method is a simple one pot synthesis, with a straightforward work up involving DCM, which is easy to remove afterwards to give the product. The method is also very straightforward to scale up, making it possible to synthesise a library of different sized methacrylated sugars.

From GPC of the methacrylated mono-, di-, tri- and tetra- saccharides there was a clear discrete distribution of molecular weight between the four sugars (figure 3.1.1). As the size of the sugar increased so did the molecular weight, causing a decrease in retention time. Any discrepancies between the expected molecular weight and the molecular weight found by GPC are due to the differences in hydrodynamic volume between the functionalised sugar 'star-like' molecules and the linear polymer calibration standards. All methacrylated sugars had low polydispersities; significantly lower than those which would be expected using a controlled radical technique.

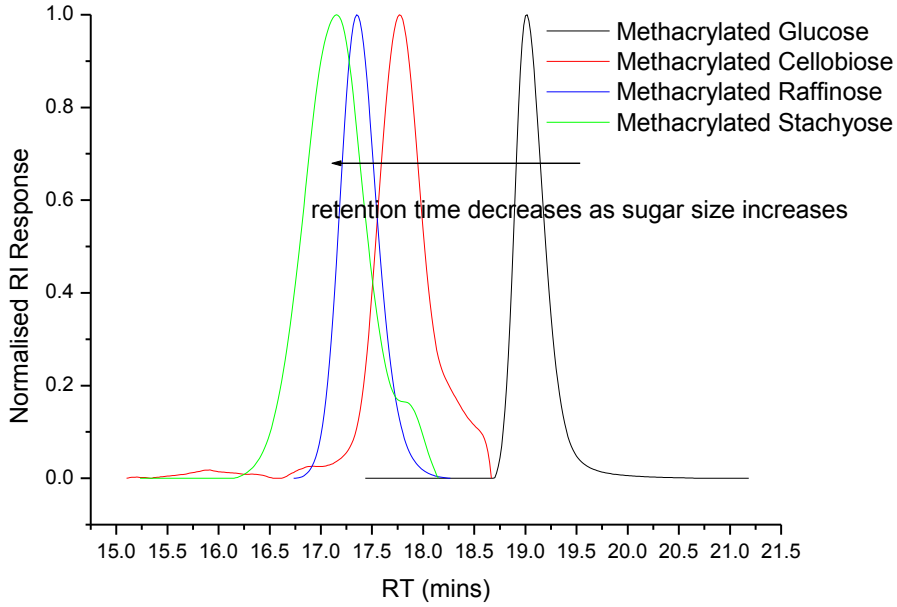


Figure 3.1.1: GPC traces showing evolution of molecular weight as size of sugars increases.

Methacrylation of Glucose

From the COSY and HMQC NMRs of the product it was clear that the 2 vinyl protons were not equivalent, there were two separate regions: between 6.27-6.03ppm and 5.76-5.44ppm which both showed coupling with the CH_3 region at 2.01-1.84ppm (figure 3.1.2) and the vinyl carbons at ~126ppm (figure 3.1.3).

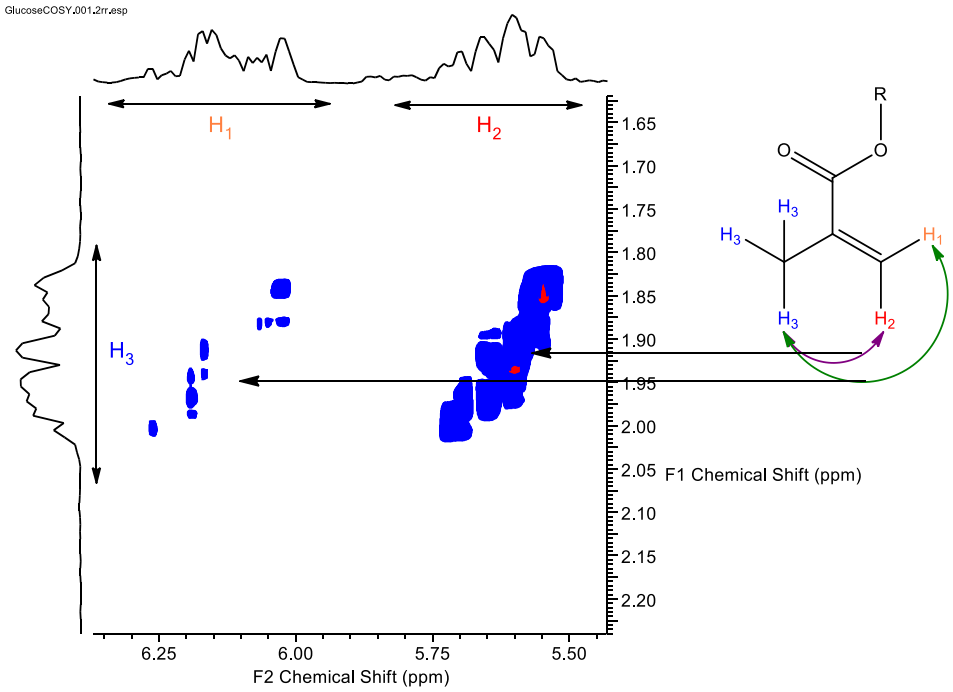


Figure 3.1.2: COSY demonstrating the coupling between the CH_3 region at 2.01-1.84ppm and the vinyl regions at 6.27-6.03ppm and 5.76-5.44ppm showing that the both vinyl protons are not equivalent. R represents the rest of the methacrylated glucose molecule.

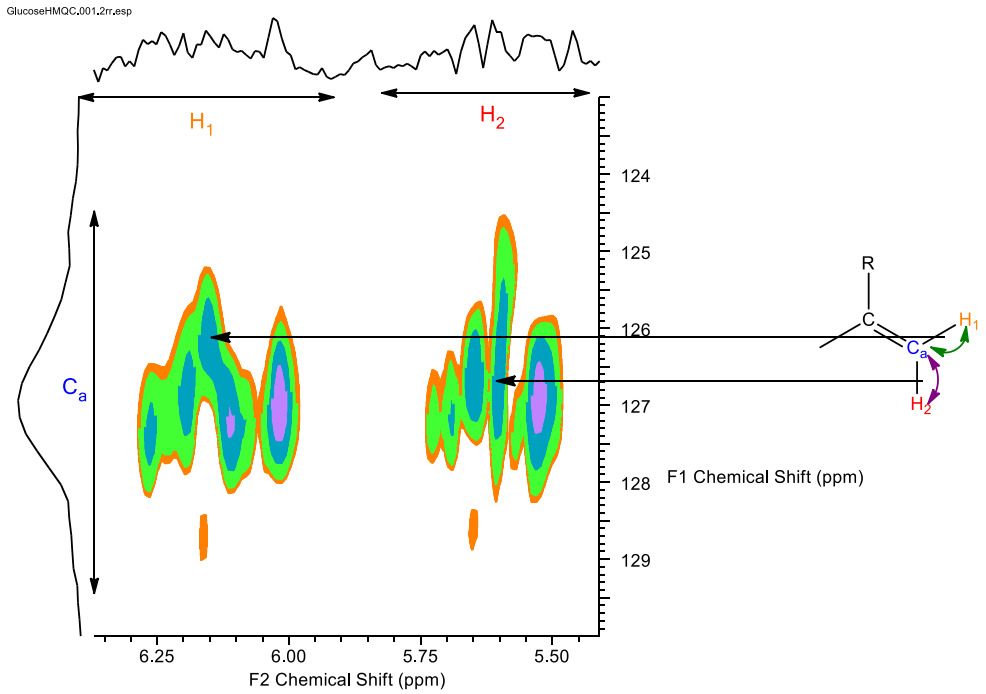


Figure 3.1.3: HMQC showing coupling between carbons at ~126ppm and the vinyl proton regions at 6.27-6.03ppm and 5.76-5.44ppm again showing that the both vinyl protons are not equivalent. R represents the rest of the methacrylated glucose molecule.

A plot of the IR of glucose (figure 3.1.4) compared with the product shows a significant reduction in the size of the peak for O-H, as well as an increase in the size of the peak corresponding to C=O, as would be expected if the reaction was successful.

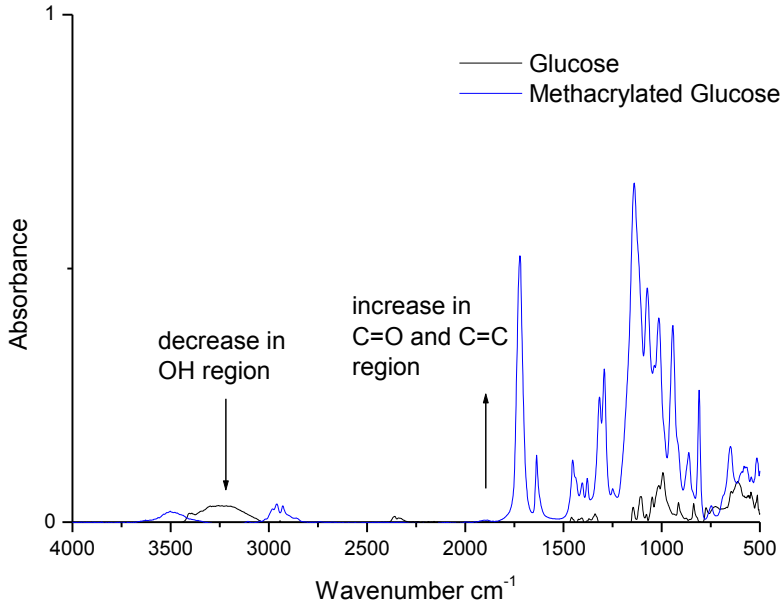


Figure 3.1.4: IR showing the reduction in size of the OH region of glucose as methacrylation occurs.

Methacrylation of Cellobiose

From the COSY and HMQC NMRs of the product it was clear that the 2 vinyl protons were not equivalent, there were two separate regions: between 6.25-6.00ppm and 5.73-5.53ppm which both showed coupling with the CH_3 region at 2.01-1.81ppm (figure 3.1.5) and the vinyl carbons at ~126ppm (figure 3.1.6).

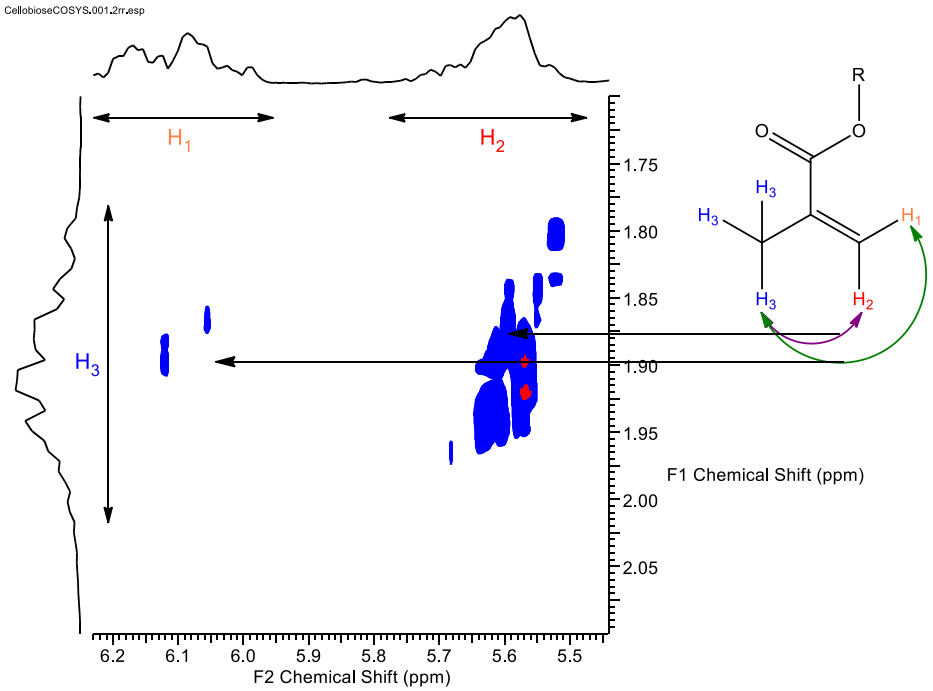


Figure 3.1.5: COSY showing coupling between CH_3 region at 2.01-1.81ppm and the vinyl regions at 6.25-6.00ppm and 5.73-5.53ppm showing that both vinyl protons are not equivalent. R represents the rest of the methacrylated cellobiose molecule.

