The synthesis of a maltose responsive switch

Kristian Hollingsworth

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Abstract

A cells interaction with its surroundings is governed by the flora of the cell surface. This complex landscape of structures provides an opportunity for the re-engineering of the surface and so the cells properties without the use of genetic modification. Applying the principles of supramolecular chemistry; surface proteins can be targeted with carbohydrate based ligands to form both stable and metabolite-responsive non-covalent complexes. This redecoration of the surfaces of bacteria will make it possible to control the interactions that a bacterium makes with its environment, whether in a patient or a bioreactor.

In this project the transport protein maltoporin and maltose binding protein (MBP) will be utilised in the construction of a maltose responsive switch. Both proteins will be targeted with a maltose-based polymer which can thread through maltoporin on the cell surface to interact with MBP in the periplasm. In addition, the synthesis of molecules to probe the binding of maltoporin through biophysical experiments will be investigated.

Abbreviations

APS	Ammonium persulfate
BLA	β-lactamase
Boc	*tert*-butoxylcarbonyl
Bp	Base pairs
DCM	Dichloromethane
DDM	n-Dodecyl-β-D-maltoside
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleotide
ConA	Concanavalin A
CRD	Carbohydrate recognition domains
CuAAC	Copper-promoted azide-alkyne cycloaddition
DGL	*Dioclea grandiflora* lectin
DIPEA	N,N-Diisopropylethylamine
DMAP	4-Dimethylamino pyridine
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electron spray ionisation mass spectrometry
FITC	Fluorescein isothiocyanate
Fmoc	Fluorenylmethyloxycarbonyl
gal	Galactose
Gb_3	Globotriaosylceramide
glu	Glucose
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HCTU	1H-Benzotriazolium 1-[bis(dimethylamino)methylene] -5chloro-,hexafluorophosphate (1-),3-oxide
HFIP	Hexafluoroisopropanol
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Infrared
ITC	Isothermal titration calorimetry
LacNAc	N-Acetyl-D-lactosamine
LB	Lysogeny broth
LCMS	Liquid chromatography mass spectrometry
man	Mannose
MOS	Malto-oligosaccharide
MBL	Mannose binding lectin
MBP	Maltose binding protein
MeCN	Acetonitrile
MS	Mass spectrometry
NEB	New England biolabs
NMR	Nuclear magnetic resonance
nt	Nucleotides
Octyl-POE	n-Octylpolyoxyethylene
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
r.t.	Room temperature
SDS	Sodium dodecyl sulphate

SOC	Super optimal broth
SPPS	Solid phase peptide synthesis
TAE	Tris, acetic acid and EDTA
TAMRA	Tetramethylrhodamine
TBAI	Tetrabutylammonium iodide
TBDMS	*tert*-butyldimethylsily
TCEP	Tris(2-carboxyethyl)phosphine
TEMED	Tetramethylethylenediamine
TEN	Tris, EDTA and NaCl
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic anhydride
TLC	Thin layer chromatography
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TMS_2O	Hexamethyldisiloxane
Tris	tris(hydroxymethyl)aminomethane
UV	Ultra violet

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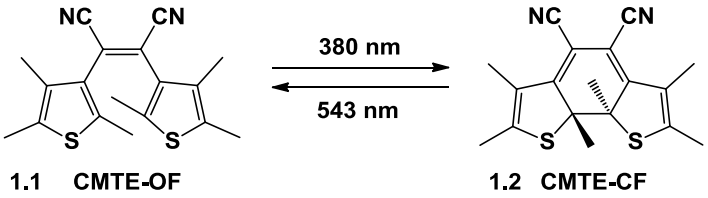
Chapter I: Introduction

1.1 Molecular Switches

Molecular switches are molecules or complexes that on interacting with an outside force can reversibly switch between two stable states.[1,2] This outside force can come in the form of light, pH, temperature, electric current or a binding ligand. The unique properties of molecular switches have made them attractive synthetic targets and a wide variety of uses have been found for them.[3-5]

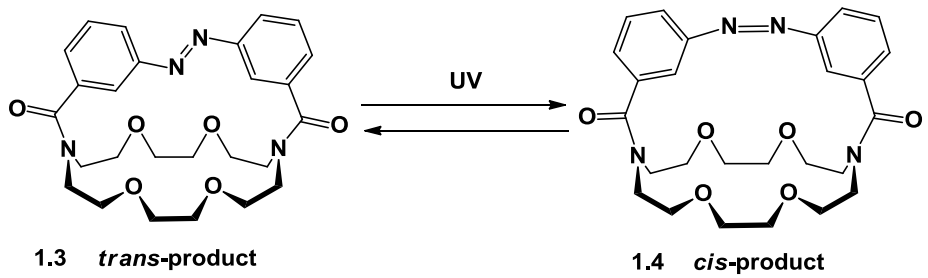
The simplest switches are small molecules such as *cis*-1,2-dicyano-1,2-bis(2,4,5-trimethyl-3-thienyl)ethane (CMTE) which is a yellow isomer **1.1** until irradiation with UV light at a wavelength of 380 nm which produces a red isomer **1.2** (Scheme 1-1).[6] This can be converted back to the original yello isomer with irradiation at 543 nm. This switch has subsequently been used for 3D data storage as the photochromatic molecule can be absorbed onto a surface and precisely switched between states with a laser.[7]



1.1 CMTE-OF **1.2 CMTE-CF**

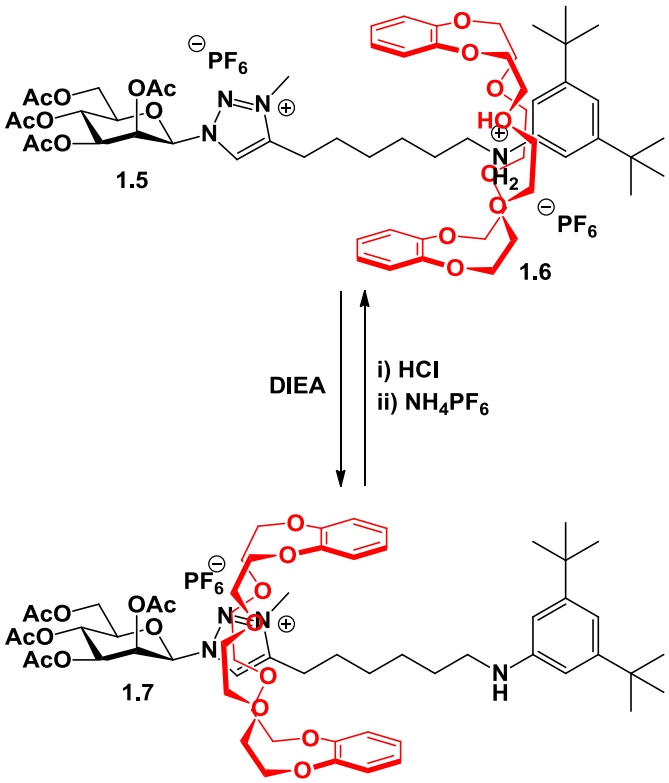
Scheme 1-1 The reversible electrocyclisation of CMTE between a yellow and red isomer

The binding of photochromatic compounds can also be influenced by light-induced conformational change. This is the case with photo responsive crown ethers.[8] Crown ethers are known to act as hosts for positive ions with the size of the cavity determining which ions can act as a guest.[9,10] Shinkai et al., bridged the two sides of a crown ether with a *trans*-azobenzene moiety which on irradiation with UV light converted to the *cis*-conformer **1.4** (Scheme 1-2). The product could be switched back to the trans-form **1.3** by leaving the molecule in the dark. This change led to an increase in the diameter of the crown and this was reflected in the ability of the crown ether to act as a host to cations. The trans-product **1.3** had a preference for the smaller Na^+ ions while the cis-product **1.4** was selective for the larger K^+ ions.[8]



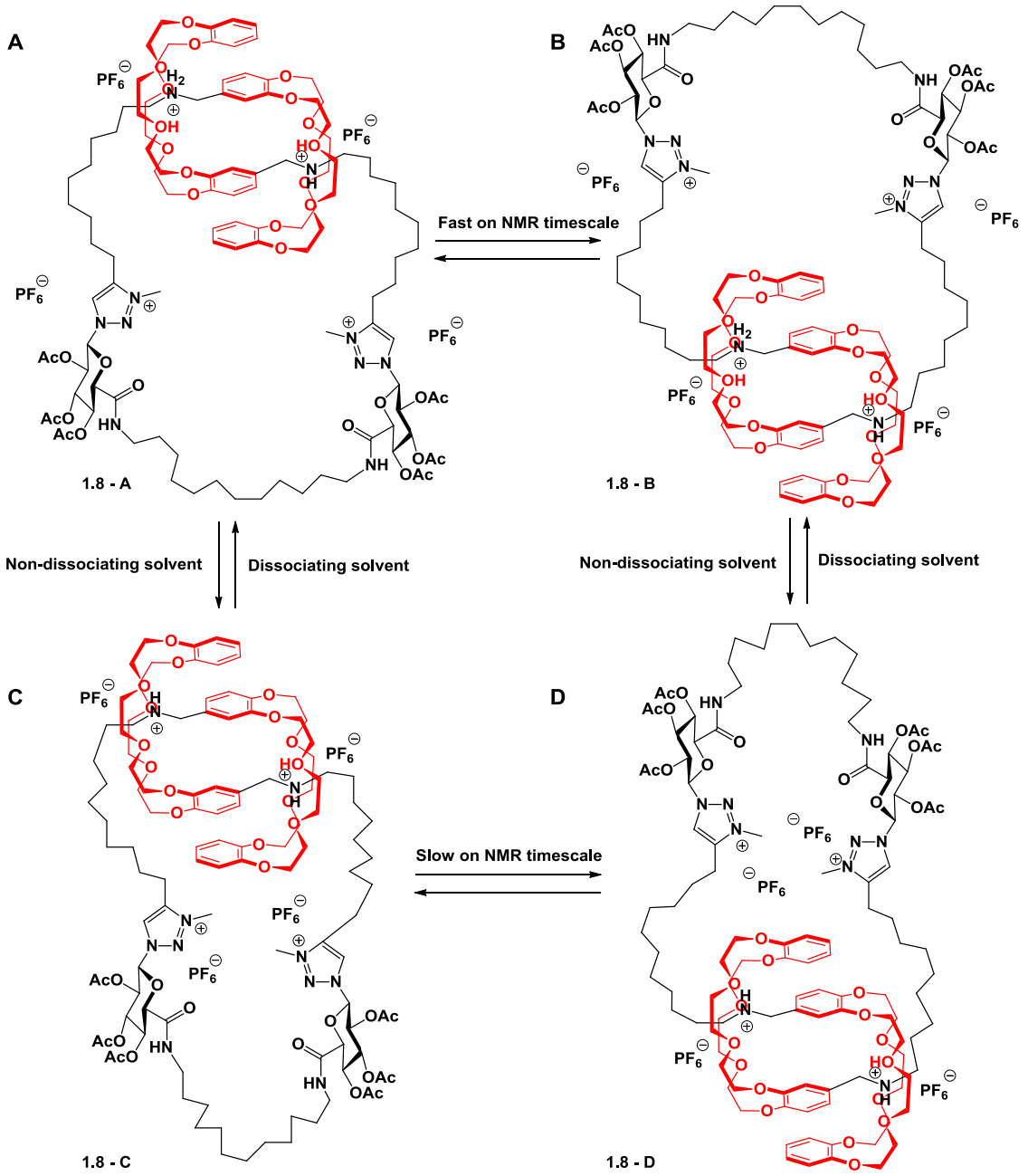
Scheme 1-2 After irradiation with UV light the azo-group switches to the cis-form increasing the diameter of the crown ether.

Some of the most complex molecular switches are based on rotaxanes.[11,12] Here the macrocycle is often locked on a single section of the axle either by switchable steric bulk[13] or electrostatic interactions. In the case of Busseron et al., the position of a macrocycle **1.6** could be exchanged with a variation in pH (Scheme 1-3).[14] At low pH the crown ether **1.6** preferentially surrounds the anilinium moiety of the axle **1.5** while at a higher pH the crown ether **1.6** switches to the opposite end of the axle **1.7** interacting with the triazolium station.



Scheme 1-3 A crown ether **1.6** was switched between from an anilinium station **1.5** to a triazolium station **1.7** by the use of DIEA and the process could be reversed by acidifying the solution.

Molecular machines based on rotaxanes can also be made to change state dependent on solvent. Coutrot et al. developed a molecular switch **1.8** where a 'rope' could swing around the macrocycles (Scheme 1-4). This was found to occur on a fast timescale in non-dissociating solvents but when the solvent was exchanged for a dissociating solvent the speed at which the 'rope' swung round was reduced to enable two distinct rotamers **1.8-C** and **1.8-D** to be seen by NMR.[15] In this experiment a dissociating solvent is one which can solvate the hexafluorophosphate ions. When these ions are solvated the positive charges on the triazolium ions repel each other keeping the two 'ropes' of the molecule **1.8-A** and **1.8-B** untangled allowing for the rope to rotate easily. Once in a non-dissociating solvent the hexafluorophosphate ions can sit in-between the two triazolium ions causing the 'rope' to tangle and therefore it can no longer rotate.



Scheme 1-4 The 'jump rope' mechanism. In sections A and B the molecule **1.8** is in a dissociating solvent therefore the hexafluorophosphate ions are solvated and the 'rope' can swing freely around the macrocycles. In C and D the hexafluorophosphate ions are not solvated so they interact with both triazolium ions tangling the 'rope' meaning it swings much more slowly.

As well as solvents external stimuli such as different cations can be used to induce a change in conformation of rotaxanes. In an example by Sauvage et al. two interlinking rotaxanes **1.9** are used with the macrocyle of one attached to the axis of the other.

Here two binding sites are included in the axis one with a tridentate binding motif and the other a bidentate. When copper (I) is added to the solution the bidentate site is preferred forming the expanded conformer **1.9-A** while the introduction of zinc (II) leads leads to binding of the tridentate site and a contracted conformer **1.9-B**.[16] This change in binding leads to a widening and shortening of molecule **1.9** much like a muscle cell (Figure 1-1).