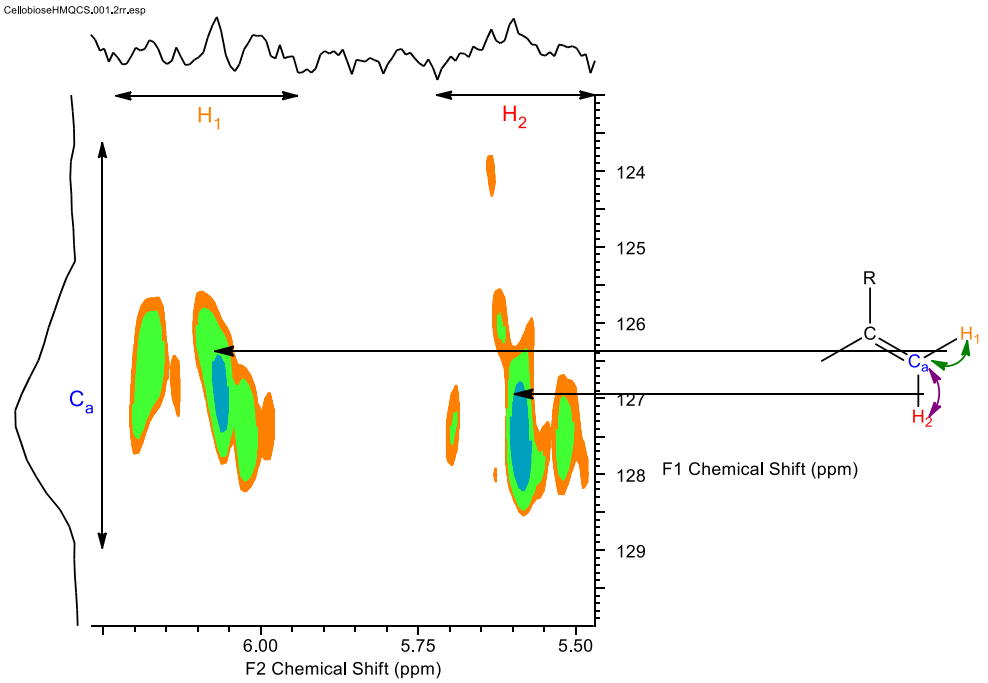


Figure 3.1.6: HMQC showing coupling between vinyl carbons at ~126ppm and vinyl protons at 6.25-6.00ppm and 5.73-5.53ppm, again showing that the vinyl protons are not equivalent. R represents the rest of the methacrylated glucose molecule.

And again, by comparing the IR of cellobiose and methacrylated cellobiose it was clear that the peak corresponding to OH had reduced (figure 3.1.7). Integration of the peaks before and after methacrylation also showed that the OH region had decreased by approximately the same amount that the C=O region had increased by, showing that the OH groups were being replaced by groups containing a C=O.

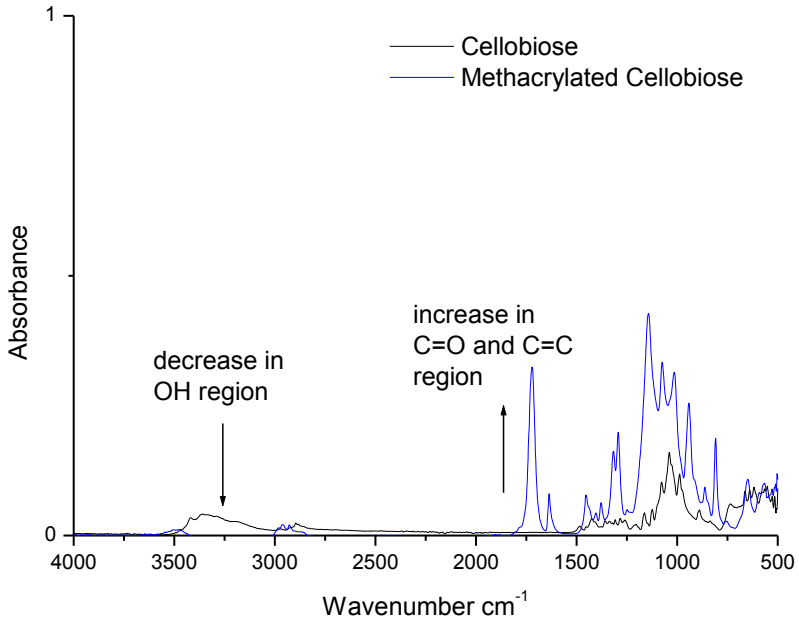


Figure 3.1.7: IR showing differences in OH region between cellobiose and methacrylated cellobiose.

Methacrylation of Raffinose

From comparing IRs of raffinose and methacrylated raffinose there is a reduction in the size of the region corresponding to OH (figure 3.1.8). Integration of the peaks before and after methacrylation also showed that the OH region had decreased by approximately the same amount that the C=O region had increased by, showing that the OH groups were being replaced by groups containing a C=O.

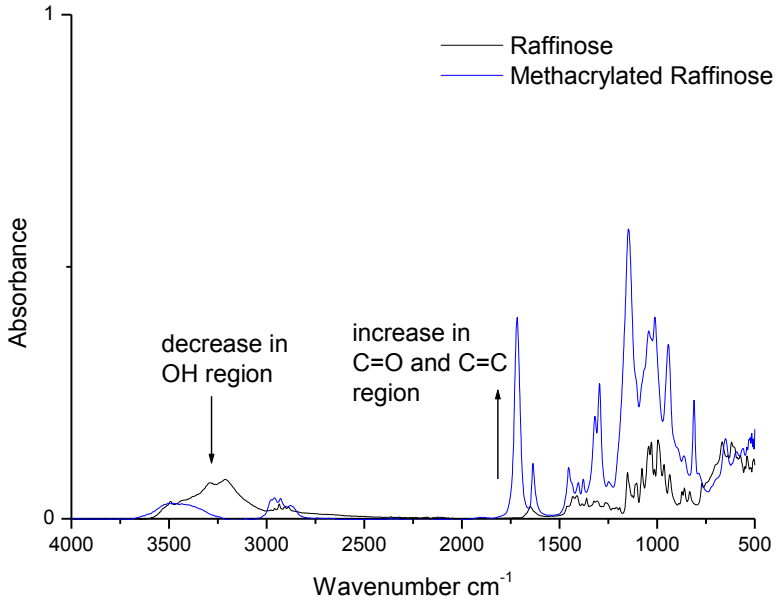


Figure 3.1.8: IR showing differences between raffinose and methacrylated raffinose.

Methacrylation of Stachyose

From comparing IRs of stachyose and methacrylated stachyose there is a reduction in the size of the region corresponding to OH (3.1.9). Integration of the peaks before and after methacrylation also showed that the OH region had decreased by approximately the same amount that the C=O region had increased by, showing that the OH groups were being replaced by groups containing a C=O.

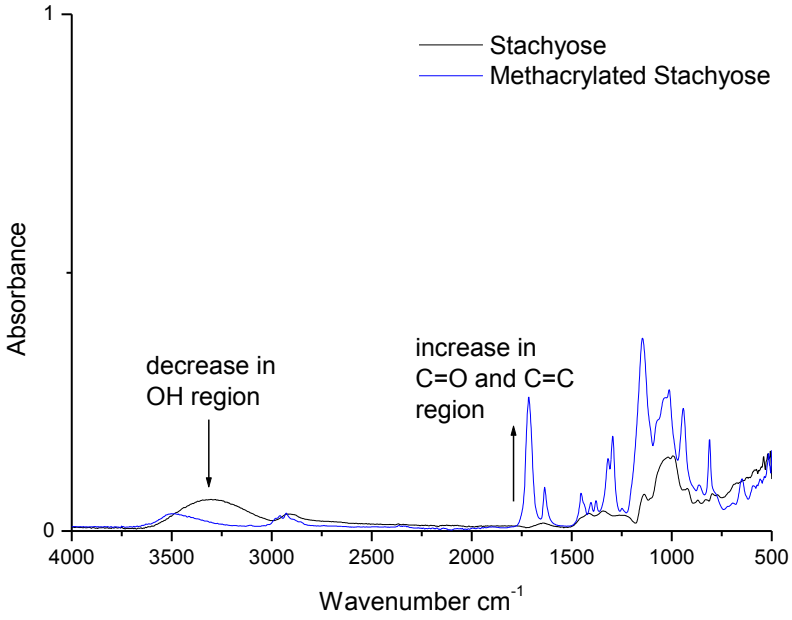
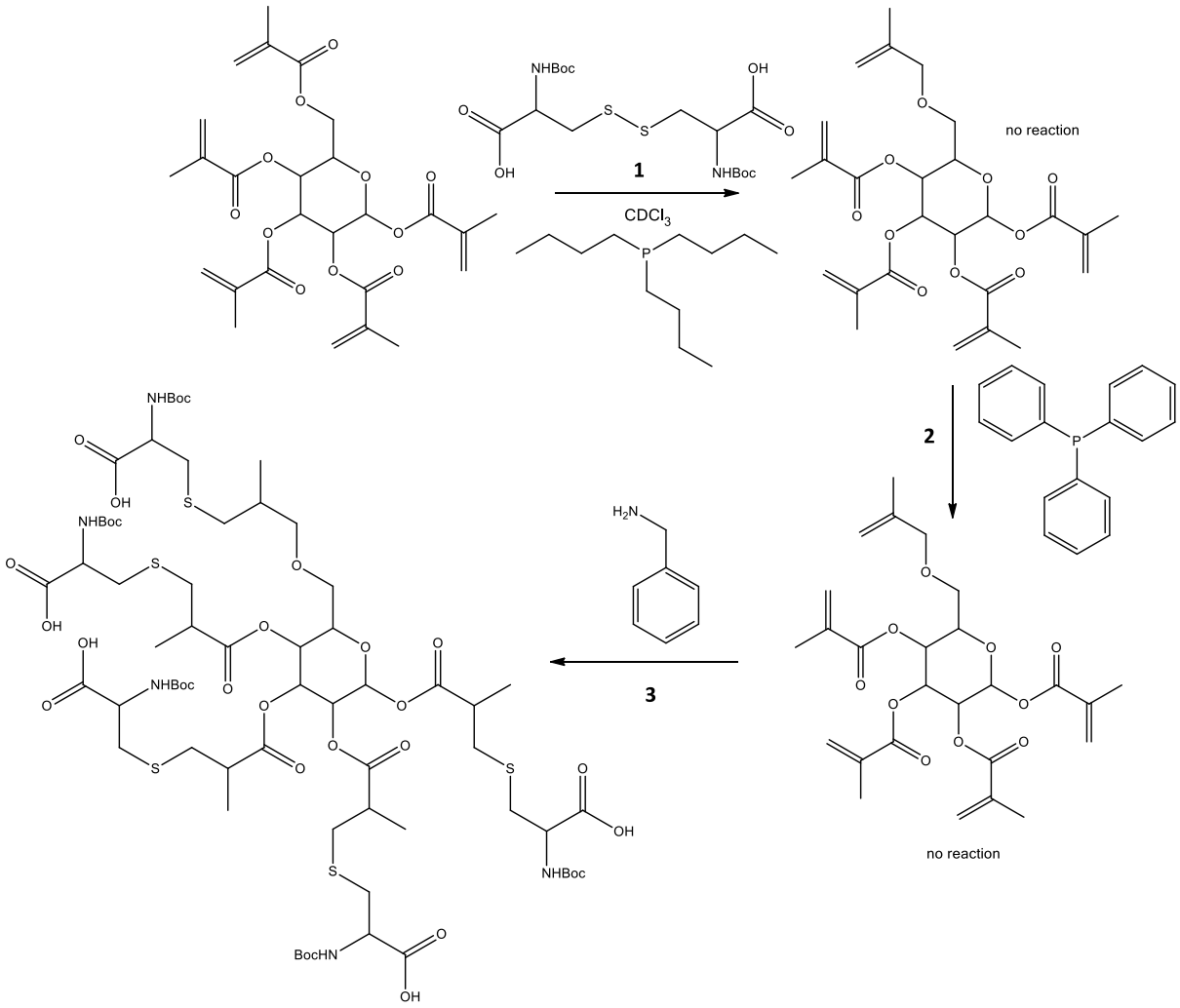


Figure 3.1.9: IR showing differences between stachyose and methacrylated stachyose.

3.2. Michael Addition of Boc-Cysteine to Methacrylated Sugars

Michael Addition in NMR Tube

The main aim of this project was to functionalise the methacrylated sugars scaffolds with thiogalactose for testing as an inhibitor of galactosyl transferase. However, due to the high price of thiogalactose the Michael addition step was first optimised with cysteine, which is significantly cheaper. The differences in solubility between cysteine and the methacrylated sugars made optimisation difficult so a protected cysteine (boc-cysteine) was used instead as it is soluble in DCM, as were the methacrylated sugars. A number of methods for the Michael addition step were tested, including borax[87] and tertiary phosphines[82], although none of them seemed to work. It was unclear if this was due to the reaction being unsuccessful, or down to product being lost during the work up stages. Therefore the reaction was carried out in an NMR tube, where changes in the size of the vinyl peaks could be monitored, and different reagents could be added to see their effect on the reaction (scheme 3.2.1). From this tributyl phosphine and benzylamine were chosen as reagents for the rest of the Michael addition reactions.



Scheme 3.2.1: Reaction scheme for optimisation of Michael addition reaction in NMR tube.

During all steps of the reaction the reaction was monitored by ^{1}H NMR and the integral of the vinyl peaks compared to the integral of the peak at 1.01-0.85ppm which represents the CH_3 groups present in boc-cysteine, of which there are 3, making this peak equal to 9 protons in total. After monitoring during step 1 (20 hours in total) and step 2 (2 hours in total) there was no difference in the relative size of the vinyl peaks.

After the addition of benzylamine (step 3) the reaction was then monitored for 144 hours and it was found that the relative size of the vinyl peaks decreased (figure 3.2.1), as would be expected if the reaction was successful. The region corresponding to the alkyl peaks increased, as would be expected as the reaction causes the vinyl carbons to become alkyl carbons (figure 3.2.2).

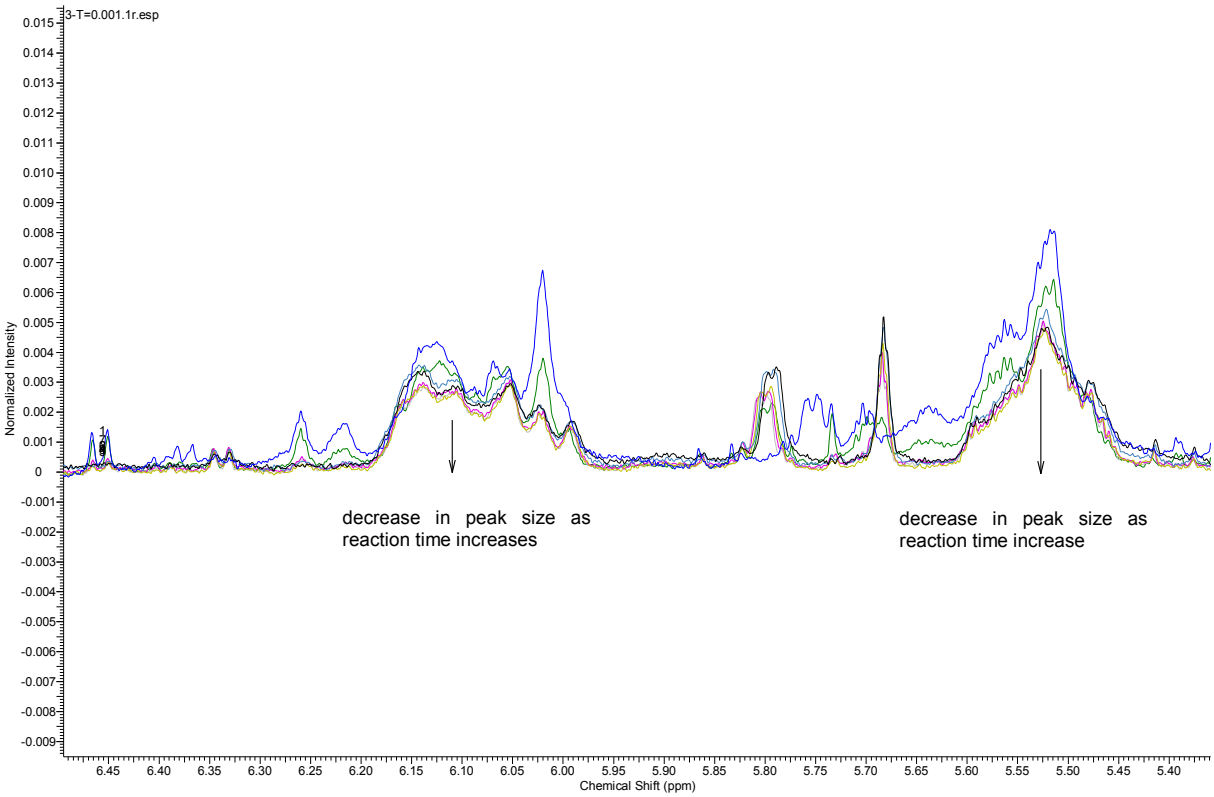


Figure 3.2.1: ^{1}H NMR showing the reduction in size of the vinyl peaks as the reaction time increased, step 3, after the addition of benzylamine.

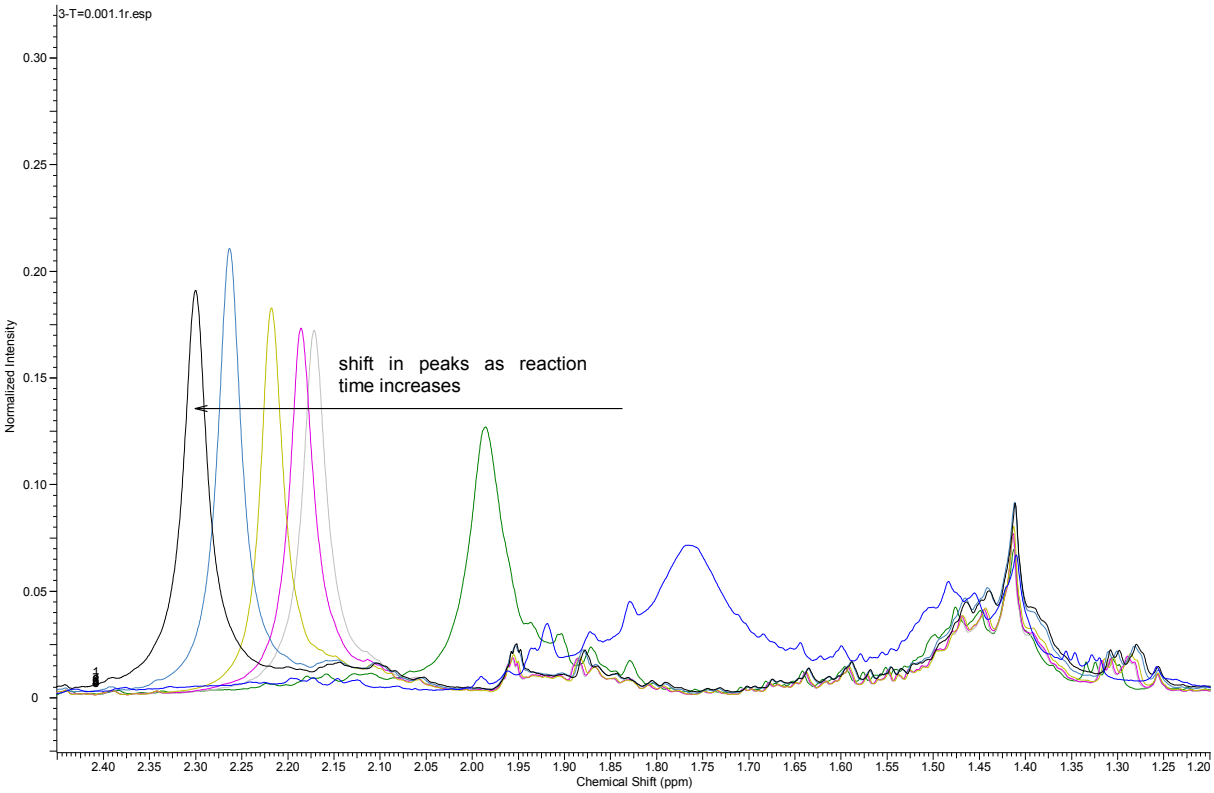
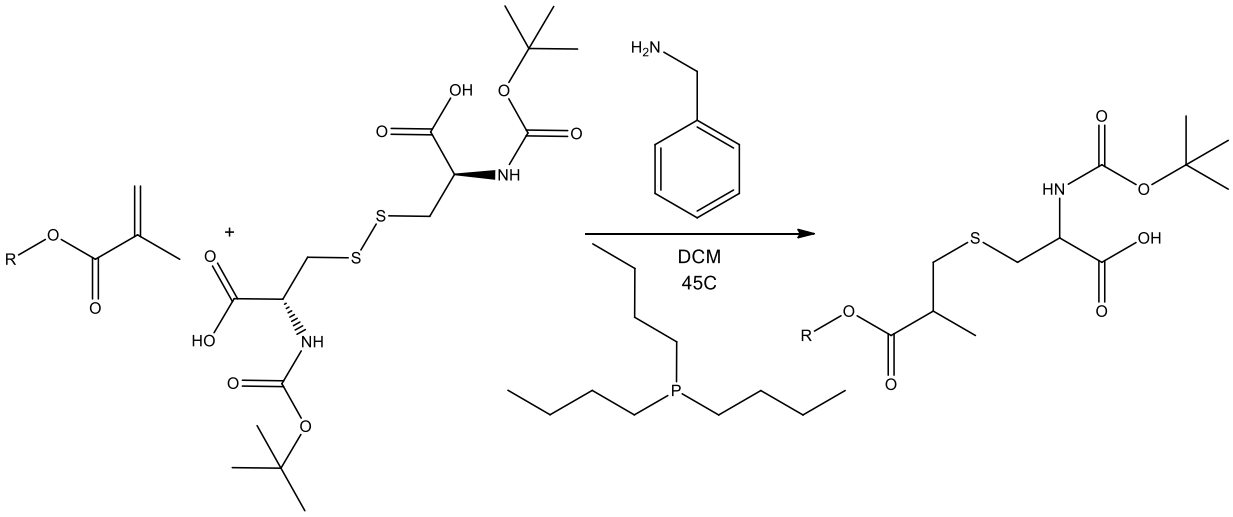


Figure 3.2.2: ^{1}H NMR showing evolution of the aliphatic peaks as reaction time increased, step 3, after addition of benzylamine.

Michael Addition of Boc-Cysteine to Methacrylated Glucose



Scheme 3.2.2: Reaction scheme for Michael addition of boc-cysteine to methacrylated sugars showing optimised procedure. R is the rest of the sugar molecule.

Comparing the GPC traces for methacrylated glucose and methacrylated glucose after the Michael addition of boc-cysteine shows a longer retention time for methacrylated glucose, meaning a smaller molecular weight (figure 3.2.3). This suggests that something has been added to the molecule in the Michael addition step, showing that the reaction was successful. However, the GPC trace for the boc-cysteine functionalised glucose exhibits 'high' polydispersity of 1.26. This is most likely due to the functionalised sugar being at the lower molecular weight end of the calibration curve for the GPC equipment, and therefore everything, whether it is unreacted starting material, or sugars that aren't fully functionalised having a similar retention time.