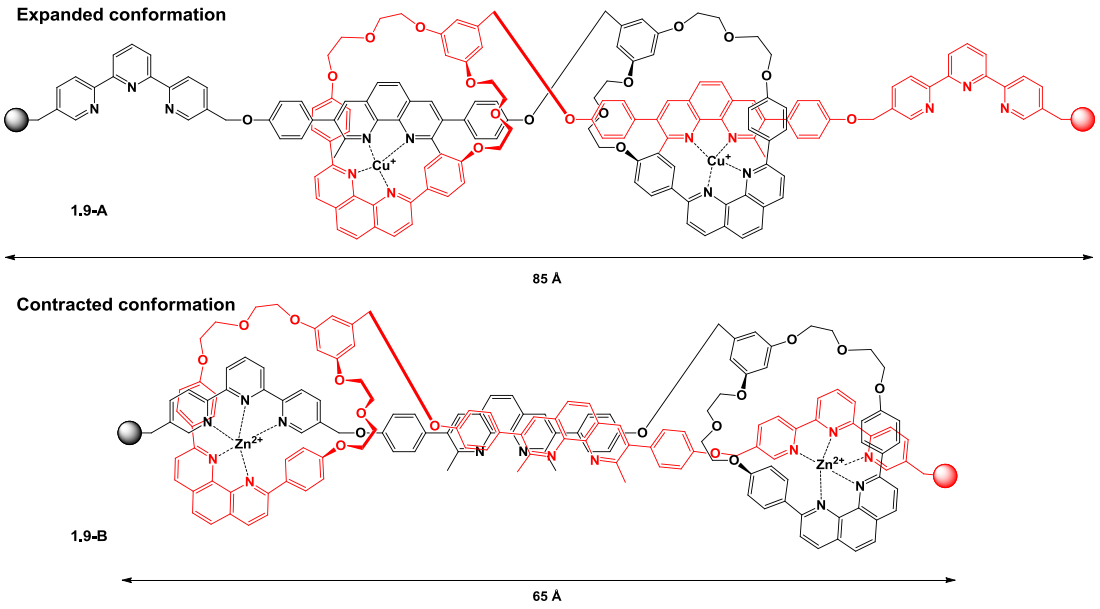


Figure 1-1 A molecular muscle. In the expanded conformation **1.9-A** a copper (I) ion is bound in a tetradentate site. When the copper is replaced with a zinc (II) ion it is instead chelated by five sites contracting the molecule **1.9-B**.

These kinds of rotaxanes can act as sensors for specific cations and this research has also extended to anions.[17-19] Molecular switches using proteins have also been created.[20,21] These switches have the advantage of the selectivity being built into the proteins and can therefore be used to sense much more nuanced changes in the ligand than simply the size of the ion.

A maltose sensor has been developed with the use of MBP and β-lactamase (BLA). β-lactamase was circularly permuted in a random fashion and then randomly inserted into the gene encoding maltose binding protein. This created a library of proteins from which one was found where the catalytic activity of BLA was dependent on MBP

binding maltose. This was due to the conformational change of MBP on binding maltose which resulted in a subsequent change in conformation of BLA restoring its enzymatic activity (Figure 1-2).[22,23]

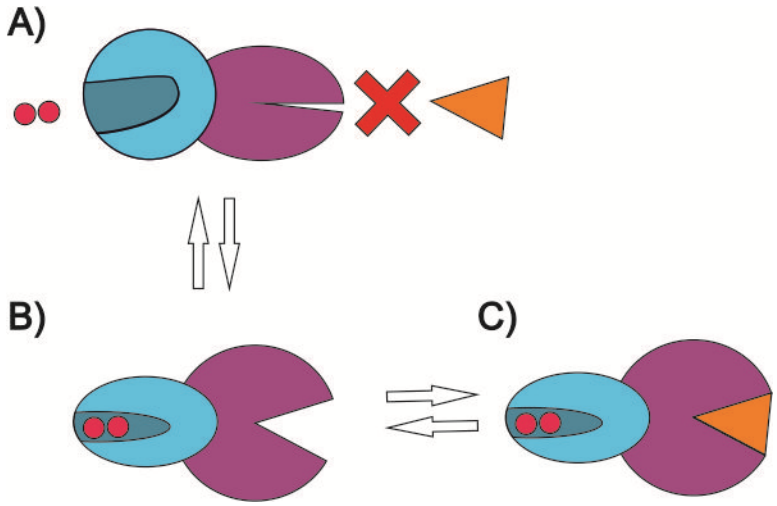


Figure 1-2 An MBP BLA fusion switch. A) When maltose (red) is not bound to MBP (blue), MBP is predominantly in the open conformation this causes the binding site of BLA (purple) to close turning off its enzymatic activity. B) When MBP binds maltose it changes to the closed conformation this induces BLA to also change conformation opening its binding site. C) With BLA in a functional conformation it is able to bind lactose (orange).

The use of proteins in a molecular switch conveys a large degree of selectivity such as the ability to differentiate between maltose and lactose. Whereas molecular switches based on synthetic molecules are typically only able to detect more distinct differences in ligands such as the radius of an ion. Although the degree of complexity and fine tuning that can be achieved using synthetic rotaxanes is impressive and a molecular switch implementing both proteins and synthetic molecules would yield the best of both worlds. The aim of this project is to investigate whether a combination of synthetic molecules and natural proteins can be used to make a functional molecular switch.

1.2 Protein-Carbohydrate interactions

Now the concept of a molecular switch has been introduced the specific interactions that lie behind the fundamental design of a maltose-responsive switch will be investigated.

Protein-carbohydrate interactions are involved in many different processes in biological systems including signal transduction[24], host-pathogen recognition[25,26] and inflammation.[27,28] Carbohydrates are found on the surface of all cells and act as the recognition system through which other cells, viruses and toxins may interact. Carbohydrate binding sites are typically shallow indentations on the protein surface, and thus protein-carbohydrate interactions are inherently weak.[29] Binding is driven by a favourable enthalpy: interactions typically involve a multitude of hydrogen bonds to carbohydrate hydroxyl groups and van der Waals interactions between the faces of the carbohydrate rings and aromatic residues in the protein.[30] However, this is offset by an unfavourable entropy term which may stem from losses of carbohydrate flexibility.[31]

Nature has many examples of protein-carbohydrate interactions that overcome these limitations on binding strength. Concanavalin A (ConA), the best studied of the lectins[32,33] (i.e., proteins which can specifically recognise oligosaccharides but which are neither an enzyme nor an antibody[34]) interacts with many cell receptors and has been shown to agglutinate red blood cells and some cancer cells.[35] ConA commonly binds non-reducing terminal and internal α-D-mannosyl and α-D-glucosyl groups. The requirements for a saccharide to bind in ConA's binding site are referred to as the Goldstein Rules and require the sugar to have free equatorial hydroxyl groups in the 3 and 4 positions and a free primary hydroxyl group in the 6 position (Figure 1-3).[36] For disaccharides such as man(α1–2)man **1.10** this allows either sugar ring to bind in the monosaccharide binding site. This ability for the disaccharide to bind in more than one conformation has been proposed to increase the probability of binding and gives a more favourable entropy contribution so in part overcoming the inherent weakness of protein-carbohydrate interactions.[34,37]

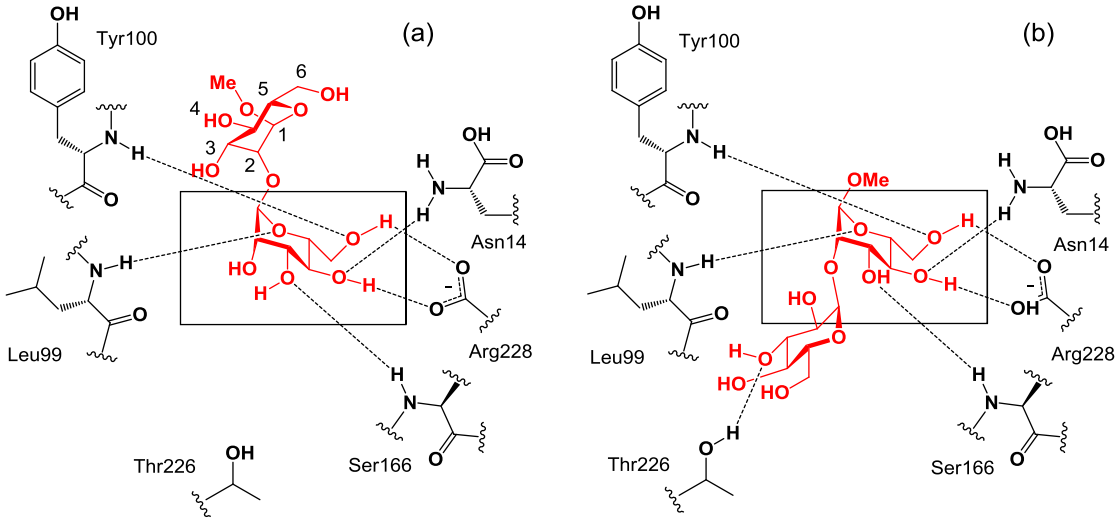


Figure 1-3 Schematic diagram of the bonding between ConA and Man α1-2 Man in the two possible conformations. The monosaccharide binding site is highlighted in the box and the positions of one of the mannose rings have been labelled.

Mannose-binding lectin (MBL) is another important carbohydrate-binding protein and is part of the innate-immune system; it recognises and binds to patterns of carbohydrates found on the surface of a range of pathogens.[38-40] MBL forms a variety of multimers (Figure 1-4) with the larger multimers having lower dissociation constants than smaller ones. Each MBL monomer contains three carbohydrate recognition domains (CRD) which mainly bind mannose and fucose residues. Multimers increase the number of binding sites on the MBL allowing it to take advantage of multivalent effects and so improve binding strength.[41] HIV has been shown to contain many high mannose carbohydrate clusters on its surface which MBL can recognise.[42] Once the pathogen is bound by MBL it activates the lectin pathway, a system similar to the classic complement pathway but not antibody dependent; or simply by binding MBL can render some viruses inactive.[43,44]

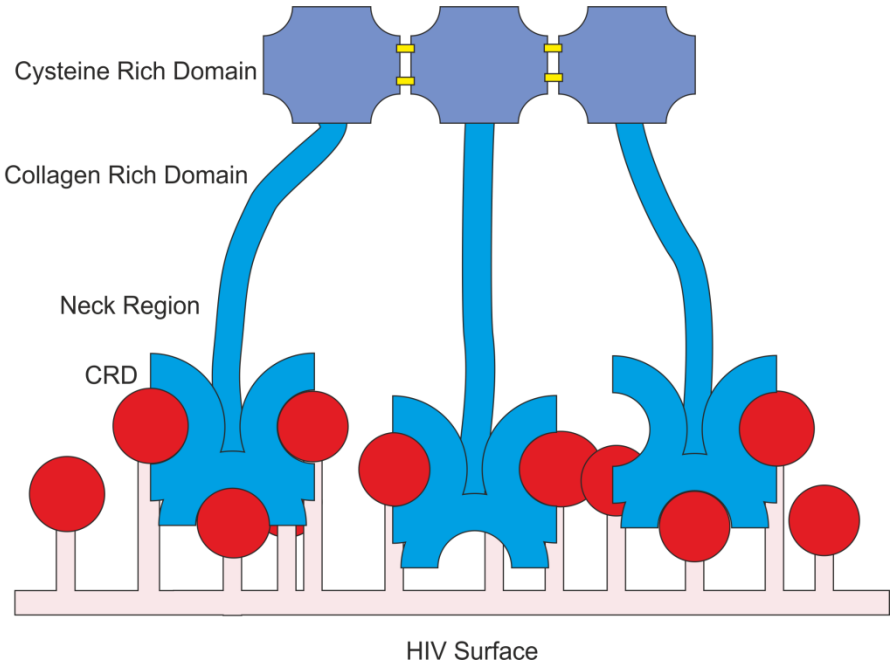


Figure 1-4 Schematic of MBL binding HIV, the MBL monomers (blue) are bound by sulphide bridges (yellow) in the cysteine rich domain to form a trimer. The MBL binds an area of high mannose (red) concentration on the surface of HIV.

Multivalency in carbohydrate-protein interactions is also used by some bacterial toxins to gain entry into cells. A classic example is Shiga toxin which compromises two subunits; the toxic A-subunit and the B-subunit which delivers the A-subunit into the cell.[45] The B-subunit binds to the glycolipid globotriaosylceramide (Gb_3) which is found on the cell surface and allows the A-subunit to enter the cell by receptor-mediated endocytosis.[46] The binding between the B-subunit and Gb_3 is enhanced by mulitvalency as the B-subunit has five binding domains that can interact simultaneously with the cell surface (Figure 1-5).[47] Furthermore, the five subunits in the Shiga toxin each have three binding sites for Gb_3 giving the B-subunit a total of 15 binding sites.[48] In this way, individual interactions with K_d = 1 mM can reinforce one another to achieve a sub-nanomolar avidity.

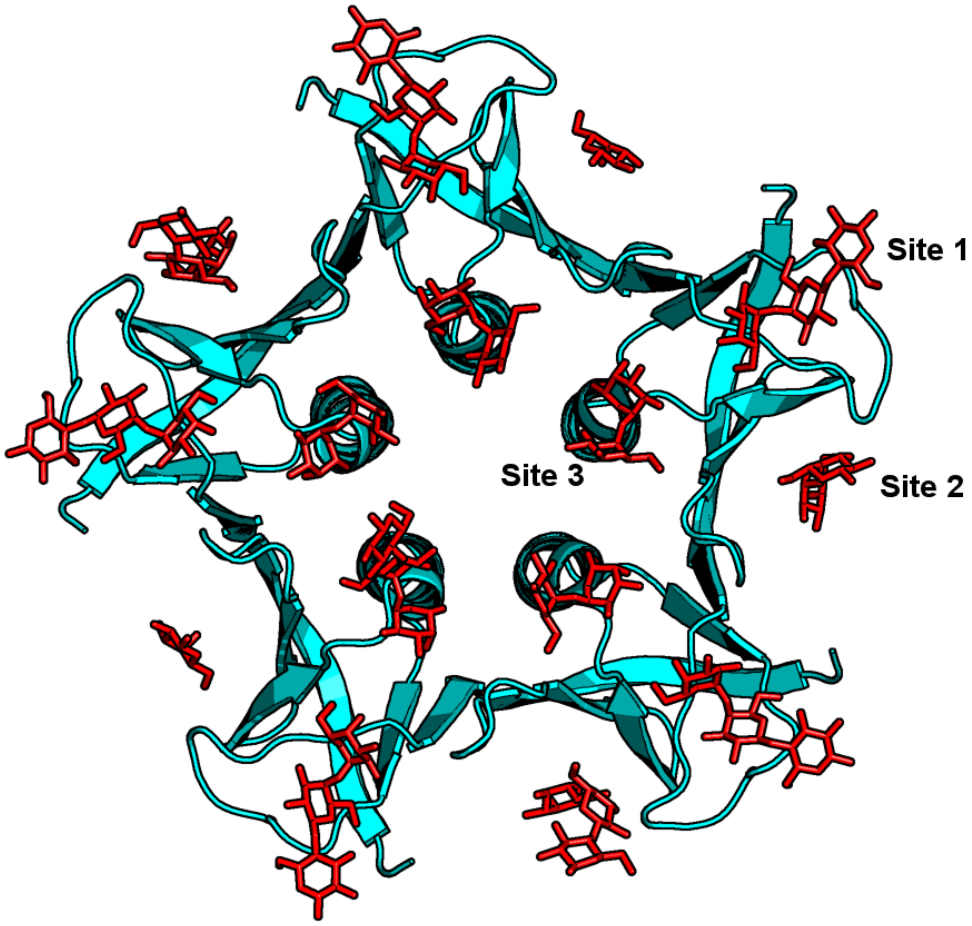


Figure 1-5 The structure of Shiga-like toxin (cyan) binding to Gb3 (red) with the three different binding sites per protomer illustrated. (PDB: 1BOS).