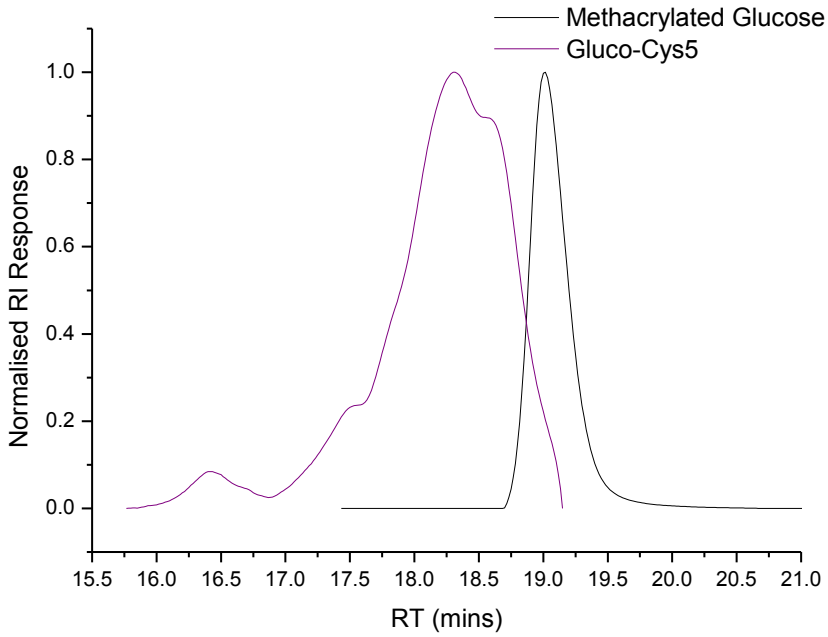


Figure 3.2.3: GPC traces showing differences in retention time (and therefore molecular weight) between methacrylated glucose and the Michael addition product.

IR analysis of methacrylated glucose and the Michael addition product showed a decrease in the size of the peak corresponding to C=C and an increase in the peak corresponding to C-S (figure 3.2.4). However, as these peaks were in similar regions to other peaks it was not possible to compare the integrals for the individual peaks to quantify if they had decreased/increased by the same amount.

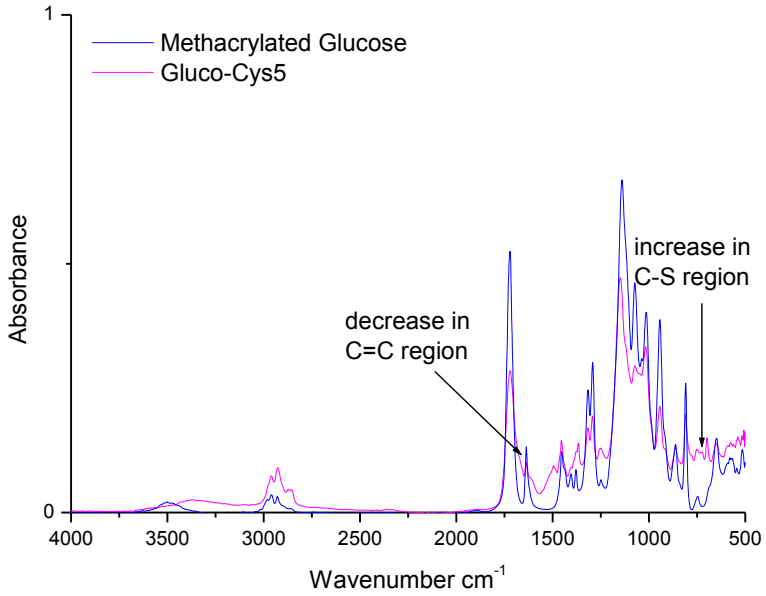


Figure 3.2.4: IR showing differences between methacrylated glucose and the Michael addition product of methacrylated glucose and boc-cysteine.

Michael Addition of Boc-Cysteine to Methacrylated Cellobiose

There is a clear decrease in retention time between the methacrylated cellobiose and the boc-cysteine Michael addition product, meaning an increase in molecular weight, suggesting the reaction has been successful (figure 3.2.5).

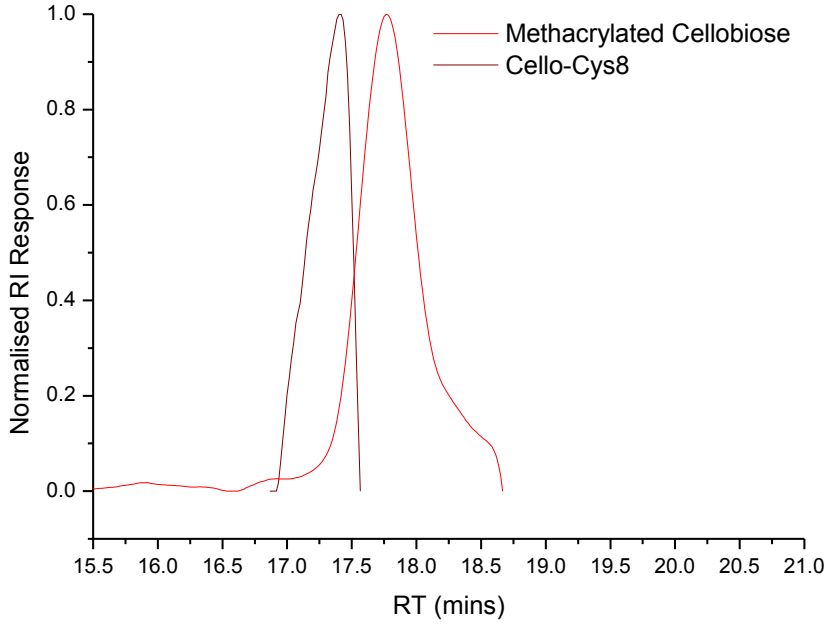


Figure 3.2.5: GPC traces showing retention time before and after Michael addition of boc-cysteine.

Michael Addition of Boc-Cysteine to Methacrylated Raffinose

Whilst it is not apparent from the comparison of the GPC traces (figure 3.2.6) the molecular weight of the sugar increased after the addition of the boc-cysteine group. The actual traces not showing this difference could just be down to the fact that both traces were analysed according to calibration curves run on different days. The differences could also be down to the changes in hydrodynamic volume between the methacrylated sugar scaffold and Raffi-Cys11.

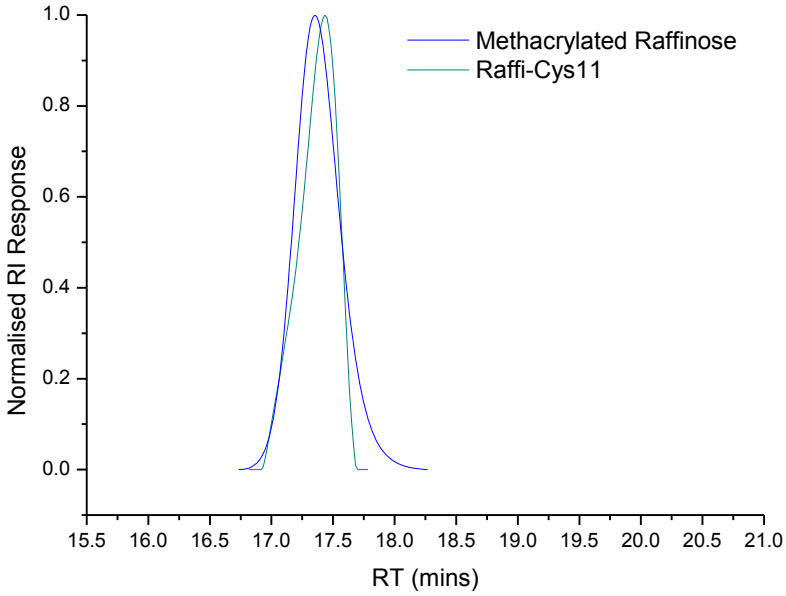


Figure 3.2.6: GPC traces showing retention times before and after Michael addition of boc-cysteine.

Michael Addition of Boc-Cysteine to Methacrylated Stachyose

Again, the difference in molecular weight (figure 3.2.7) between the two compounds (methacrylated stachyose and the boc-cysteine Michael addition product) is not evident by comparing the traces, again likely due to the different calibration standards used.

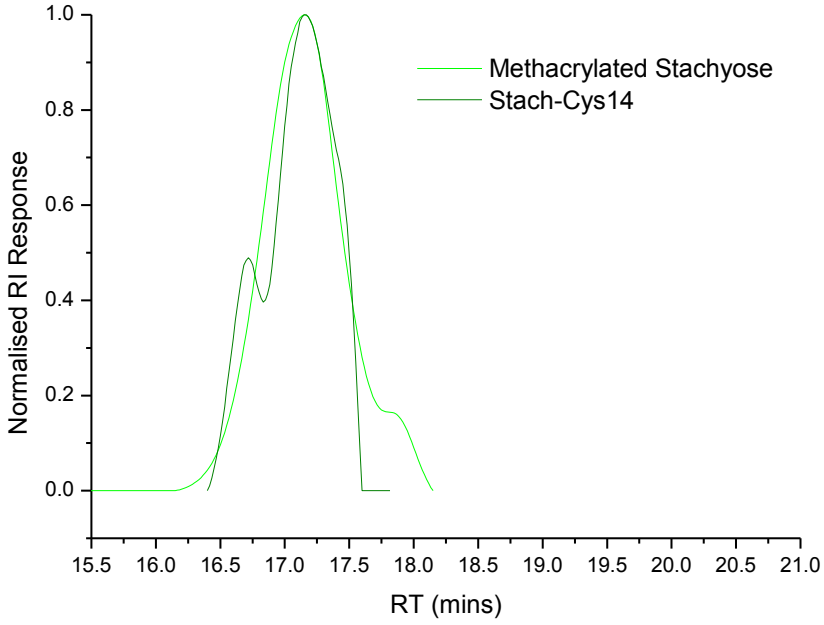
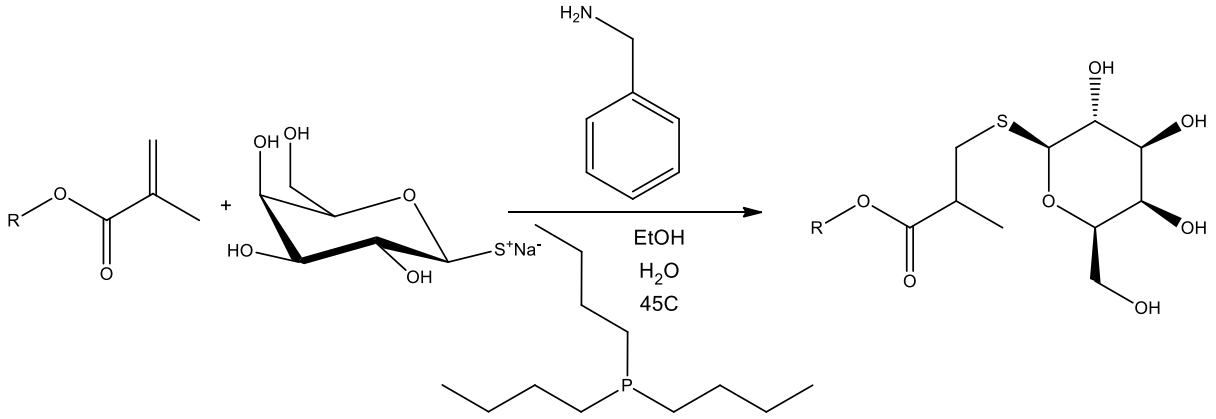


Figure 3.2.7: GPC traces showing retention time before and after Michael addition of boc-cysteine.

3.3. Michael Addition of Thiogalactose to Methacrylated Sugars



Scheme 3.3.1: General reaction scheme for Michael addition of thiogalactose to methacrylated sugars where R is the rest of the sugar molecule.