1.3 Multivalency

As is evident from the examples above, multivalency is a frequent feature of protein-carbohydrate interactions. While monovalent interactions are typically very weak, multivalent interactions are often found to increase the effective affinity of binding to a point where the interaction is functionally useful.

Clusters of carbohydrate epitopes are common in many naturally occurring glycoconjugates.[49] Lectins have been shown to have higher affinities for clusters of carbohydrate epitopes compared to a single oligosaccharide.[50-52] The binding of a lectin with multiple subsites to a carbohydrate cluster is relatively well understood as is the increase in binding affinities. An example is the binding of the asialoglycoprotein receptor to a trivalent carbohydrate with terminal LacNAc residues (Figure 1-6) which results in a ~10^6-fold increase in binding affinity relative to a single LacNAc residue.[53,54]

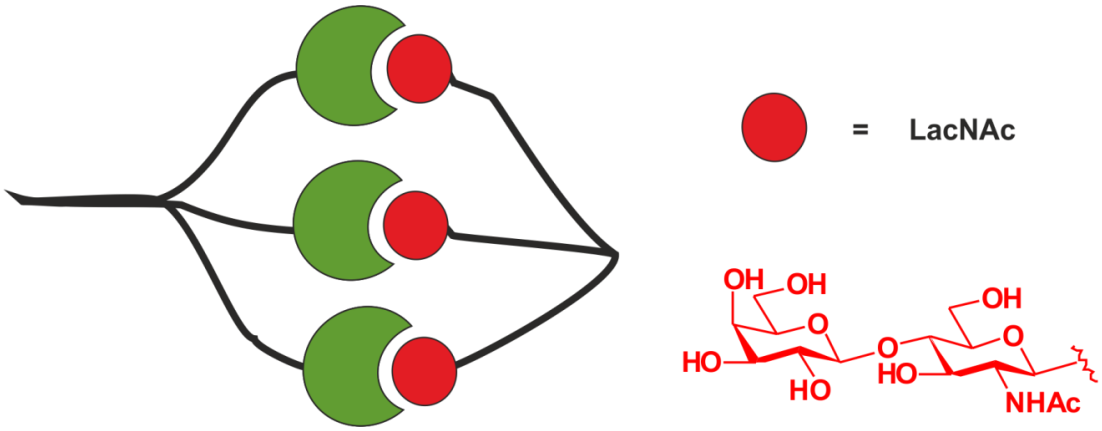


Figure 1-6 Schematic of asialoglycoprotein (green) binding to a trivalent carbohydrate with terminal LacNAc residues (blue) in the 'face-to-face' model.

In the case of asialoglycoprotein, the increase in affinity is the sum of the free energies of binding of the subsites. This is called the chelation model, and is the basis for the enhanced binding of the Shiga toxin mentioned above.[55]

Increases in affinity have also been shown for the tetravalent lectins ConA and *Dioclea grandiflora* lectin (DGL) binding to multivalent saccharides.[49] ConA was shown to bind

multivalent carbohydrates **1.11**, **1.12** and **1.13** with 6-, 11- and 35-fold increases in affinity, respectively, compared to trisaccharide **1.10** (Figure 1-7). Similar results were found for DGL which showed 5-, 8- and 53-fold increases in affinity for **1.11**, **1.12** and **1.13** respectively.[56] This could not be due to chelation as the binding sites in ConA and DGL are too far apart for the glycosides to bind more than one at a time.

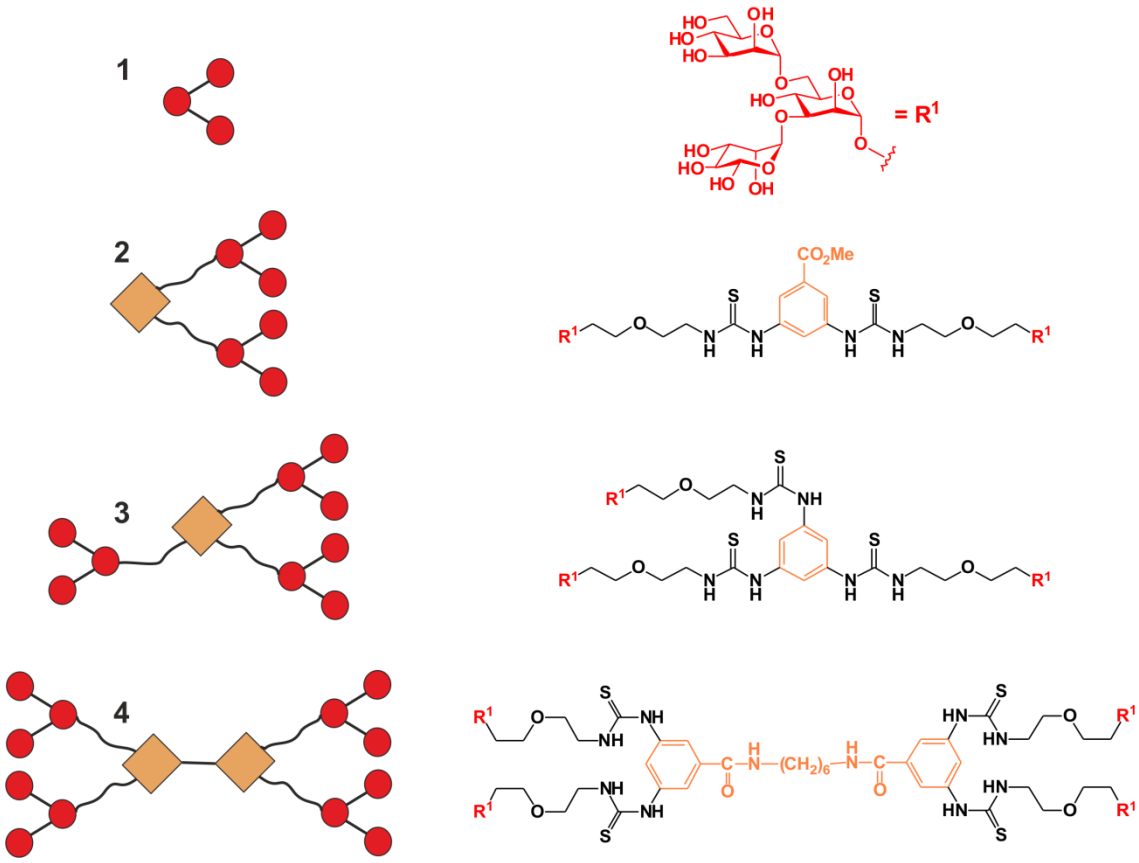


Figure 1-7 Schematic representations of **1.10** a trisaccharide and cluster glycosides **1.11**, **1.12** and **1.13**. With the structures also shown.

The increases in affinity for ConA and DGL were also found to be much lower than those expected from the chelation model. The increase in binding affinity in this case is proposed to be due to an increase in entropy of binding to the multivalent ligand. As the multivalent ligand has another epitope in close proximity to the bound epitope the effective concentration of the ligand around the binding site and so chances of rebinding when the first epitope dissociates is increased (Figure 1-8).[56]

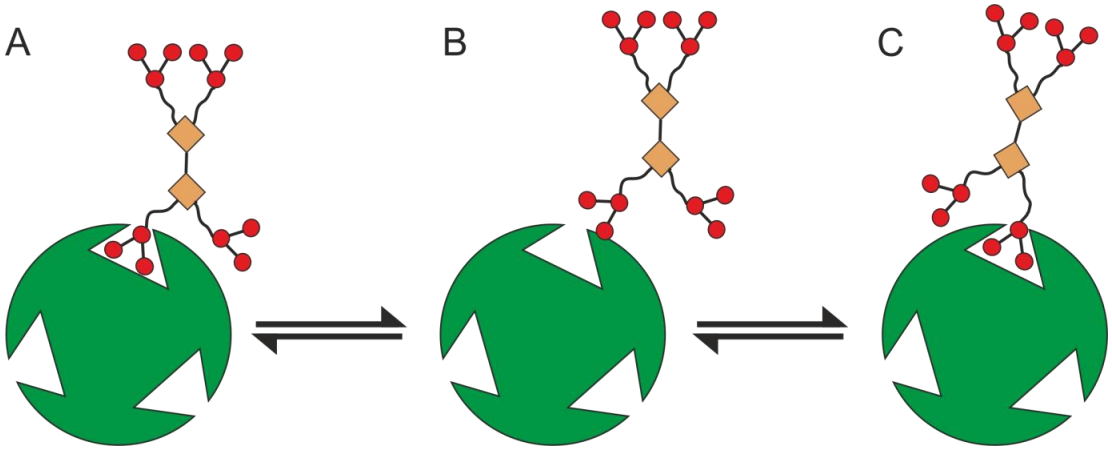


Figure 1-8 Schematic representations of binding of a tetravalent ligand **1.13** to ConA (green), in A ConA binds one epitope which then disassociates (B). ConA can then either rebind the original epitope (A) or bind a different epitope (C), this increase in entropy leads to an increase in binding strength.

This use of repeated epitopes to improve binding by recapturing the ligand is repeated elsewhere in biology but with affinities much closer to those achieved in chelation binding. An area where this process has been investigated is in the binding between DNA regulatory proteins and DNA. Evidence suggests that the DNA regulatory proteins bind at a non-specific sequence and then diffuse along the backbone of the DNA until reaching the site for which the protein has the highest affinity.[57,58] The energy barrier for moving from base pair to base pair is so small that the regulatory protein can traverse 10,000 base pairs every second. This is called the 'bind and slide' mechanism (Figure 1-9).[59] This mechanism allows the regulatory protein to find faults in the DNA and repair them before mutations occur and is much faster than if it was to detach from the DNA and then reattach at each different site along the strand, which is referred to as 'bind and hop'.

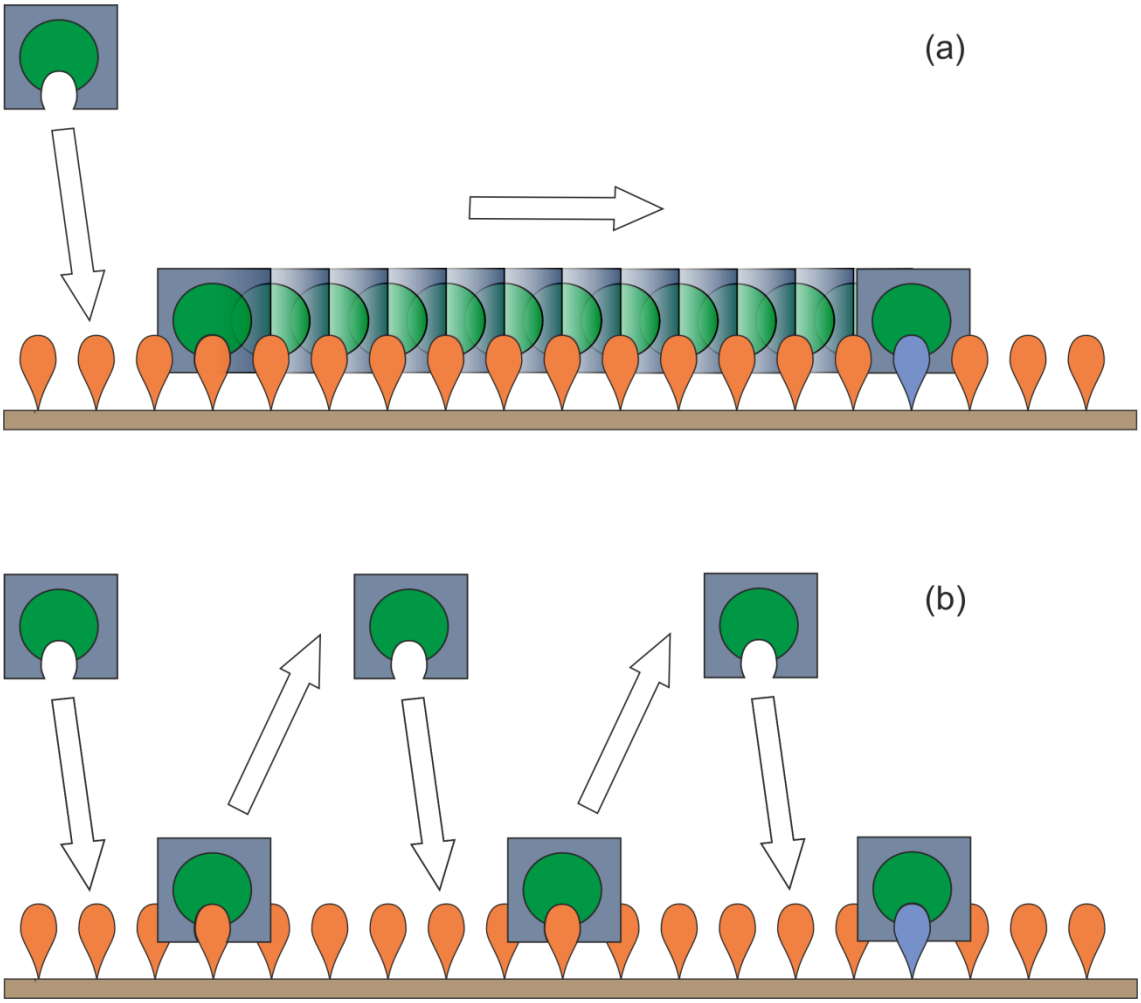


Figure 1-9 Schematic of (a) the 'bind and slide' mechanism and (b) the 'bind and hop' mechanism. The regulatory protein is shown in green/grey and the DNA backbone in orange/brown.