After optimisation of the Michael addition conditions it was then possible to carry out the Michael addition of thiogalactose to the methacrylated sugars (figure 3.3.1). As thiogalactose is insoluble in DCM the reaction was carried out in a 1:1 mixture of ethanol and water to dissolve both the thiogalactose and the methacrylated sugar. The work up for this reaction was also straightforward due to the product being water soluble. After removal of the ethanol the reaction solution was filtered to remove any undissolved material and then any remaining water soluble reactants were removed by dialysis before the water was removed by freeze drying. The ease of purification makes this method suitable for use on a larger scale, as it requires very little input from the chemist.

From GPCs of all the thiogalactose Michael addition products there was a decrease in retention time compared to the methacrylated sugar starting materials (figures 3.3.1-3.3.4). A decrease in retention time is concomitant with an increase in molecular weight showing that the Michael addition reactions have been successful. All the thiogalactose addition products also exhibited low polydispersities, showing that these products would be suitable for an environment where reproducible properties are required such as biomedical applications.

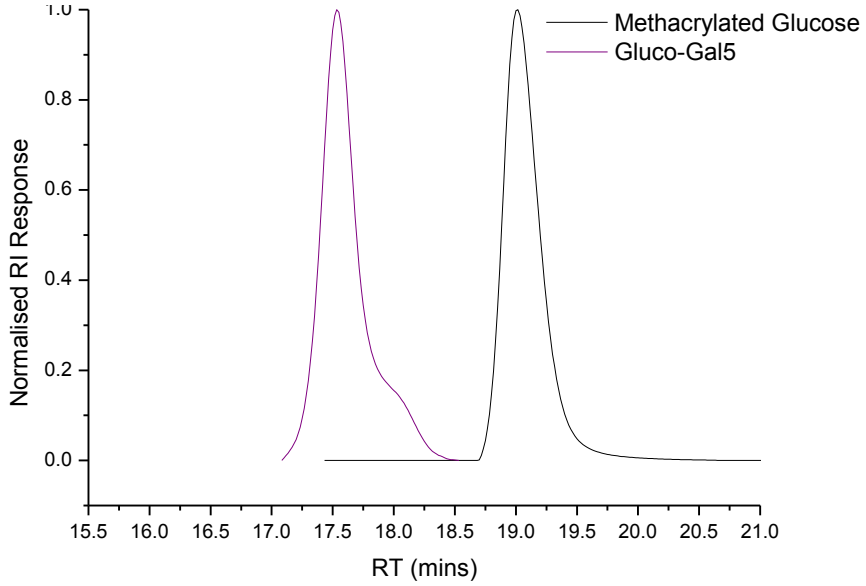


Figure 3.3.1: GPC traces showing retention time of methacrylated glucose before and after Michael addition of thiogalactose.

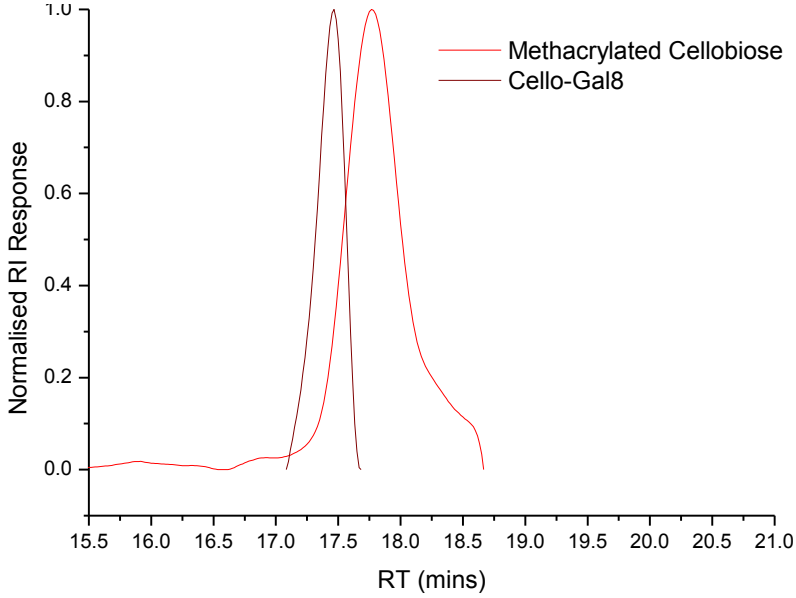


Figure 3.3.2: GPC traces showing retention time of methacrylated cellobiose before and after Michael addition of thiogalactose.

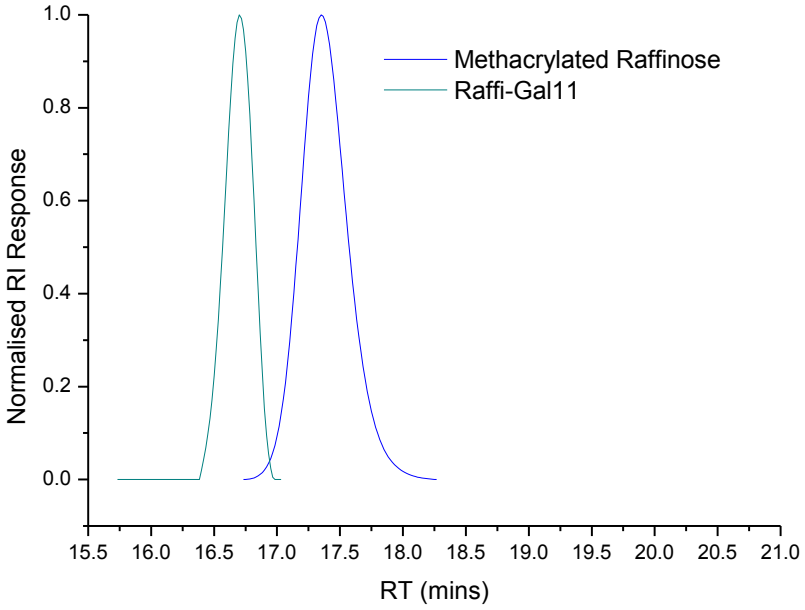


Figure 3.3.3: GPC traces showing retention time of methacrylated raffinose before and after Michael addition of thiogalactose.

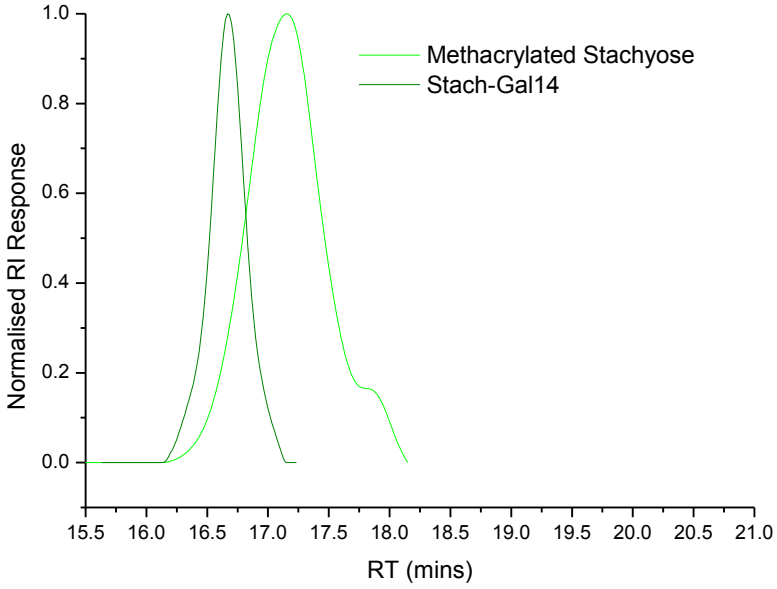
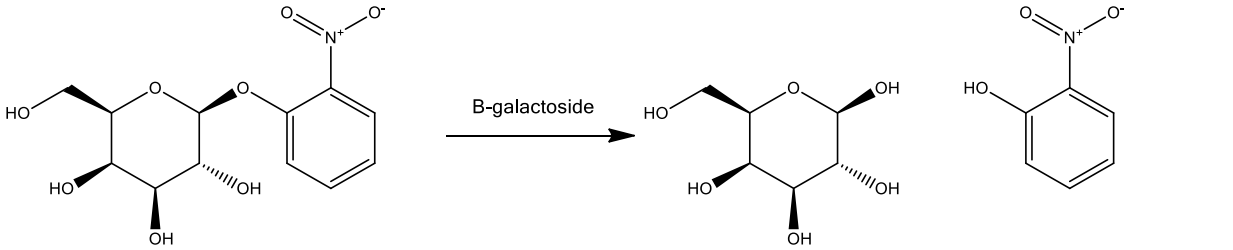


Figure 3.3.4: GPC traces showing retention time of methacrylated stachyose before and after Michael addition of thiogalactose.

3.4. Galactosyl Transferase Assay



Scheme 3.4.1: Galactosyl transferase assay procedure showing colourless starting material and yellow product.

Single substrate enzymes tend to obey Michaelis Menten kinetics whereby a plot of time vs. product concentration (or a property related to product concentration such as absorbance) is initially linear with a gradient of v, or the initial reaction rate (equation 3.4.1).

$$v = \frac{V_{max}[S]}{K_m[S]}$$

Equation 3.4.1: Michaelis Menten equation showing the relationship of initial reaction rate v to substrate concentration [S] where V_{max} is the maximum reaction rate and K_m is the substrate concentration at half V_{max}.[88]

Taking the reciprocal of both sides gives the Lineweaver Burk equation (equation 3.4.2) which can be used to determine whether inhibition is competitive (gradient x V_{max} increases as inhibitor concentration increases) or non competitive (lower V_{max}).[89]

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Equation 3.4.2: Lineweaver Burk equation, reciprocal of Michaelis Menten equation.

The figure below (figure 3.4.1) shows the intercepts/ lines that would be expected on a Lineweaver Burk plot with different types of inhibition: no inhibition, non-competitive inhibition (the inhibitor binding somewhere other than the active site and affecting the shape of the active site so the substrate can't bind), competitive inhibition (the inhibitor binds to the active site, preventing binding of the substrate) and full inhibition.

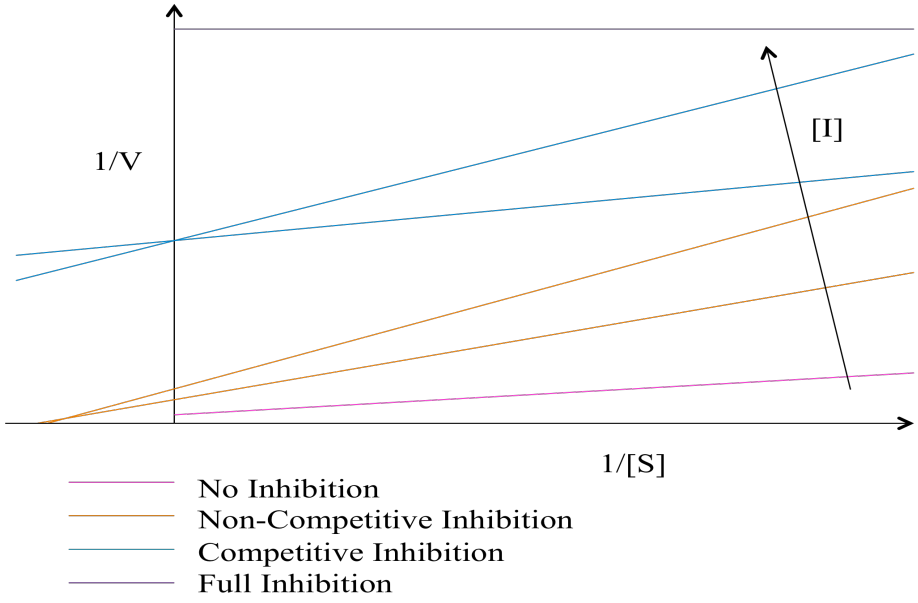


Figure 3.4.1: Graph showing the expected plots on a Lineweaver Burk plot for no inhibition, non-competitive inhibition, competitive inhibition and full inhibition.