A similar bind and slide mechanism has been observed for lectins binding to mucins, these are heavily glycosylated gel forming proteins produced in epithelial cells intrinsic to most gel-like secretions. In this interactions it was shown that an increase in carbohydrate chain length led to a greater affinity.[60,61] The enhancement arises from the lectin having a much greater length of mucin to travel along before either reaching the chain end or meeting another lectin and thus having to dissociate and rebind.[62]

These three multivalent effects; chelation, repeated epitopes and 'bind-and-slide' are some of nature's most efficient methods of increasing the strength of protein-carbohydrate interactions.

1.4 Maltoporin

Maltoporin is a bacterial outer membrane protein that forms a trimeric channel through the membrane to allow starch fragments to enter the periplasm of the bacterium. The protein was first identified in 1973 as the receptor for bacteriophage-λ, hence it also being referred to as LamB.[63] Maltoporin is a maltodextrin-specific channel as well as acting as a diffusion pore for ions and other small hydrophilic solutes. Its structure has been determined by X-ray crystallography.[64] Maltoporin is a trimeric channel with C_3 symmetry, in which an 18-strand beta-barrel forms each channel (Figure 1-10). The beta-sheet has an all next-door neighbour anti-parallel fold and is formed of 246 residues (in *Salmonella typhimurium*). At the extracellular edge the beta-strands are connected by long loops (L1-L9) averaging 17 residues. On the periplasmic side the beta-strands are connected by much shorter turns averaging only 4 residues.

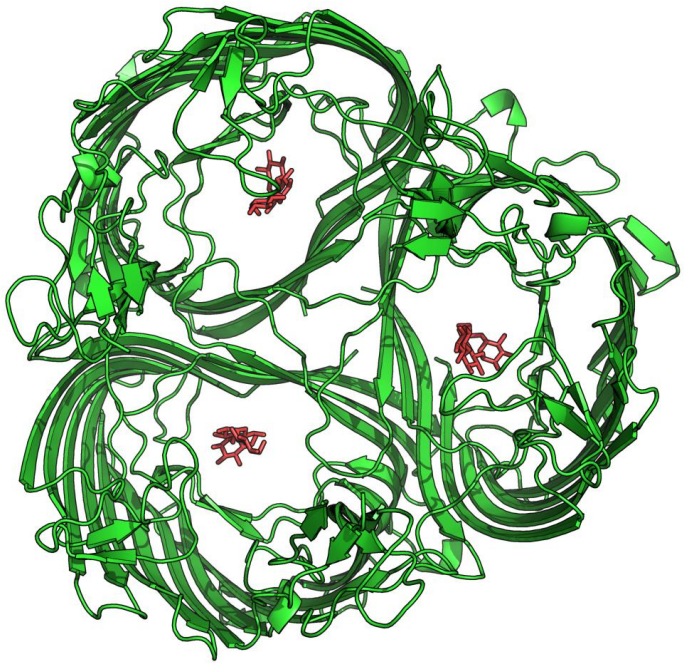


Figure 1-10 The structure of maltoporin (green) shown from extracellular space showing a trimeric structure with the maltotriose (red) bound in all three pores. (PDB: 2MPR).

Loop L2 is positioned near the trimer axis and reaches over into an adjacent subunit placing Trp74 into the adjacent subunit's pore. Loops L4, L5, L6 and L9 form a

protrusion at the extra cellular edge while loops L1, L3 and the beginning of L6 fold into the beta-barrel and pack against the pore wall, creating a constriction within the pore (Figure 1-11). The inner loops L1, L3 and the beginning of L6 are rigidified by the only disulfide linkage in the subunit which is found between two cysteine residues of L1. This rigid constriction is involved in the selectivity of the pore for maltoligosaccharides (MOS).

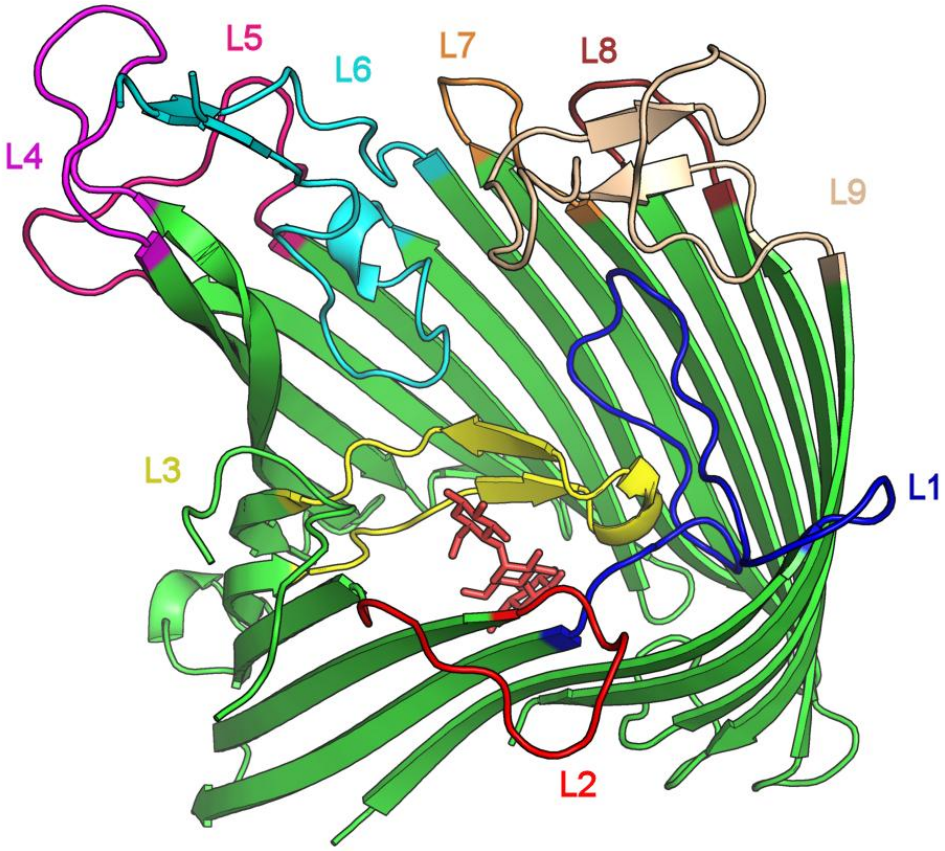


Figure 1-11 The inside of maltoporin's (green) beta-barrel shown from extracellular space with all the loops labelled. Loops 1, 3 and 6 can been seen folding into the beat-barrel. Maltotriose (red) is also shown in the constriction site. (PDB: 2MPR).

Within each pore there is a row of aromatic side chains, beginning with Trp74 from the L2 loop of an adjacent subunit. These side chains follow a left handed helix along Tyr41, Tyr6, Trp426, Trp368 to Tyr227 which sits at the periplasmic end of the subunit. These residues together are referred to as the 'greasy slide' (Figure 1-12).[65] It has been shown that Tyr41, Tyr6 and Trp426 bind to maltodextrin while the other residues

act as the ‘slide’ allowing maltodextrin to pass through the pore and into the periplasm. There also exists a multitude of polar residues on either side of the ‘greasy slide’ which form hydrogen bonds with the sugar. The polar residues line the length of the ‘greasy slide’ allowing the hydroxyl groups of the sugar to ‘bind and slide’ into the constriction site. When combined with the ‘greasy slide’, the polar residues ensure that there is no large energy barrier to be overcome for the sugar to access the pore. Tyr118, separated from the ‘greasy slide’ on the opposite side of the constriction site is thought to act as a steric barrier only allowing specific molecules such as maltodextrin to pass through the pore.

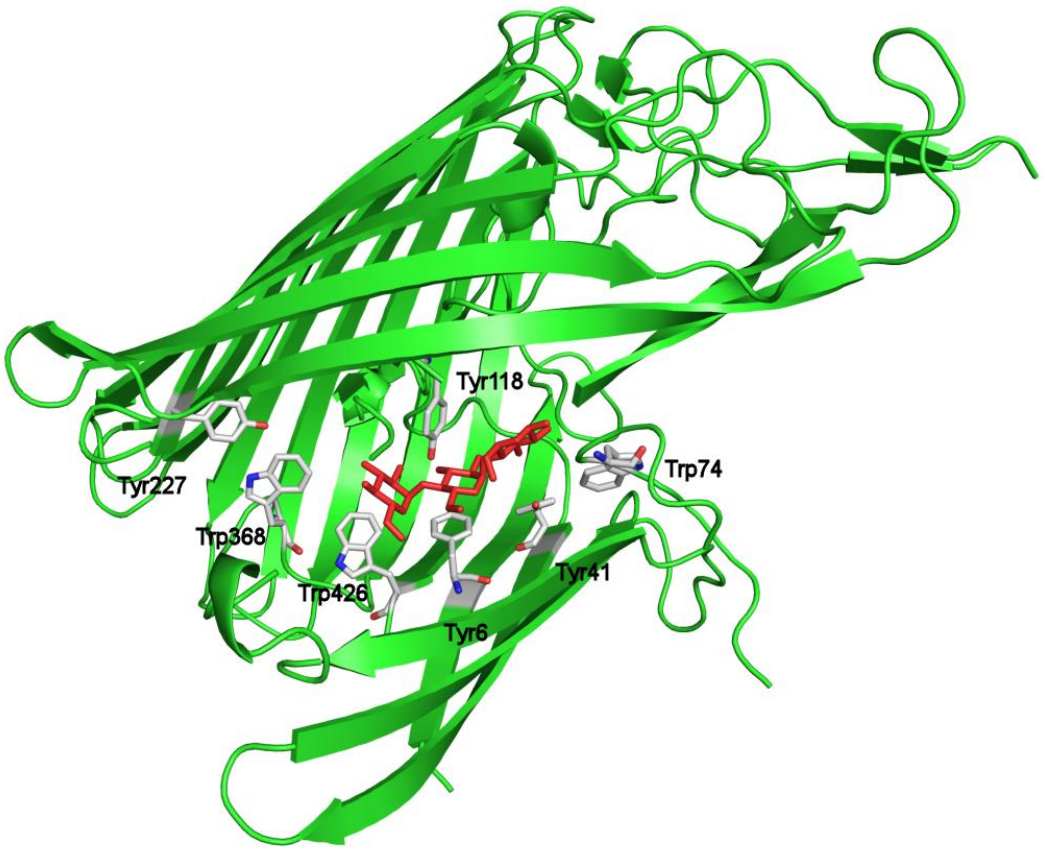


Figure 1-12 The inside of maltoporin’s (green) beta-barrel showing the greasy slide (white) with maltotriose bound (red). Maltotriose is shown interacting with residues Trp426, Tyr6 and Tyr41. Tyr118 is not part of the greasy slide instead acting as a steric barrier. The front face of the pore has been removed for clarity (PDB: 2MPR).

Crystal structures of maltoporin binding to maltodextrin as well as maltodextrin mimics have been determined.[64,66,67] It has been shown that maltodextrin enters the pore with its non-reducing end leading from the extracellular side. There are significant van der Waals interactions between g4, g3 and g2 (rings of MOSs from g1 at the reducing end (Figure 1-13)) and Trp426, Tyr6 and Tyr41 respectively.[64,67] Maltodextrins longer than three residues do not show additional binding interactions to the greasy slide as the left handed helix of maltodextrin does not exactly match the helix of the greasy slide. The maltodextrin also binds through a series of hydrogen bonds to polar residues in the pore eyelet. When unligated the constriction site is occupied by water molecules which are visible by crystallography and are therefore tightly bound.[67] As these water molecules are displaced on binding, only a ligand capable of replacing the lost hydrogen bonds is likely to enter the pore. There are only minor conformational changes in the pore eyelet on binding maltodextrin as the charged residues in the constriction site form salt-bridges between themselves adding to the rigidity of the structure.

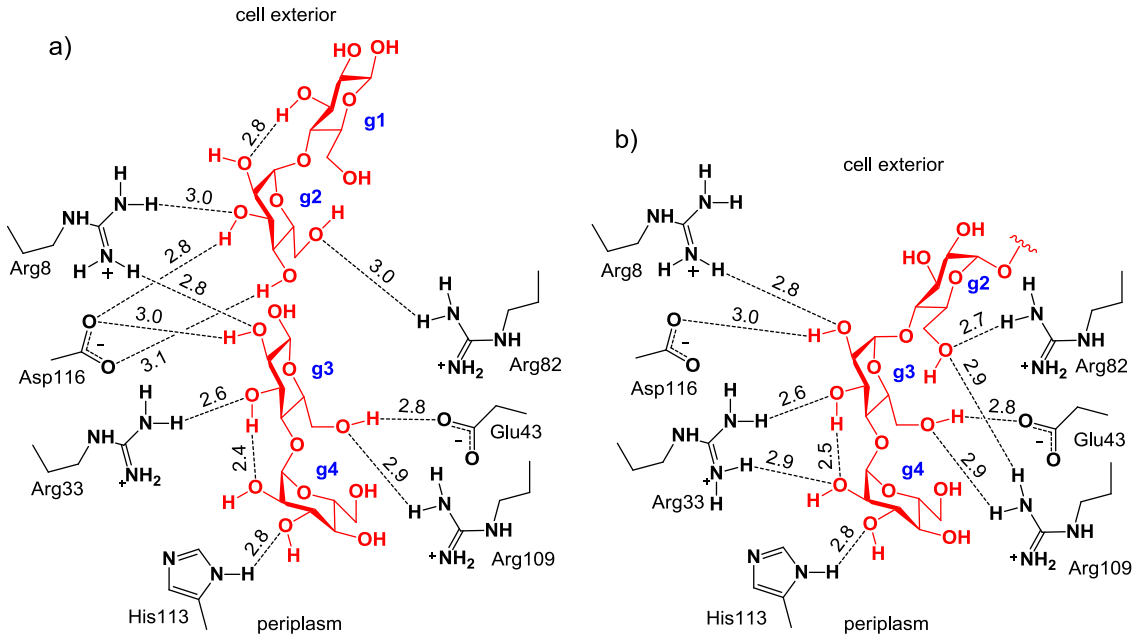


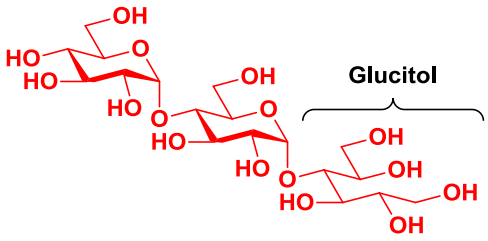
Figure 1-13 Here the binding of MOSs (blue), maltose (a) and heptamaltose (b) within maltoporin (black) is shown, g4 and g3 bind in a similar manner for both molecules while g2 is found twisted towards Arg82 in (b).