Due to enzyme degradation it was not possible to carry out assays of some of the materials, including assays for Gluco-Gal5, a blank ONPG and a control sample for ONPG and galactosyl transferase. Therefore any results that have been gained from the assays are purely qualitative, and it is not possible to quantify them. It was also not feasible to take repeat measurements, due to the small quantities of thiogalactose functionalised sugars synthesised and the enzyme degradation.

The figure below (figure 3.4.2) shows the rise in absorbance at 405nm as the concentration of ONPG increases. This is as would be expected, as the concentration of ONPG increases, the amount of o-nitrophenol produced will increase, and therefore there will be a higher absorbance at 405nm. All of the measurements for the varying concentrations of ONPG were all taken at the same concentration of Raffi-Gal11 (0.42mM) so that a direct comparison could be seen.

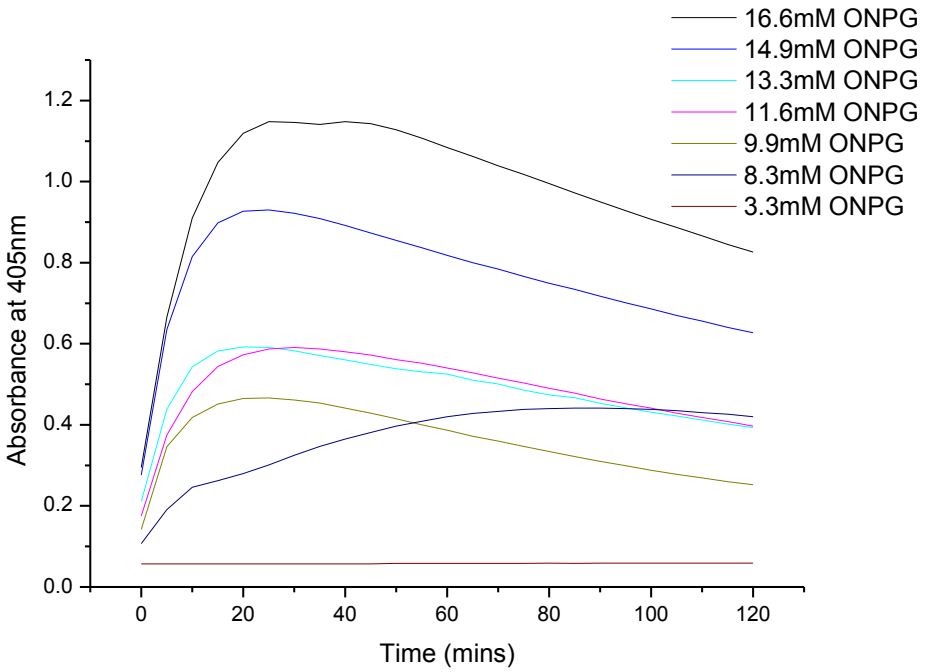


Figure 3.4.2: Graph showing rise in absorbance at 405nm, and therefore amount of o-nitrophenol produced as concentration of ONPG increases.

The next graph (figure 3.4.3) shows the changes in absorbance at different concentrations of Cello-Gal8. All measurements were taken using a concentration of 16.6mM ONPG. The graph shows that there was a general decrease in absorbance as the concentration of Cello-Gal8 increased, suggesting inhibition. There is, however, a slight discrepancy in the results for 346.2μM and 173.1μM Cello-Gal8, as they are showing a higher absorbance at higher Cello-Gal8 concentration. The results were normalised by subtraction of the absorbance for spontaneous decomposition of ONPG as well as the absorbance of the thiogalactose functionalised sugars, as they were an orange/yellow colour and would therefore affect the absorbance. It follows that the higher absorbances would be expected for the control samples of Cello-Gal8 with the highest concentrations; however the control value for 173.1μM Cello-Gal8 seemed unnaturally high, which is probably why the results for this assay seem slightly skewed. Further repeats of this experiment would most likely lead to more accurate results.

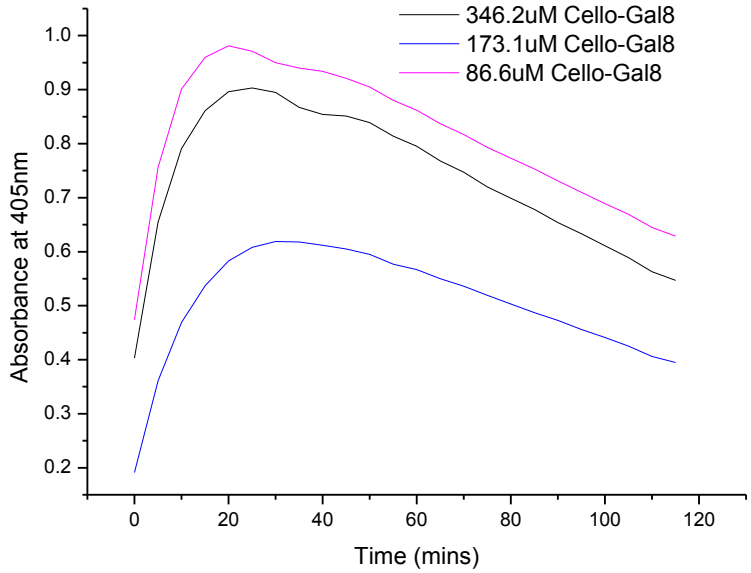


Figure 3.4.3: Plot of time vs. absorbance showing absorbances at different concentration of Cello-Gal8.

The next graph (figure 3.4.4) shows the changes in absorbance at different concentrations of Raffi-Gal11, again all carried out at a concentration of 16.6mM ONPG. The graph shows a clear decrease in absorbance at the concentration of Raffi-Gal11 increases, again suggesting that the Raffi-Gal11 is acting as an inhibitor of the galactosyl transferase enzyme.

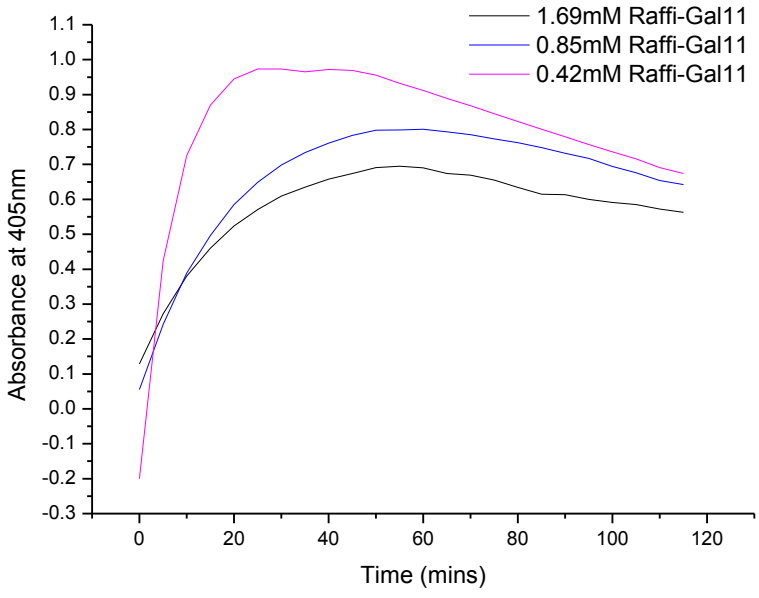


Figure 3.4.4: Graph of time vs. absorbance for different concentrations of Raffi-Gal11, suggesting that Raffi-Gal11 is an inhibitor of galactosyl transferase.

The figure below (figure 3.4.5) shows the changes in absorbance at 405nm with change in Stach-Gal14 concentration. If inhibition was occurring then the absorbance would be expected to decrease as the inhibitor concentration increased, which is the opposite of what

is shown in this graph. This could be due to only one assay being carried out for each concentration, which if carried out incorrectly would lead to incorrect results, or possibly could be due to the size of the Stach-Gal14 molecule. It seems likely that the mode of inhibition, if there was any from the thiogalactose functionalised sugars would be competitive as they so closely resemble the natural substrate of the enzyme. However, the active site of an enzyme is a highly sterically controlled environment and the lack of inhibition seen by Stach-Gal14 could be down to the bulky functionalised tetrasaccharide not fitting into the active site.

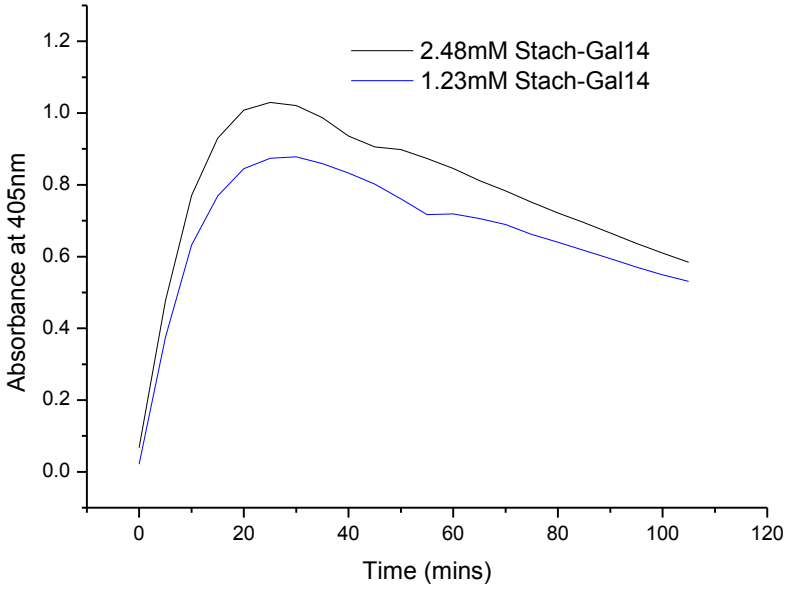


Figure 3.4.5: Graph showing absorbance vs. time for different concentrations of Stach-Gal14.

The data that it was possible to obtain was analysed by taking the initial gradient of a plot of time vs. absorbance as the initial reaction rate v. 1/v and 1/[S] could then be calculated and plotted on a Lineweaver Burk plot. Whilst this method is a poor estimate for K_m and V_{max}[90] for the purposes of looking at the data qualitatively it is acceptable.

The figure below (figure 3.4.6) is a Lineweaver Burk plot for different concentrations of Cello-Gal8. As was the case for the absorbance plots for Cello-Gal8 the values for 173.1μM Cello-Gal8 seem very high. Further repeats would hopefully provide better data to reach a more meaningful conclusion but from the plots of 86.6μM and 346.2μM Cello-Gal8 it appears that Cello-Gal8 is inhibiting the action of galactosyl transferase as the values of 1/v increase as the concentrations of Cello-Gal8 increase. The lines don't intercept at the y axis as would be expected if the inhibition was competitive, but as the lines are so close to intersecting at the y axis further repeats may well show that inhibition is competitive. As the

inhibitor so closely resembles the substrate of the enzyme it is likely that inhibition is competitive.

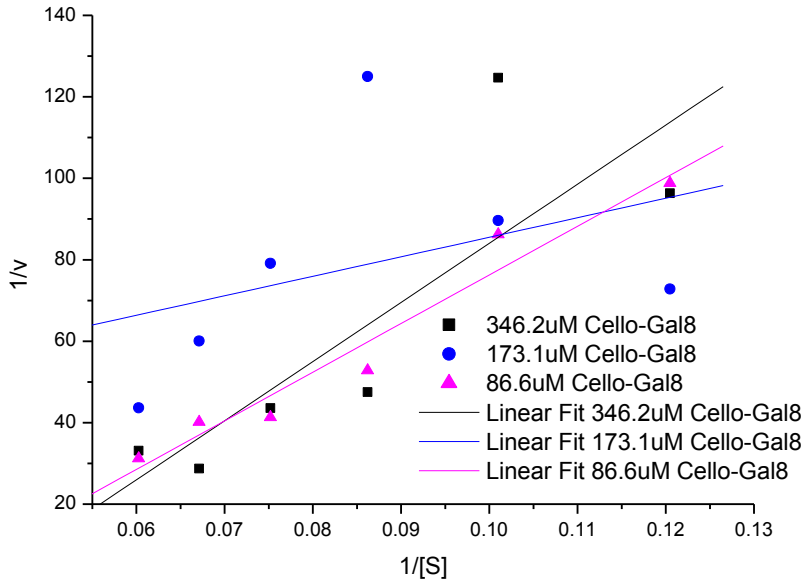


Figure 3.4.6: Lineweaver-Burk plot showing reaction rates at different substrate and Cello-Gal8 concentrations.