While g3 and g4 are found to bind in very similar positions whatever the size of the MOS, the position of g2 varies depending on the number of glycosyl moieties in the MOS (Figure 1-13). This is due to the large amount of space available in the entrance to the pore compared to the constriction site leading to an increased variability of the position of the tail end of the molecule. Interactions of glycosyl moieties on either side of g2 thus determine its position in the channel.

The three protomers that form the maltoporin channel bind sugars independently of each other.[68] Electrical conductance measurements across a black lipid membrane have shown that the association rates of maltodextrins are largely independent of the chain length while the dissociation constant decreases with chain length (Table 1-1).[69] In other words, the dissociation constants decrease from di- to hexasacchardies. This observation appears to contradict the structural data which suggests that all MOS make similar interactions with the pore. However, once the sugar is bound it can be thought to behave in a similar manner to the 'bind and slide' mechanism: maltoporin can bind equally well to any section of the MOS, and as dissociation only occurs once it reaches the end of the sugar, longer MOSs will have lower dissociation constants. This is similar to the mechanism previously observed for lectins binding to mucins.

Table 1-1 The affinities of maltoporin and MBP for relevant oligosaccharides.[70,71]

K_d / µM maltoporin	K_d / µM maltose binding protein (MBP)
10,000 Maltose	1.0 Maltose
	75.0 Maltotriitol
59 Maltopentaose	2.9 Maltohexaitol



Maltotriitol

1.5 Maltose Binding Protein (MBP)

Maltose Binding Protein (MBP) is a monomeric protein that binds MOSs in the periplasm and transfers them to transporter proteins in the inner membrane.[71] MBP consists of two globular domains separated by a groove where the MOS is bound.[72] These two domains are referred to as the C- and N-domains, containing the C- and N-termini respectively, and are connected by three segments that form a 'hinge' between the C- and N-domains (Figure 1-14).[73,74] Both globular domains exhibit similar structures that consist of a beta-pleated sheet flanked on both sides by two or three parallel alpha helices.[75]

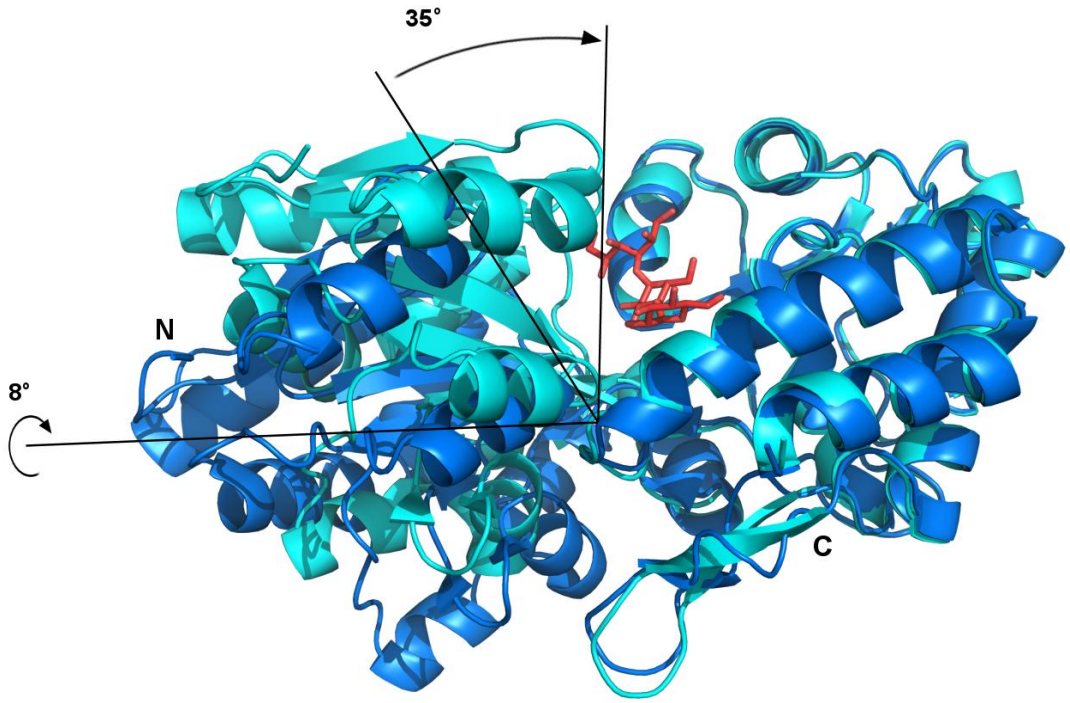


Figure 1-14 An overlay of MBP shown in the open form (blue) and the closed form (cyan), a hinge angle of 35° and a rotation of 8° is shown between the two conformations. Maltotriotol (red) is shown bound in the open conformation. (PDB: 1OMB).

MBP binds MOS in two different modes which can depend on the length of the oligosaccharide. The different modes are shown above. The change between them is

characterised as a closing of the two globular domains by 35° around the hinge. In both binding modes the ligand is bound in the cleft between the N and C domains (Figure 1-14). The two modes are referred to as 'end on' and 'side on'. In 'end on' binding MBP binds to the reducing terminus of the sugar, allowing the protein to adopt a closed conformation. MBP can also bind to the middle of longer oligosaccharides, 'side on'; in this binding mode MBP adopts an open conformation.[76] Reduced, oxidized, or cyclic maltodextrin derivatives can only bind 'side on', only linear maltodextrin up to 8 glucose units long can bind 'end on'.[71,77] As MBP can only dock to the transporter protein at the inner membrane when it assumes the closed conformation it is only these linear maltodextrins that can be transported. In the open conformation the binding site is open to solvent while in the closed conformation the two globular domains move around the hinge to surround the bound MOS allowing no contact with the solvent (Figure 1-15).

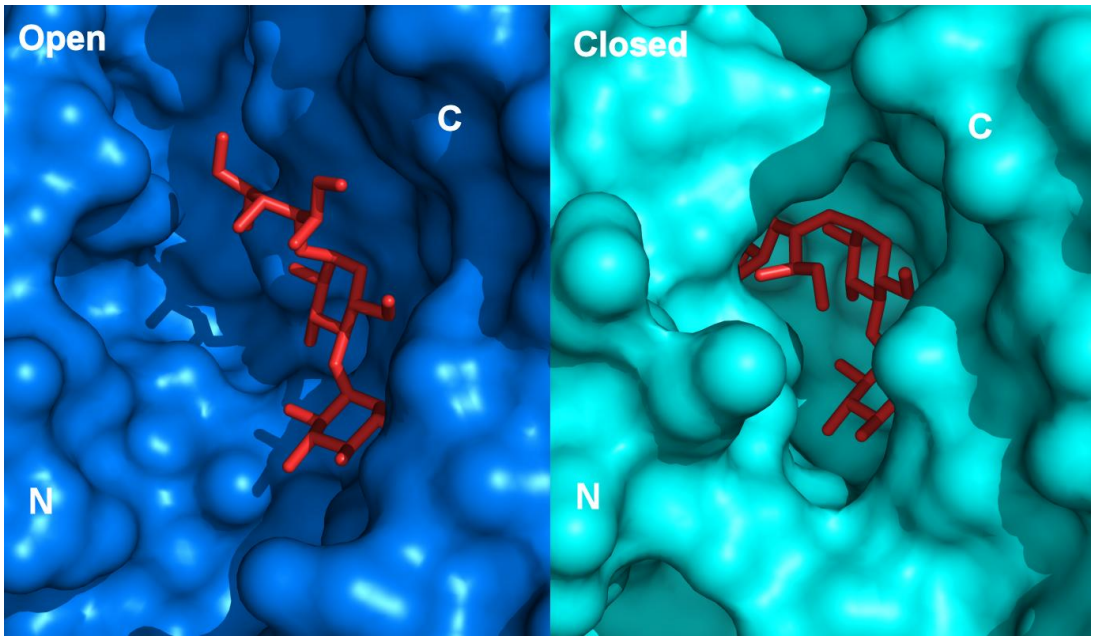


Figure 1-15 Maltotriitol (red, shown below) binding in the open conformation (left) and the closed conformation (right), it occupies nearly identical binding sites except that in the open case the C domain (on the right) is not involved in binding. The glucitol group of maltotriitol (Figure 1-16) is shown extending in to the s-1 binding site in the open conformation while it bends round in the closed conformation. (PDB: 1FQC and 1FQB).

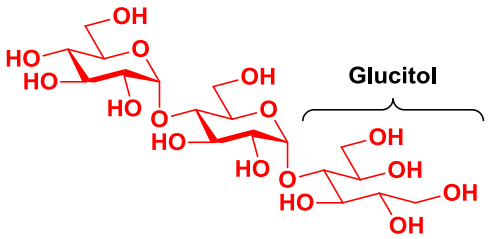


Figure 1-16 Maltotriitol

Both globular domains contribute to MOS binding by forming a binding site of flat aromatic residues on the top and bottom faces (Figure 1-17) with polar residues along the edges. This design is complementary to the shape of maltodextrins which adopt a conformation like a twisted ribbon with all their hydroxyl groups along the edges and the apolar ring faces making up the flat surface of the ribbon. Charged side chains line the edges of the binding site and hydrogen bond to the sugar. All the hydroxyl groups on glucose residues g1, g2 and g3 (beginning from the reducing end) are involved in hydrogen bonding (Figure 1-17). Residue g4 is partially exposed to solvents and any residues beyond this point would be entirely outside the binding cleft. The aromatic residues stack against the apolar ring faces of the MOS creating van der Waals interactions.[75,78]

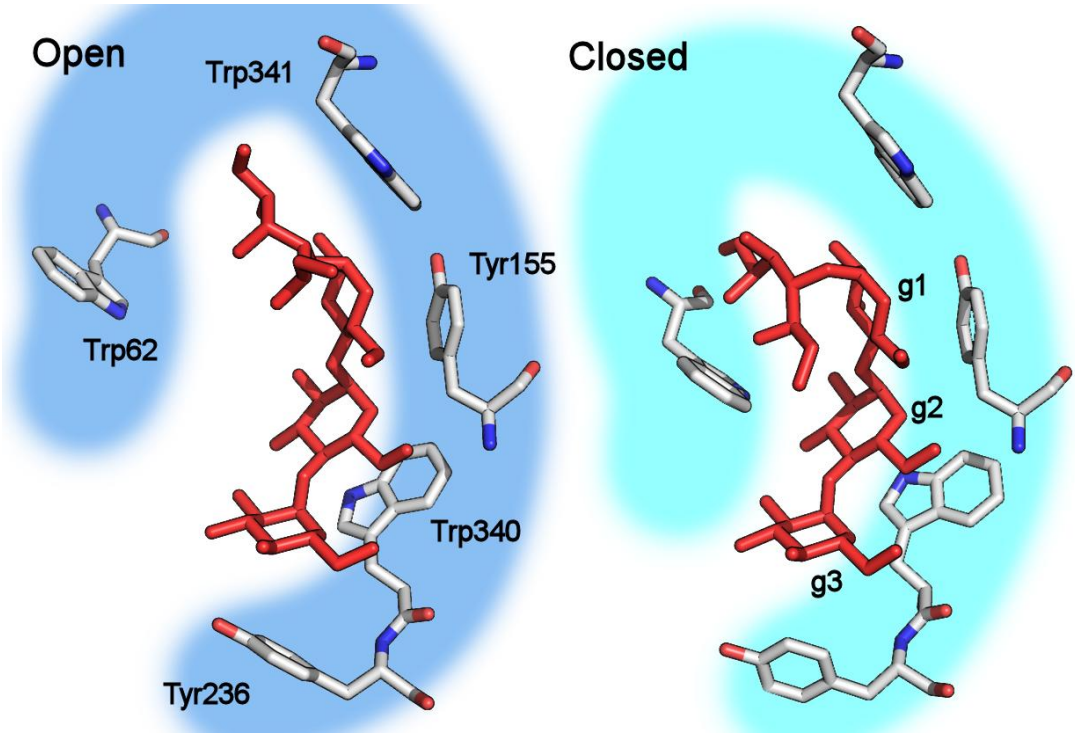


Figure 1-17 Aromatic stacking between maltotriitol and aromatic residues in both the open and closed conformations. Little difference is observed between the stacking of g1, g2 and g3 in both binding modes.

The K_d values for MBP binding to three different MOSs (maltose, maltotriose and maltotetraose) have been determined as 3.5, 0.16 and 2.3 µM respectively, which are some of the tightest protein-carbohydrate interactions known.[66] The g1-4 residues occupy four distinct subsites in MBP with the glycosyl residues g1 and g2 having the greatest interaction with the MBP binding site (Figure 1-18). These sites are occupied in both the closed and open binding conformations. It has been shown with maltotriitol (Figure 1-16) that a -s1 binding site exists beyond the s1 binding site which is bound by the glucitol residue of maltotriitol.[76]

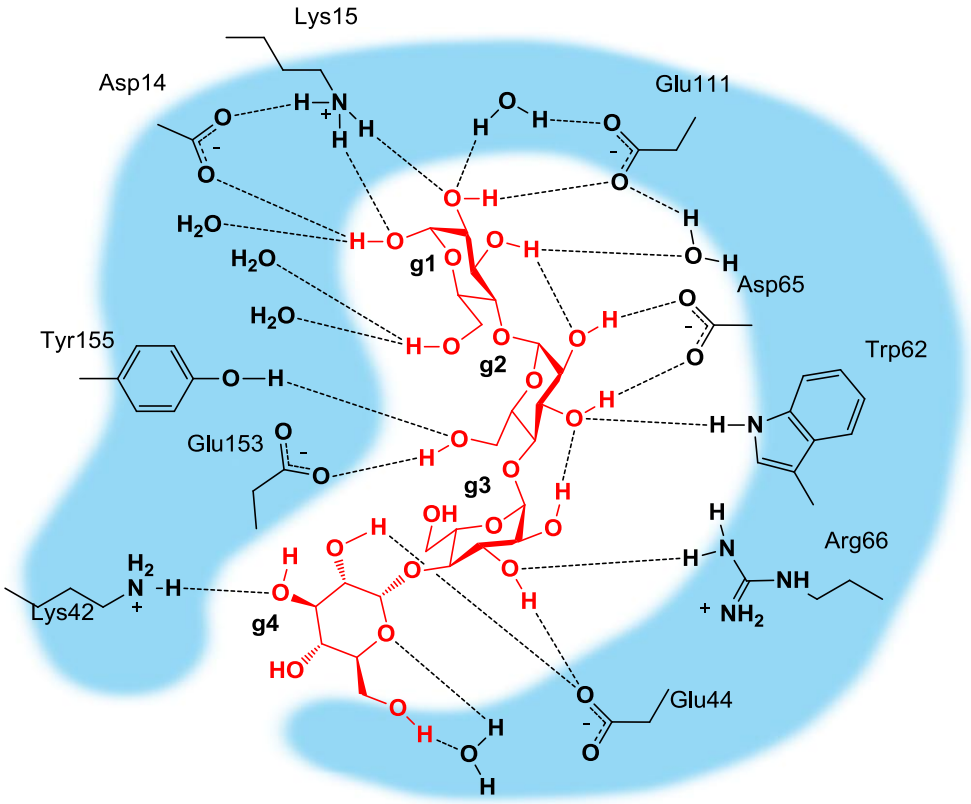


Figure 1-18 Hydrogen bonding between MBP and maltotetraose (red). This is an example of closed binding.

In the open conformation the MOSs bind in the same subsites as closed but the two globular domains do not move around the hinge to bury the ligand (Figure 1-14). Binding to the N-domain is greatly diminished,[76] reducing much of the hydrogen bonding as the N-domain contains the majority of the polar side chains. The open conformation makes the –s1 binding site more accessible and molecules such as maltotriitol can then occupy this site.

1.6 Aims and Objectives

1.6.1 Aims

In this project we aim to use maltoporin as a receptor to attach biomolecules at a bacterial cell surface. This approach will use MOSs as part of a ligand taking advantage of the multivalent binding models previously discussed as chelation and 'bind and slide'. The chelation effect (Figure 1-19) could be used to anchor a molecule to maltoporin through a trimeric ligand while the 'bind and slide' mechanism could allow for the production of a maltose responsive switch (Figure 1-21) that could be used as a sensor for the presence of MOS in solution. Feasibly, such a sensor could be used to alter the surface properties of the bacterium, for example to allow it to bind reversibly to a surface.

This method would allow for the modification of bacterial cell surfaces without the need for genetic modification. With the development of supramolecular engineering at the cell surface, it could be possible to bestow temporary adhesive properties to therapeutic bacteria allowing them to colonise a patient during a course of treatment. It may also be possible to attach antigenic groups to "inert" bacteria to create live vaccines without the risk of causing serious infections. New opportunities such as this to modify and control bacteria will ultimately lead to the medicines of the future.

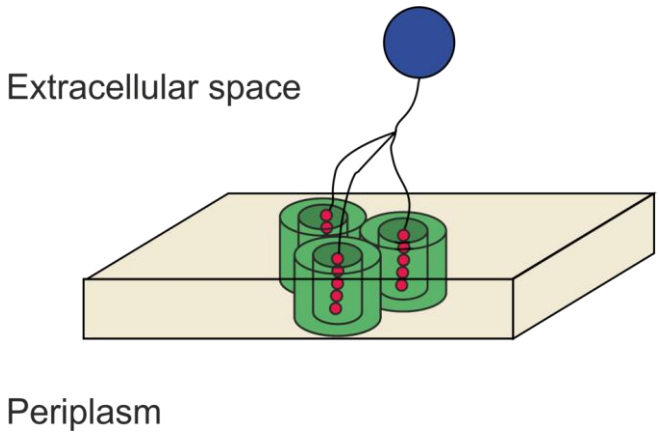


Figure 1-19 A cartoon representation of a multivalent ligand anchoring a biomolecule to a cell surface with malto-oligosaccharide (red), biomolecule (blue) and maltoporin (green).

It was anticipated that this molecular switch would be made with a single chain ligand that comprises maltosyl units linked together by PEG chains (Figure 1-20) that could bind to maltoporin at different points along its length. This ligand would be able to thread through maltoporin to form a pseudorotaxane. At the “reducing end”, a large molecule of interest could be attached, which would act as a stopper to prevent the molecule from passing through maltoporin into the periplasm.

Figure 1-20 A schematic of the ligand.

Once the ligand had passed through maltoporin it would become bound by MBP in the periplasm. As MBP binds more tightly to the MOS than does maltoporin (Table 1-1), the ligand would effectively be “pulled” through maltoporin until the stopper comes into contact with the cell surface and prevents anymore of the ligand passing through. This arrangement should be relatively stable until the system comes into contact with maltose which has higher affinity than MOS for MBP. In the presence of maltose, MBP would thus release the ligand allowing it to move back through maltoporin. When the maltose concentration decreases, for example, after it has been transferred into the cytoplasm of the bacterium, MBP would once again bind the ligand bringing the appended biomolecule close to the opening of maltoporin. This mechanism would act like a switch that can be activated by the presence of maltose (Figure 1-21).

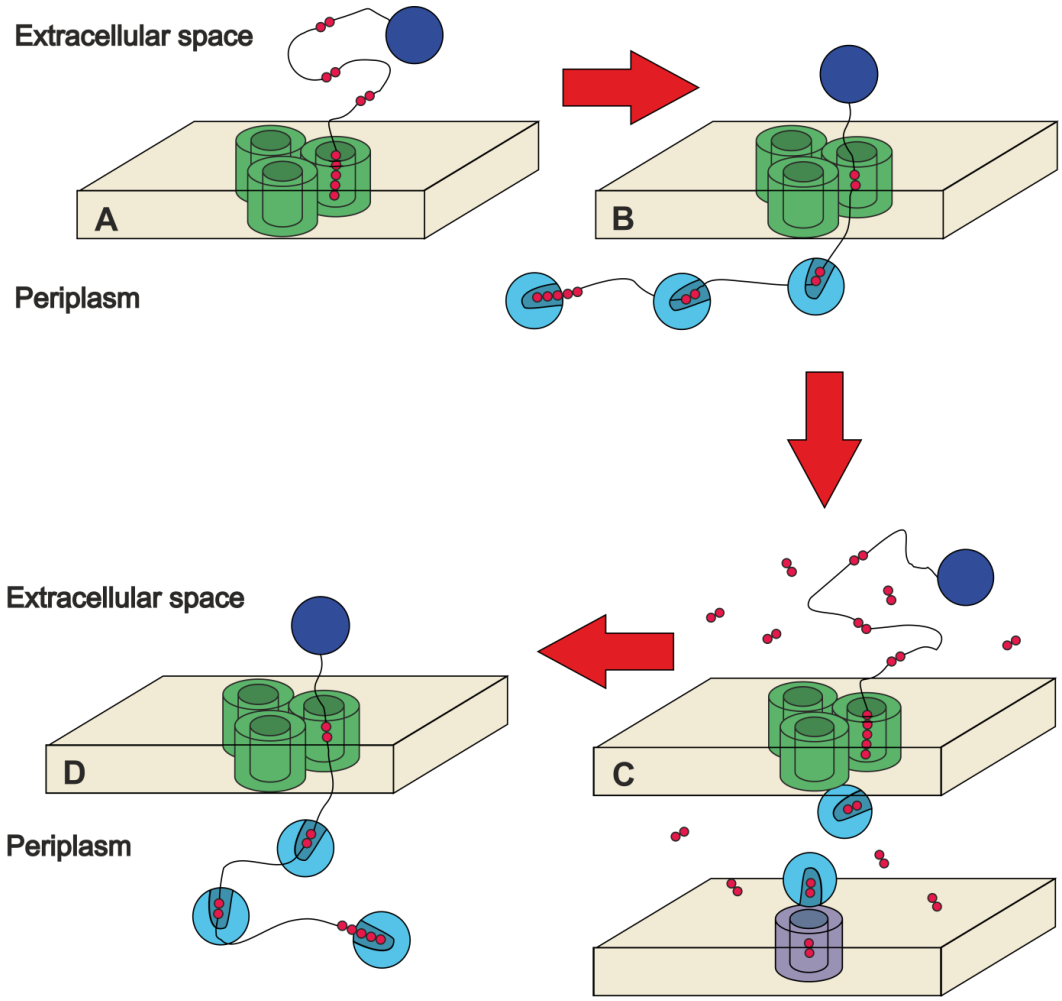


Figure 1-21 Cartoon representation of molecular switch. Glucose moiety (red), maltoporin (green), MBP (blue), maltose transporter (purple), biomolecule (dark blue) and the outer and inner membranes (cream). (a) The ligand initially binds maltoporin (b) MBP binds the the ligand pulling the biomolecule close to the cell membrane. (c) On addition of maltose MBP releases the ligand, preferentially binding maltose. The biomolecule is free to return to its original position. (d) Once all the maltose has been removed MBP again binds the ligand holding the biolmolecule close to the cell surface.

The design for the molecular switch is based on the different affinities of maltoporin and MBP for different MOS. In the polymeric ligand, the reducing end of each sugar will be attached to the non-reducing terminus of the next maltose unit; therefore only the open conformation of MBP binding will be possible.[71,76] Comparison of binding affinities of reduced MOSs to MBP and maltoporin (i.e., in the side-on binding mode) shows that MBP should bind with a greater affinity than maltoporin to the MOSs within the ligand

(Table 1-1). For example, MBP binds maltohexaitol 20 times and maltotriitol 130 times more tightly than maltoporin binds maltopentaose and maltose, respectively.[70,71] This means as the ligand threads through maltoporin it will become preferentially bound by MBP in the periplasm, effectively pulling the ligand through until the stopper on the extracellular side of the ligand prevents any more passing through (Figure 1-21). The result will be a rotaxane-like structure holding the appended biological molecule close to the cell surface.

MBP has an affinity 75 times higher for maltose than it does for maltotriitol (a good analogue for the maltose mimics in the ligand). On addition of maltose, the MBP will preferentially bind maltose releasing the ligand and allowing the pseudorotaxane to move back through maltoporin. Maltoporin has an affinity 170 times higher for maltopentaose than maltose. The proposed ligand will take advantage of this by having a maltopentaose at the non-reducing terminus. After release by MBP the ligand can move through maltoporin in a 'bind and slide' manner.[30] This allows the ligand to assume the position with the greatest binding affinity and so place the maltopentaose residue in the constriction site of maltoporin. As this residue is at the non-reducing terminus it places the appended biomolecule as far out into extracellular space as possible (Figure 1-21).

After MBP binds maltose it takes it to a transporter in the inner membrane where it is removed from the periplasm.[79] This means if no more maltose is added to the system the concentration of maltose will decrease allowing MBP to rebind the ligand. Thus the absence/presence of maltose can be used as a reversible switch to trigger a change in the cell surface of the bacterium. This movement could be converted into a signal for the presence of maltose with use of fluorescence. If a fluorophore was attached to the reducing end of the ligand and its FRET pair attached to maltoporin the fluorescence of the fluorophore would change according to their proximity.

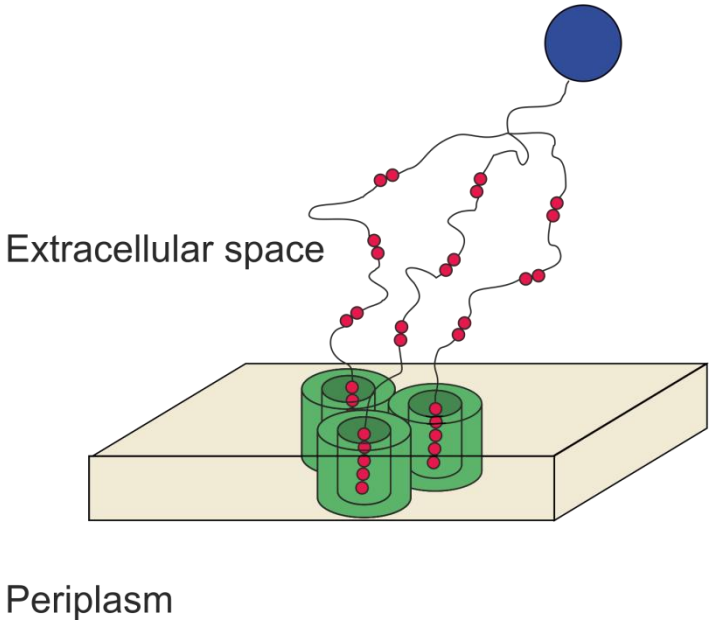


Figure 1-22 Schematic of a multivalent maltose responsive switch.