The graph below (figure 3.4.7) is a Lineweaver Burk plot for different concentrations of Raffi-Gal11 It appears that the value for 1/v at the lowest substrate concentration (highest 1/[S]) for 1.69mM Raffi-Gal11 is incorrect as it doesn't seem to follow the general trend of the others. Taking this value out and plotting lines of best fit (figure 3.4.8), the graph shows the general trend expected if inhibition is occurring i.e. 1/v will increase as the inhibitor concentration increases. Again, it is difficult to tell the mode of inhibition, but as all the lines intercept close to the y axis it seems likely that the inhibition is competitive.

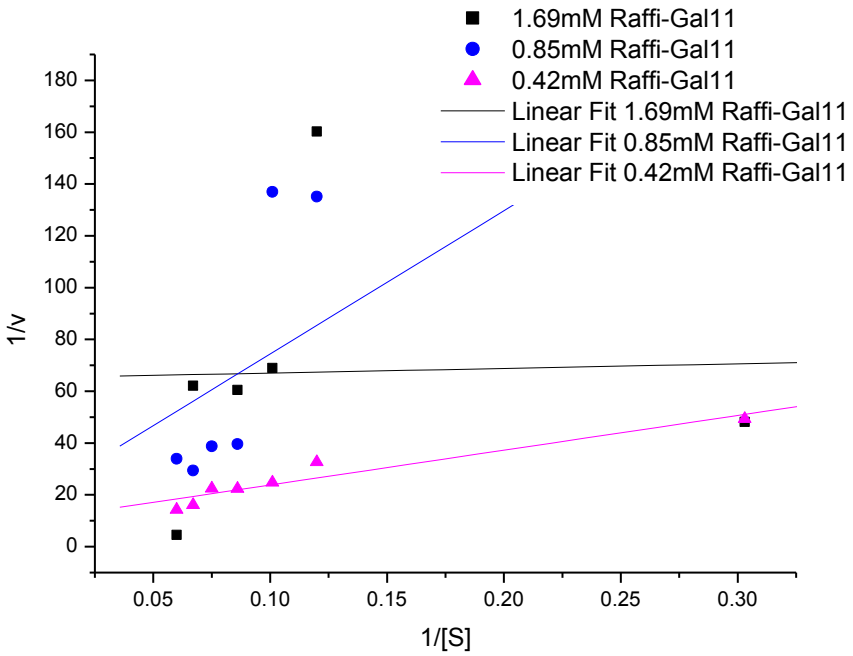


Figure 3.4.7: Lineweaver Burk plot showing reaction rate at different substrate and Raffi-Gal11 concentrations.

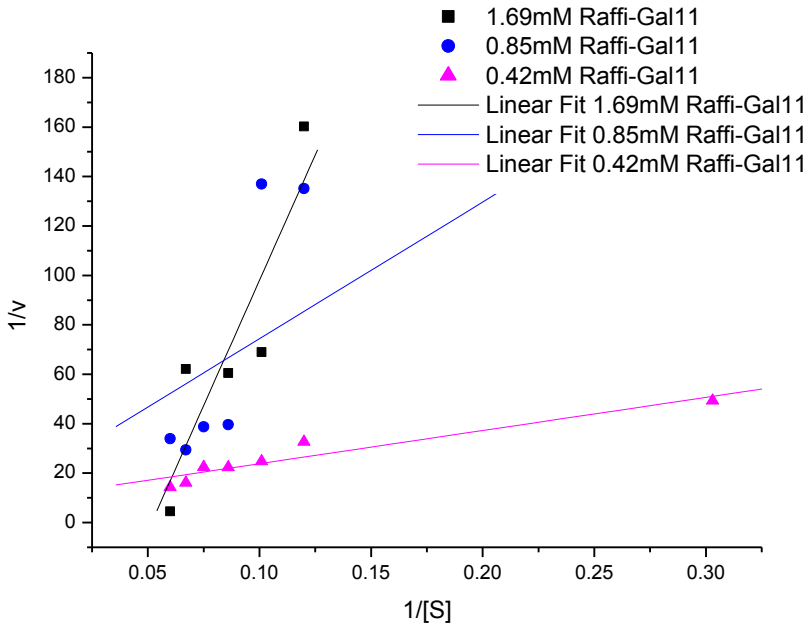


Figure 3.4.8: Lineweaver Burk plot showing reaction rate at different substrate and Raffi-Gal11 concentrations without anomalous result for 1.69mM Raffi-Gal11.

The graph below (figure 3.4.9) is a Lineweaver Burk plot for different concentrations of Stach-Gal14. This does not exhibit the trend that would be expected if inhibition is occurring: 1/v decreases with increasing Stach-Gal14 concentration rather than increasing. The reasons why Stach-Gal14 might not act as an inhibitor have been outlined earlier and include the steric constraints that it has by being such a large molecule. However, as only one

repeat has been carried out, it is impossible to tell whether the trend exhibited is true for Stach-Gal14 or whether it is just due to a mistake in the assay procedure.

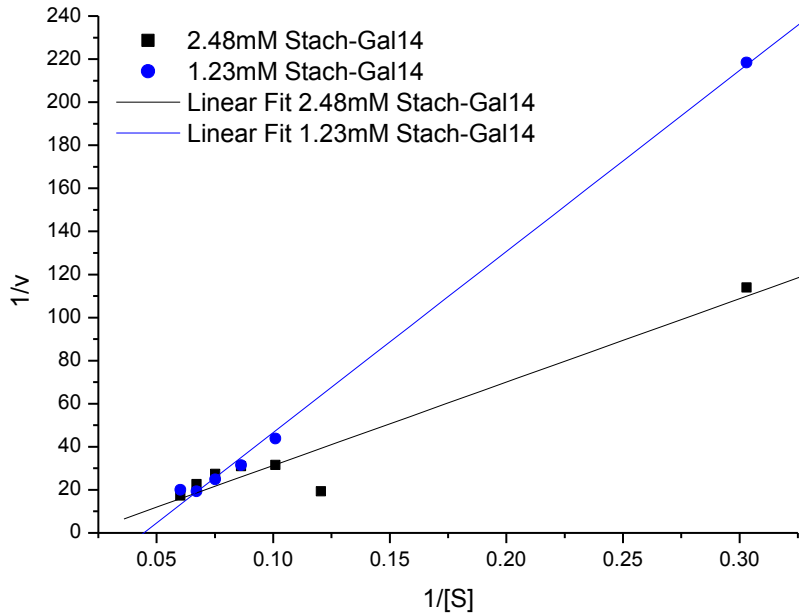


Figure 3.4.9: Lineweaver Burk plot showing reaction rate at different substrate and Stach-Gal14 concentrations.

However, as well as the lack of repeats/ a full set of data making it difficult to calculate any real values for enzyme kinetics there is of course the fact that Michaelis Menten kinetics only apply for single substrate enzymes. Whilst the substrate enzyme reaction for galactosyl transferase is single substrate i.e. the enzyme takes one molecule of ONPG and converts it to one molecule of galactose and one molecule of o-nitrophenol, the inhibitors all have more than one site that the enzyme could bind to. For example, for fully functionalised Cello-Gal8 there are 8 galactose groups that an enzyme could bind to, and therefore, sterics permitting, a Cello-Gal8 molecule could inhibit the action of 8 galactosyl transferase enzymes. With more inhibition data it could be possible to model how many enzymes are binding to each sugar molecule, which would help in the development of better inhibitors.

4. Conclusion

4.1. Project Overview

This project has shown that it is possible to make 'polymerisable' sugars using a method that is less time consuming and using more readily available reagents than conventional controlled polymerisation techniques. These sugars exhibited low polydispersities, much lower than those which could be expected with controlled polymerisation techniques, although the techniques need further optimisation for better yields.

It was also shown that it was possible to functionalise these sugars with different thiol based groups, boc-cysteine and thiogalactose, using Click chemistry techniques. The functionalised sugars also exhibited low polydispersities.

The thiogalactose sugars were then tested for inhibition of the enzyme galactosyl transferase. There were signs of inhibition, but without repeats it is difficult to draw a real conclusion. However, the results do appear to show that multivalent enhancement works for enzyme inhibition. Previously multivalent enhancement has only been shown to work for lectins and for one example of enzyme inhibition.

By managing to make controlled size sugar (and therefore biocompatible) polymers I have shown that these could be used as a viable alternative to controlled radical glycopolymers in biomedical applications. However, the poor yields during all stages of the polymer synthesis are a concern, and the techniques all require further optimisation to rectify this.

4.2. Further Research

One of the first areas to target for further research would be to synthesise the thiogalactose functionalised sugars in larger quantities in order to carry out the galactosyl transferase assay with enough data to make a quantitative analysis of the results, as well as carrying out a full kinetic analysis of the inhibitors. It would also be interesting to see if the stereochemistry of the sugar polymers had any effect on their inhibitory properties, as this may have an effect on the multivalent enhancement for example if the stereochemistry of neighbouring groups prevented them both from binding to enzyme active sites at the same time. If this was successful an enzyme inhibition assay could then be carried out on bacterial

cells, although to find out if the functionalised sugars were effective against the peptidoglycan glycosyl transferases the thiogalactose group would probably need to be changed to one that more resembled the natural substrate, i.e. the growing peptidoglycan chain.

Another area for future research would be to methacrylate different sugars to produce a library of monodisperse polymerisable scaffolds. As the Michael addition reaction is applicable to not only thiol containing groups but other nucleophilic groups there is a large range of compounds that could be added on to the scaffolds. Here surely the only limits are those of experimental optimisation, sterics and the chemists' imagination.