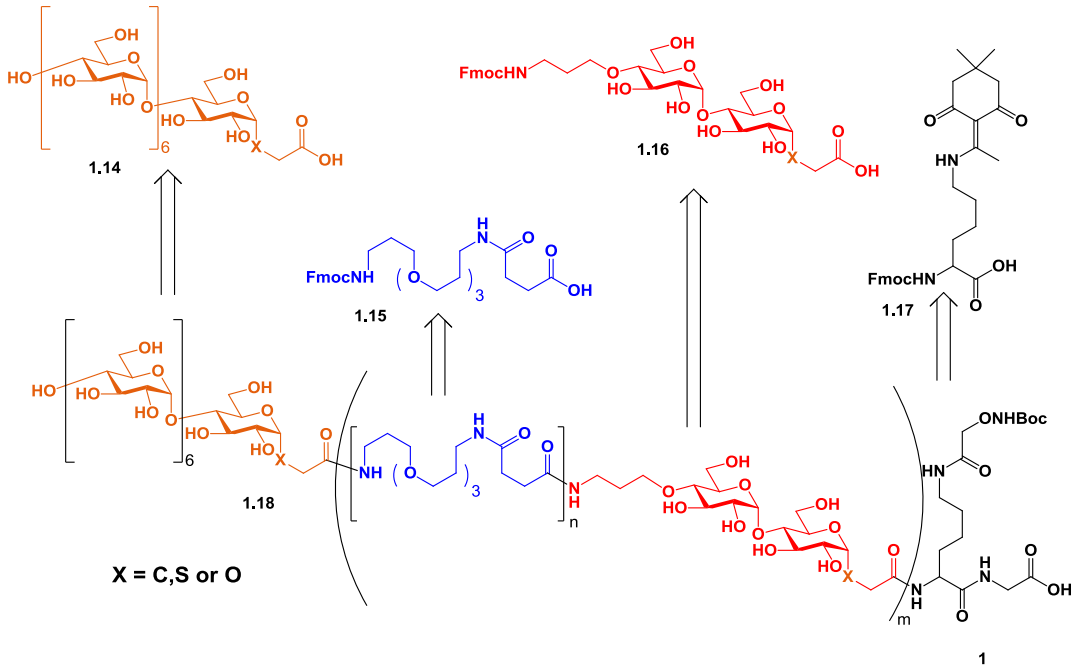
As protein-carbohydrate interactions are very weak, the binding of the ligand through maltopentaose may be too small to anchor it to the bacterium. If this proved to be the case a multivalent approach could be used, in which a trivalent ligand will bind to all three of the channels in maltoporin (Figure 1-19). A multivalent ligand would have affinities in the nM as opposed to mM or μM range, greatly increasing the binding strength.[49] As well as the molecular switch described above, a multivalent approach could be used to redecorate the surface of bacteria. With the use of three MOS ligands a biological molecule could be bound stably to maltoporin (Figure 1-22). This would allow the attachment of a wide range of molecules to the surface of bacteria without needing to genetically engineer the bacterium.

1.6.2 Objectives

1.6.2.1 Design of the switch axle

The axle for the switch was to be developed based on solid phase peptide synthesis (SPPS). This method would allow for formation of a variety of ligands using simple building blocks.[80] A retrosynthesis of the axle showed four sections that would need to

be synthesised (Scheme 1-5). They are a maltoheptaose fragment **1.14** at the non-reducing end, a central maltose fragment **1.16**, a capping lysine derivative **1.17** and PEG-linkers to connect the fragments **1.15**.



Scheme 1-5 The switch axle **1.18** with the maltose mimic **1.16** (red), PEG amino acid linker **1.15** (blue) and the maltopentaose moiety **1.14** (orange) highlighted.

Maltoheptaose was chosen as the high affinity ligand as it could be purified from β-cyclodextrin. [81] Maltoheptaose and the central maltose fragment were designed to be preferably *C*-glycosides or *S*-glycosides as these avoid any risk of cleavage by glycosidases in the periplasm.[82] It was anticipated that the axle would be synthesised from the reducing to the non-reducing end, therefore the carboxylic acid residues were placed at the reducing ends of the sugars.

The central maltose fragment **1.16** was to be derivatised at the 4'-position because maltoporin's natural substrates are connected via this position. As MOSs are connected by α-1,4-glycosidic linkages, this motif was to be repeated in the switch axle **1.18** to give the highest probability of the axle being able to thread through maltoporin. Therefore, it was anticipated that the sugars within the molecules would all be α -glycosides.

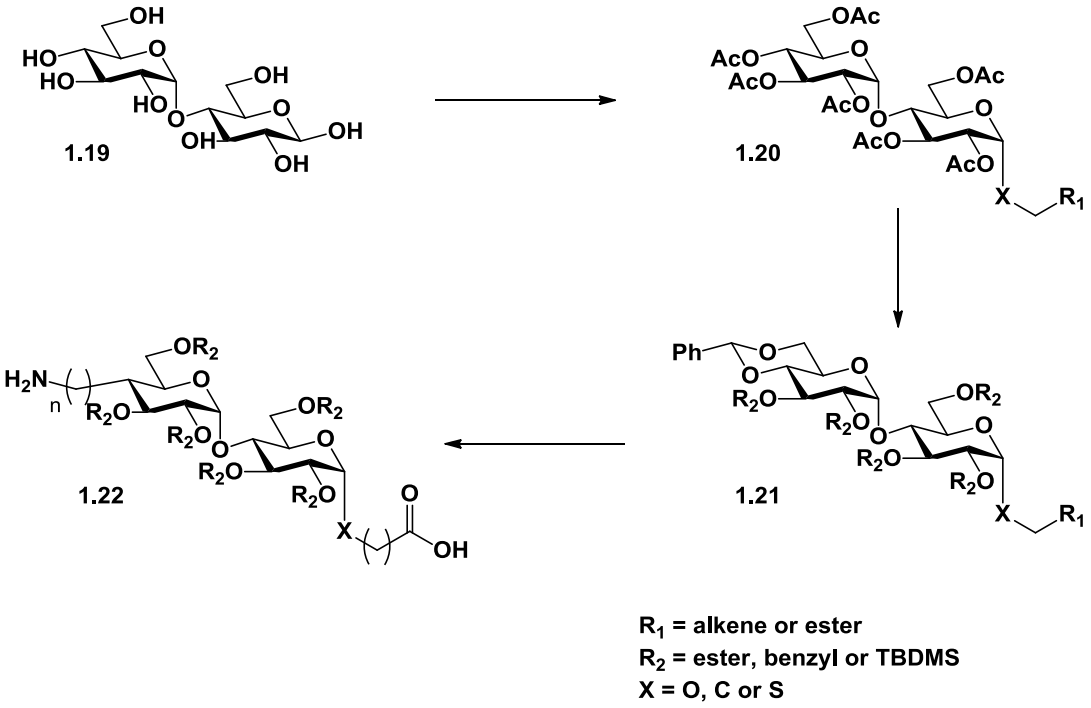
The PEG-linker **1.15** and similar linkers of different lengths are commercially available and therefore could be easily varied. The final section of the axle would be constructed using a Dde-protected lysine **1.17**. This was chosen as it would be resistant to piperidine Fmoc deprotection and could be orthogonally deprotected with hydrazine at the end of the synthesis. Once deprotected it could be functionalised with a variety of moieties, for example an oxyamine for ligation to larger molecules that would act as a cap on the axle.

The modular nature of SPPS would allow for variations in the number of maltose fragments **1.16** and PEG linkers **1.15** between them. This method could be used to generate several different ligands which can then be tested separately.

An alternative to SPPS is 'Click' chemistry which could be used to link the fragments together in an efficient solution-based method.[83,84] Copper-catalyzed alkyne amine cycloaddition (CuAAC) is a well-documented reaction that has been used to connect carbohydrates together.[85] This would require the introduction of an azide and alkyne at opposing ends of the saccharide and could conceivably be developed to form polymers in one-pot-syntheses.

1.6.2.2 Synthesis of ligand building blocks

The original synthetic design for the central maltosyl moiety of the axle is shown in Scheme 1-6. Following this route the anomeric position would be derivatised first before a regioselective protecting group strategy to functionalise the 4'-position.



Scheme 1-6 The important general precursors in the synthesis of the maltose mimic **1.22** starting from maltose **1.19**.

A benzylidene acetal was to be employed to selectively protect and then reveal the 4' position for introduction of the amino group. The benzylidene acetal would only protect the 4' and 6' positions as these are the only positions which allow the formation of a stable six membered ring. The benzylidene acetal **1.21** could be opened selectively to give a hydroxyl group at the 4' position which could be transformed to introduce the equatorial amino group in compound **1.22**.[86] This amino group could either be used directly for the formation of an amide linkage to another maltosyl building block or used to attach a linker with another amine group at the end if desired.

1.6.2.3 Binding studies

Electrochemical conductance measurements in black lipid membranes made from diphytanoyl phosphatidylcholine have previously been used to study sugar translocation through maltoporin.[70,87] These methods are not routinely used in the Turnbull lab so a number of other biophysical methods that would give complementary information were to be investigated.

It was anticipated that isothermal titration calorimetry (ITC) would be used to measure solution binding affinities of mono-, di- and trivalent ligands using detergent-solubilised maltoporin. A comparison of the enthalpy of binding for mono- and multivalent ligands would determine if all the ligand groups in the molecule are binding and therefore the optimum length of linker between the ligand groups.[56]

Another method useful in determining the binding affinities of carbohydrate protein interactions is fluorescence anisotropy.[88,89] When a fluorophore is excited with polarized light its emission is also polarized. The extent to which the emitted light is depolarized is related to the size of the molecule it is attached to due to the speed of tumbling in solution.

This effect could be harnessed to gain binding information as the degree of anisotropy will change if a fluorophore tagged maltoside binds to the much larger maltoporin. For fluorescence anisotropy experiments, the attachment of a fluorophore to the ligands would be necessary.

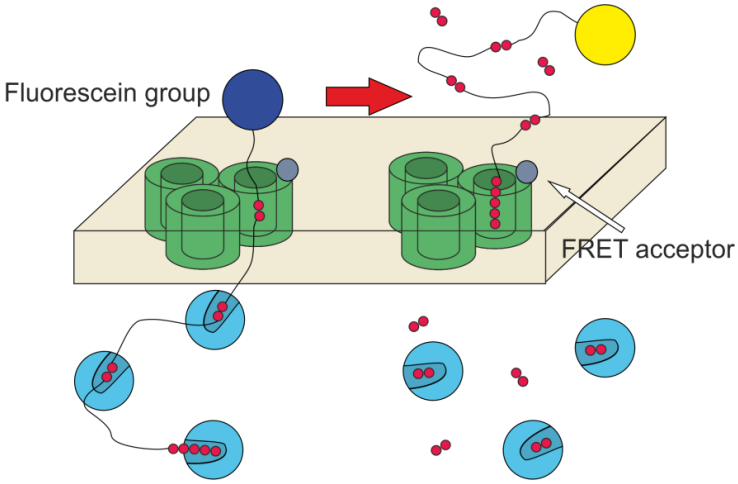


Figure 1-23 Cartoon showing the FRET experiment. (a) MBP (light blue) binds the ligand holding the fluorescein group and the FRET acceptor in close proximity. (b) On addition of maltose (red) the MBP releases the ligand allowing the fluorescein group to move away from the FRET acceptor and light up.

FRET experiments would provide a method to monitor the effectiveness of the maltose responsive switch. One fluorescein group would act as the stopper on the ligand while a complementary FRET acceptor would be attached using maleimide chemistry to the surface of a G382C mutant of maltoporin via the cysteine residue at position 382. Modification of this residue has been shown not to affect the ability of maltoporin to bind MOSs. MBP would be captured within vesicles into which the modified maltoporin has been reconstituted. The ligand would be added to the solution and in the presence of MBP should pull the fluorescein group close to the complementary FRET acceptor reducing its fluorescence (Figure 1-23). On addition of maltose, the ligand should be released and 'light up', thus signalling that it has moved away from the FRET acceptor and the cell surface. Ultimately, the FRET experiment will be reproduced in whole *E. coli* cells expressing the maltoporin G382C mutant.

Chapter II: Expression, purification and modification of proteins

2.1 Introduction

The design of the switch requires the hijacking of two members of the E. coli maltose transport system. The expression of these two proteins, maltoporin and MBP, have been recorded in the literature.[90,91] MBP is commonly used as a fusion protein to aid solubility and facilitate purification of other proteins, therefore there are standardised protocols for its expression.[92] In contrast there exist a variety of methods for maltoporin with significant differences suggesting its expression and/or purification is tempremental.

Maltoporin has been expressed using recombinant methods as well as from wild-type bacteria. Its expression from unmodified E. coli was achieved by solubilising the protein with a detergent (lauryldimethylamine oxide) and passing the fraction down an amylose affinity column.[93] To increase the protein yield, the expression of maltoporin can also be induced by using a minimal media with maltose as the carbon source which leads to up-regulation of the maltose transport system.[94] Although this method has proved successful, there is only a finite amount of space in the bacterial outer membrane which limits the amount of protein that can be expressed. Later, groups used over-producing E. coli strains which contain the lamB gene in an expression plasmid under control of a tac-promoter.[95] This method was also transferred to E. coli strains which had a number of their outer membrane proteins knocked out to give more space in the outer membrane for the recombinant protein. This strategy was demonstrated by T. Schirmer and co-worders who expressed maltoporin from BL21(DE3)omp5 cells, a strain of E. coli with the major outer membrane proteins knocked out.[96]

Once expressed, the binding of the proteins will be investigated. Isothermal calorimetery (ITC) was selected to be the primary method for acquiring binding information. ITC has never been performed with maltoporin before and use of this technique could provide new insights into the binding thermodynamics of this protien. Fluorescence binding studies were also identified as an alternative and complementary strategy for studying the interactions of the protein. These experiments require modifications of the protein in the form of site directed mutagenesis: [97] a powerful method to specifically modify and derivatise proteins.

2.2 Expression and purification of maltoporin

Expression and purification of maltoporin in high yields was essential for its use in a variety of studies. At the time of taking on the project, expression of maltoporin had been attempted by members of the Turnbull group but with no success. The LamB gene had successfully been cloned from E. coli DH5α cells and ligated into pSAB2.2 vectors (Figure 2-2). Expression trials had been performed in E. coli BL21 cells using autoinduction media, but no overexpression had been seen.