5. References

1. B. R. Bloom and C. J. L. Murray, *Science*, 1992, **257**, 1055–1064.
2. C. Walsh, *Nature*, 2000, **406**, 775–781.
3. R. I. Aminov, *Frontiers in microbiology*, 2010, **1**, 134.
4. P. I. Lerner, *New England Journal of Medicine*, 2004, **351**, 524.
5. A. J. Alanis, *Archives of Medical Research*, 2005, **36**, 697–705.
6. Y. Chander, S. C. Gupta, S. M. Goyal, and K. Kumar, *Journal of the Science of Food and Agriculture*, 2007, **87**, 739–742.
7. S. B. Levy and B. Marshall, *Nature Medicine*, 2004, **10**, S122–S129.
8. B. H. Normark and S. Normark, *Journal of Internal Medicine*, 2002, **252**, 91–106.
9. H. S. Gold and R. C. Moellering, *New England Journal of Medicine*, 1996, **335**, 1445–1453.
10. L. Leibovici, K. Soares-Weiser, M. Paul, E. Goldberg, A. Herxheimer, and P. Garner, *Journal of Antimicrobial Chemotherapy*, 2003, **52**, 564–571.
11. R. I. Aminov and R. I. Mackie, *Fems Microbiology Letters*, 2007, **271**, 147–161.
12. M. Belanova, P. Dianiskova, P. J. Brennan, G. C. Completo, N. L. Rose, T. L. Lowary, and K. Mikusova, *Journal of Bacteriology*, 2008, **190**, 1141–1145.
13. K. Ilic, E. Jakovljevic, and V. Skodric-Trifunovic, *European Journal of Pediatrics*, 2012, **171**, 767–777.
14. H. C. Neu, *Science*, 1992, **257**, 1064–1073.
15. W. Vollmer, D. Blanot, and M. A. de Pedro, *Fems Microbiology Reviews*, 2008, **32**, 149–167.
16. B. A. Dmitriev, S. Ehlers, and E. T. Rietschel, *Medical Microbiology and Immunology*, 1999, **187**, 173–181.
17. W. Vollmer and S. J. Seligman, *Trends in Microbiology*, 2010, **18**, 59–66.
18. M. Terrak, T. K. Ghosh, J. van Heijenoort, J. Van Beeumen, M. Lampilas, J. Aszodi, J. A. Ayala, J. M. Ghuysen, and M. Nguyen-Disteche, *Molecular Microbiology*, 1999, **34**, 350–364.
19. D. Chatterjee, *Current Opinion in Chemical Biology*, 1997, **1**, 579–588.
20. R. E. Burge, A. G. Fowler, and D. A. Reaveley, *Journal of Molecular Biology*, 1977, **117**, 927–953.
21. C. Schaffer and P. Messner, *Microbiology-Sgm*, 2005, **151**, 643–651.
22. B. Dmitriev, F. Toukach, and S. Ehlers, *Trends in Microbiology*, 2005, **13**, 569–574.
23. C. Fraipont, F. Sapunaric, A. Zervosen, G. Auger, B. Devreese, T. Lioux, D. Blanot, D. Mengin-Lecreulx, P. Herdewijn, J. Van Beeumen, J. M. Frere, and M. Nguyen-Disteche, *Biochemistry*, 2006, **45**, 4007–4013.
24. S. Wang, L. Furchtgott, K. C. Huang, and J. W. Shaevitz, *Proceedings of the National Academy of Sciences of the United States of America*, 2012, **109**, E595–E604.
25. K. C. Huang, R. Mukhopadhyay, B. Wen, Z. Gitai, and N. S. Wingreen, *Proceedings of the National Academy of Sciences of the United States of America*, 2008, **105**, 19282–19287.
26. R. J. Doyle and R. E. Marquis, *Trends in Microbiology*, 1994, **2**, 57–60.
27. S. O. Meroueh, K. Z. Bencze, D. Hesek, M. Lee, J. F. Fisher, T. L. Stemmler, and S. Mobashery, *Proceedings of the National Academy of Sciences of the United States of America*, 2006, **103**, 4404–4409.
28. Schleife.Kh and O. Kandler, *Bacteriological Reviews*, 1972, **36**, 407–477.
29. J. M. Ghuysen, *International Journal of Antimicrobial Agents*, 1997, **8**, 45–60.

30. B. Schwartz, J. A. Markwalder, S. P. Seitz, Y. Wang, and R. L. Stein, *Biochemistry*, 2002, **41**, 12552–12561.
31. M. J. Osborn, *Annual review of biochemistry*, 1969, **38**, 501–538.
32. J. van Heijenoort, *Glycobiology*, 2001, **11**, 25R–36R.
33. B. A. Dmitriev, F. V Toukach, K. M. Schaper, O. Holst, E. T. Rietschel, and S. Ehlers, *Journal of Bacteriology*, 2003, **185**, 3458–3468.
34. J. van Heijenoort, *Natural Product Reports*, 2001, **18**, 503–519.
35. Y. Yuan, D. Barrett, Y. Zhang, D. Kahne, P. Sliz, and S. Walker, *Proceedings of the National Academy of Sciences of the United States of America*, 2007, **104**, 5348–5353.
36. C. M. Gampe, H. Tsukamoto, E. H. Doud, S. Walker, and D. Kahne, *Journal of the American Chemical Society*, 2013, **135**, 3776–3779.
37. A. K. Pathak, V. Pathaka, L. Seitz, S. S. Gurcha, G. S. Besra, J. M. Riordan, and R. C. Reynolds, *Bioorganic & Medicinal Chemistry*, 2007, **15**, 5629–5650.
38. S. J. Projan, *Current Opinion in Pharmacology*, 2002, **2**, 513–522.
39. A. L. Lovering, L. H. de Castro, D. Lim, and N. C. J. Strynadka, *Science*, 2007, **315**, 1402–1405.
40. E. S. A. Derouaux M. Terrak, *Frontiers in immunology*, 2013, **4**, 78.
41. A. Erb, H. Weiss, J. Haerle, and A. Bechthold, *Phytochemistry*, 2009, **70**, 1812–1821.
42. U. M. Unligil and J. M. Rini, *Current Opinion in Structural Biology*, 2000, **10**, 510–517.
43. R. V Stick and A. G. Watts, *Monatshefte Fur Chemie*, 2002, **133**, 541–554.
44. U. Kempin, L. Hennig, D. Knoll, P. Welzel, D. Muller, A. Markus, and J. vanHeijenoort, *Tetrahedron*, 1997, **53**, 17669–17690.
45. L. L. Lairson, B. Henrissat, G. J. Davies, and S. G. Withers, *Annual Review of Biochemistry*, 2008, **77**, 521–555.
46. S. G. Withers, *Carbohydrate Polymers*, 2001, **44**, 325–337.
47. P. Gallo, S. Fabbrocino, L. Serpe, M. Fiori, C. Civitareale, and P. Stacchini, *Rapid Communications in Mass Spectrometry*, 2010, **24**, 1017–1024.
48. M. Zehl, E. Pittenauer, A. Rizzi, and G. Allmaier, *Journal of the American Society for Mass Spectrometry*, 2006, **17**, 1081–1090.
49. J. Halliday, D. McKeveney, C. Muldoon, P. Rajaratnam, and W. Meutermans, *Biochemical Pharmacology*, 2006, **71**, 957–967.
50. B. Ostash and S. Walker, *Natural Product Reports*, 2010, **27**, 1594–1617.
51. S. Dumbre, A. Derouaux, E. Lescrinier, A. Piette, B. Joris, M. Terrak, and P. Herdewijn, *Journal of the American Chemical Society*, 2012, **134**, 9343–9351.
52. S. J. Chung, S. Takayama, and C. H. Wong, *Bioorganic & Medicinal Chemistry Letters*, 1998, **8**, 3359–3364.
53. S. Perez, B. E. McJury, P. Eichhorn, and D. S. Aga, *Journal of Chromatography A*, 2007, **1175**, 234–241.
54. K. Choudhury, S. K. Chatterjee, M. Bhattacharya, and J. J. Barlow, *Biochemical Pharmacology*, 1982, **31**, 459–460.
55. R. A. Field, D. C. A. Neville, R. W. Smith, and M. A. J. Ferguson, *Bioorganic & Medicinal Chemistry Letters*, 1994, **4**, 391–394.
56. K. Hatanaka, H. Takeshige, K. I. Kanno, A. Maruyama, J. Oishi, Y. Kajihara, and H. Hashimoto, *Journal of Carbohydrate Chemistry*, 1997, **16**, 667–672.
57. F. Guette, R. Cacan, J. Montreuil, and A. Verbert, *Biochimie*, 1983, **65**, 563–567.
58. V. Ladmiral, E. Melia, and D. M. Haddleton, *European Polymer Journal*, 2004, **40**, 431–449.
59. A. Mun, *Journal of Polymer Science Part A: Polymer Chemistry*, 2011, **49**, 2627–2635.

60. A. Muñoz-Bonilla, O. León, V. Bordegé, M. Sánchez-Chaves, and M. Fernández-García, *Journal of Polymer Science Part A: Polymer Chemistry*, 2013, **51**, 1337–1347.
61. J. L. Jiménez Blanco, C. Ortiz Mellet, and J. M. García Fernández, *Chemical Society reviews*, 2013, **42**, 4518–31.
62. M. L. Cerrada and M. Ferna, *Journal of Polymer Science Part A: Polymer Chemistry*, 2008, 7238–7248.
63. Y. Gou, J. Geng, S.-J. Richards, J. Burns, C. Remzi Becer, and D. M. Haddleton, *Journal of polymer science. Part A, Polymer chemistry*, 2013, **51**, 2588–2597.
64. M. I. Gibson and N. R. Cameron, *Journal of Polymer Science Part A: Polymer Chemistry*, 2007, **45**, 2059–2072.
65. V. Vázquez-Dorbatt, J. Lee, E.-W. Lin, and H. D. Maynard, *Chembiochem : a European journal of chemical biology*, 2012, **13**, 2478–87.
66. F. Suriano, R. Pratt, J. P. K. Tan, N. Wiradharma, A. Nelson, Y.-Y. Yang, P. Dubois, and J. L. Hedrick, *Biomaterials*, 2010, **31**, 2637–45.
67. L. E. Ers, B. A. Johns, and C. R. Johnson, *Tetrahedron Letters*, 1998, **39**, 749–752.
68. a Lohse, K. B. Jensen, K. Lundgren, and M. Bols, *Bioorganic & medicinal chemistry*, 1999, **7**, 1965–71.
69. J. Diot, M. I. García-Moreno, S. G. Gouin, C. Ortiz Mellet, K. Haupt, and J. Kovensky, *Organic & biomolecular chemistry*, 2009, **7**, 357–63.
70. C. Decroocq, D. Rodríguez-Lucena, V. Russo, T. Mena Barragán, C. Ortiz Mellet, and P. Compain, *Chemistry (Weinheim an der Bergstrasse, Germany)*, 2011, **17**, 13825–31.
71. R. H. Treadway and E. Yanovsky, *Journal of the American Chemical Society*, 1945, **67**, 1038–1039.
72. C. Zandanel, C. Mioskowski, R. Baati, and A. Wagner, *Tetrahedron*, 2009, **65**, 9395–9402.
73. B. Mukhopadhyay, K. P. R. Kartha, D. A. Russell, and R. A. Field, *Journal of Organic Chemistry*, 2004, **69**, 7758–7760.
74. X. Z. Hu, Z. Zhang, X. Zhang, Z. Y. Li, and X. X. Zhu, *Steroids*, 2005, **70**, 531–537.
75. H. C. Kolb, M. G. Finn, and K. B. Sharpless, *Angewandte Chemie (International ed. in English)*, 2001, **40**, 2004–2021.
76. J. E. Moses and A. D. Moorhouse, *Chemical Society reviews*, 2007, **36**, 1249–62.
77. R. Service, *Science*, 2008, **320**, 868–869.
78. E. Lallana, F. Fernandez-Trillo, A. Sousa-Herves, R. Riguera, and E. Fernandez-Megia, *Pharmaceutical Research*, 2012, **29**, 902–921.
79. U. Mansfeld, C. Pietsch, R. Hoogenboom, C. R. Becer, and U. S. Schubert, *Polymer Chemistry*, 2010, **1**, 1560–1598.
80. C. E. Hoyle and C. N. Bowman, *Angewandte Chemie (International ed. in English)*, 2010, **49**, 1540–73.
81. M. C. de Mattos and L. Marzorati, *Quimica Nova*, 1999, **22**, 710–714.
82. C. Gimbert, M. Lumbierres, C. Marchi, M. Moreno-Manas, R. M. Sebastian, and A. Vallribera, *Tetrahedron*, 2005, **61**, 8598–8605.
83. J. H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, 1972.
84. W. Li, X. Zhao, S. Zou, Y. Ma, K. Zhang, and M. Zhang, *Applied Biochemistry and Microbiology*, 2012, **48**, 603–607.
85. K. L. Griffith and R. E. Wolf, *Biochemical and Biophysical Research Communications*, 2002, **290**, 397–402.
86. A. Giacomini, V. Corich, F. J. Ollero, A. Squartini, and M. P. Nuti, *Fems Microbiology Letters*, 1992, **100**, 87–90.

87. S. Hussain, S. K. Bharadwaj, M. K. Chaudhuri, and H. Kalita, *European Journal of Organic Chemistry*, 2007, 374–378.
88. L. Michaelis, M. L. Menten, R. S. Goody, and K. A. Johnson, *Biochem Z*, 1913.
89. H. Lineweaver and D. Burk, *Journal of the American Chemical Society*, 1934, **56**, 658–666.
90. J. Dowd, *Journal of Biological Chemistry*, 1965, **240**, 863–869.