As maltoporin is an outer membrane protein its expression is typically low yielding due to the small volume the membrane occupies compared to the rest of the cell. Membrane proteins are known to be toxic in high concentrations and it was postulated that as the concentration of maltoporin built up in the cell membrane and periplasm, the cells were becoming stressed and were ejecting the protein into the media. This effect could be mitigated if maltoporin were prevented from reaching the periplasm. Maltoporin's transportation to the outer membrane is enabled by chaperone proteins, which recognise a short peptide leader sequence of the form MMITLRKLPLAVAVAAGVMSAQAMA added on to the N-terminus. Therefore, if maltoporin were to be expressed without the leader sequence, the chaperone proteins would not recognise maltoporin and so the over-expressed protein would then be contained within inclusion bodies in the cytoplasm as unfolded aggregates.[98] It would then be possible to refold such proteins in detergent.[99]

2.2.1 Expression of maltoporin without the leader sequence

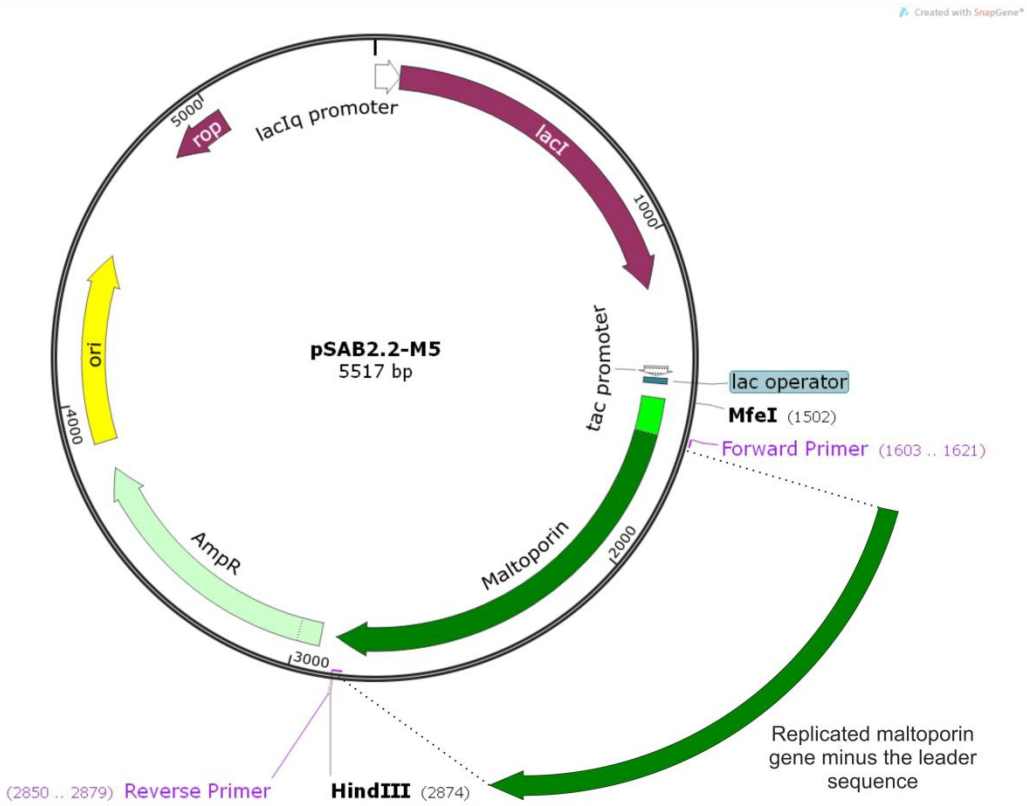


Figure 2-1 The pSAB2.2-M5 gene with maltoporin (dark green) and the leader sequence (light green) shown as well as the binding sites of the primers. The use of these primers in PCR led to replication of the maltoporin gene without the leader sequence.

The maltoporin gene was amplified from stock pSAB2.2-M5 vectors using forward primers that did not contain the leader sequence (Figure 2-1). The amplified DNA was digested with MfeI and Hind III for 1.5 hrs at 37°C to create ‘sticky ends’.

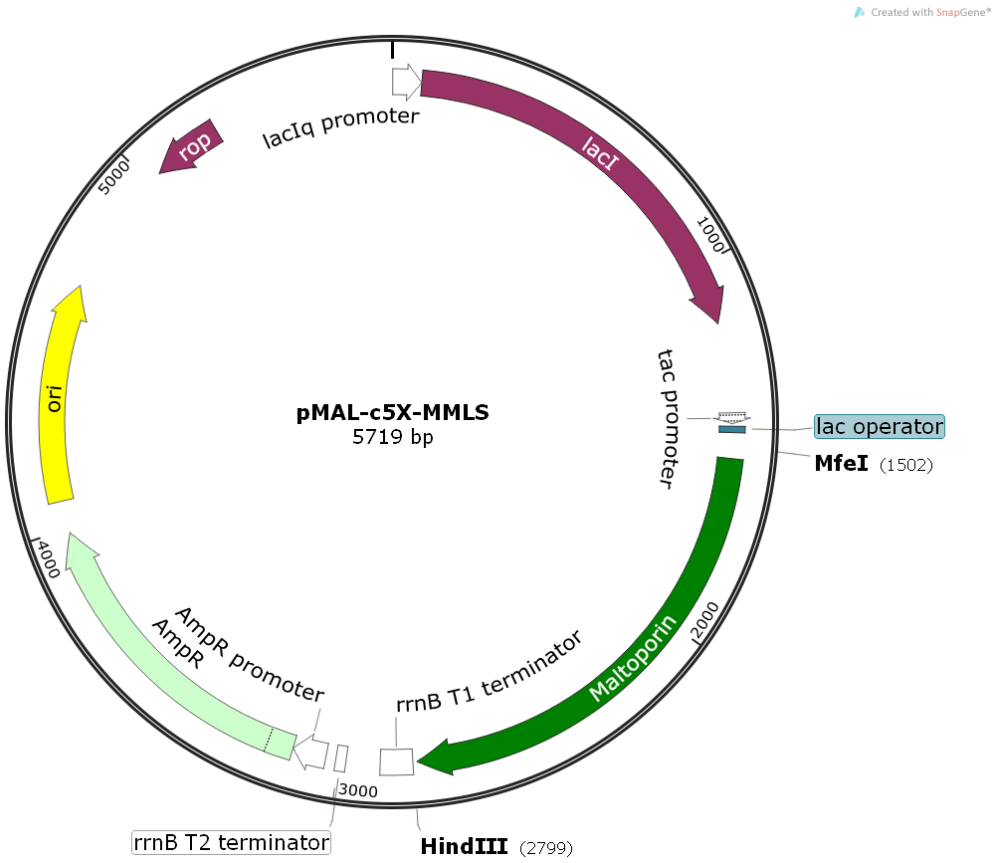


Figure 2-2 pMAL-c5X with maltoporin gene inserted in place of MBP

The digested DNA was ligated into a pMal-c5x vector in place of the MBP gene before transformation into E. coli XL10 cells (Figure 2-2). After incubation on an agar plate 8 colonies were selected. From these the plasmid DNA was extracted and digested with MfeI and Hind-III to separate the inserted *LamB* gene from the plasmid. An agarose gel of these samples showed six of the selected colonies to have the correct insert of ~1450 bp (Figure 2-3). Sequencing analysis of the five cleanest DNA bands showed that they all corresponded to the full maltoporin gene.

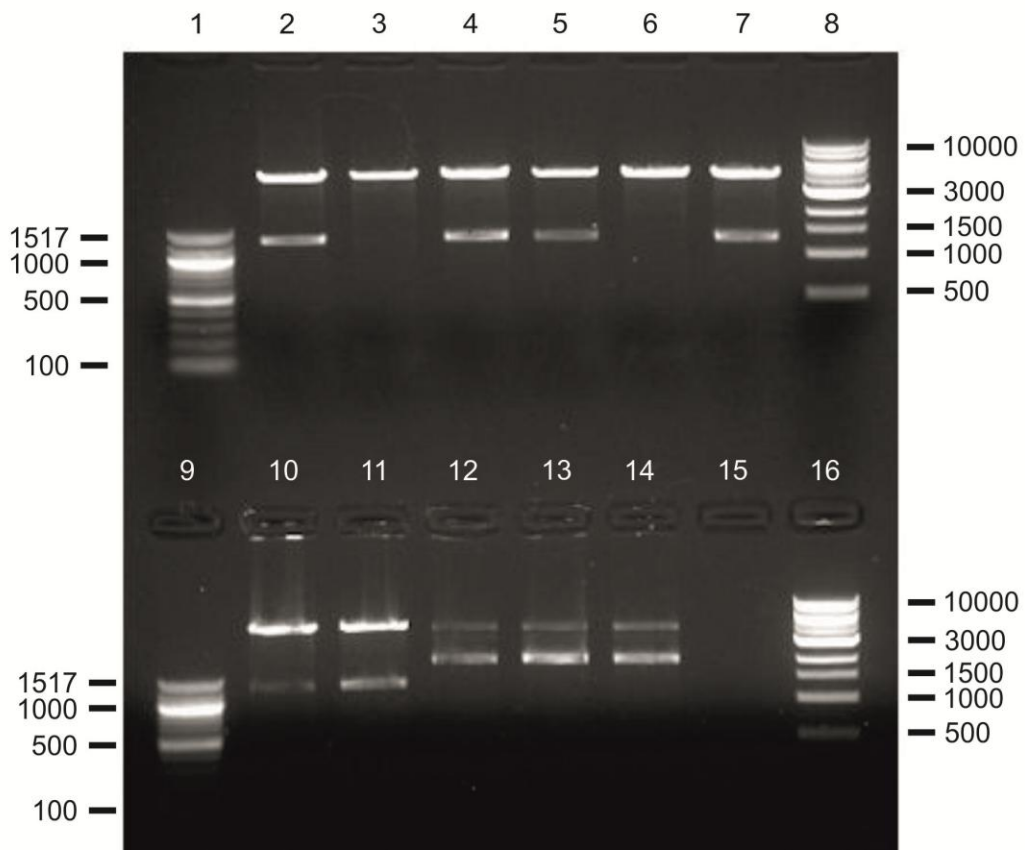


Figure 2-3 Agarose gel showing digested plasmids from lamB without leader sequence ligations. Lanes 2, 4, 5, 7, 10 and 11 all have inserts at ~1450, the weight of the correct insert. Lane 1 – 100 bp ladder; Lanes 2-7 – ligation digestion; Lane 8 – 1 K bp ladder;

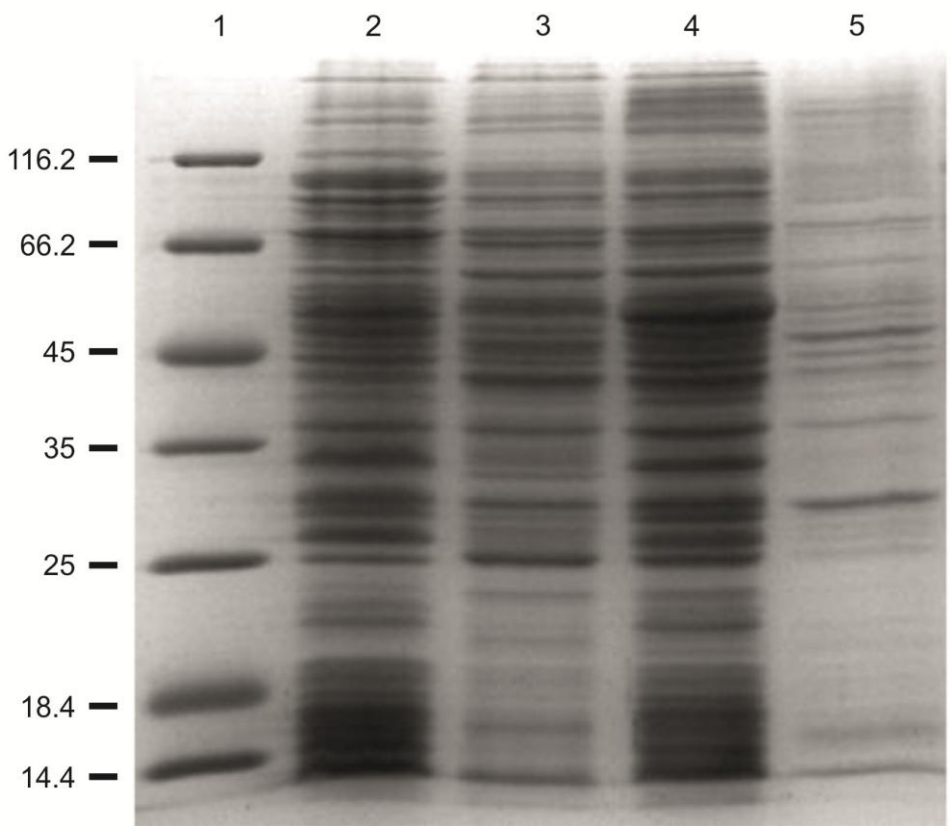


Figure 2-4 BL21 cells with plasmid expressing maltoporin minus the leader sequence with different incubation times. Lane 1 – Protein marker; Lane 2 – Inclusion bodies, 4 h; Lane 3 – Inclusion bodies, o.n.; Lane 4 – Membrane fraction, 4 h; Lane 5 – Membrane fraction o.n.

One colony with the correct gene for maltoporin without the leader sequence was transformed into *E. coli* BL21 gold cells and expressed in LB media. Samples from the culture were taken 4 h and 24 h after the cells were induced with IPTG. The samples were purified following the maltoporin purification procedure (see experimental section). An SDS PAGE showed no over-expression of maltoporin in either inclusion bodies or the membrane fraction (Figure 2-4). The leader sequence may have more important roles in the expression of maltoporin than previously thought. As this route had been unsuccessful, a different method for the expression of maltoporin was sought.

2.2.2 Expression of maltoporin in *E. coli* BL21 (DE3) ompX cells

E. coli strains with some of their outer membrane proteins knocked out have been shown to increase the yields of maltoporin expression.[96] As previously illustrated, the over-expression of membrane proteins has two major challenges. The maximum amount of protein that can be expressed is restricted by the small volume of the membrane compared to the rest of cell and overloading the membrane with one type of protein can lead to toxicity.[100] The combination of these factors means that as well as each cell containing only a small amount of protein, their exponential phase is cut-off resulting in a smaller number of cells. This results in low yields for the over-expression of proteins into the membrane.

To overcome one of these factors *E. coli* BL21(DE3)omp8 and BL21(DE3)omp9 cells were acquired from Ralf Koebnik in CNRS Montpellier (where ompX is used it refers to both sets of cells).[101] These strains of cells have had the major outer membrane proteins knocked out these consist of maltoporin, OmpC[102,103], OmpA[104] and OmpF[105,106] all of which allow passive diffusion of small polar molecules. The omp9 cells also have ompN[107] knocked out which fulfils the same function. This leaves more space in the outer membrane into which proteins may be expressed.

To transform the plasmid encoding maltoporin into the ompX cells they first had to be made heat competent. The heat competent cells were transformed using the pSAB2.2-M5 containing the intact maltoporin gene previously made in the Turnbull group by Dr James Ross (Figure 2-2). Sequencing of two colonies from the transformation showed them to be successful.

As over-expressed membrane proteins can be toxic to the cell, an expression study was performed with samples taken at different time points to ascertain the point at which expression was optimal. Expression was carried out using LB media and IPTG induction. The cells were induced at an OD_{600} of 0.6 which corresponds to the exponential growth phase of the bacteria. This optical density has been found to be optimal for a variety of membrane proteins.[108] Samples were harvested after 0, 2, 3 and 4 h, collected by centrifugation and stored at -80 °C prior to analysis.

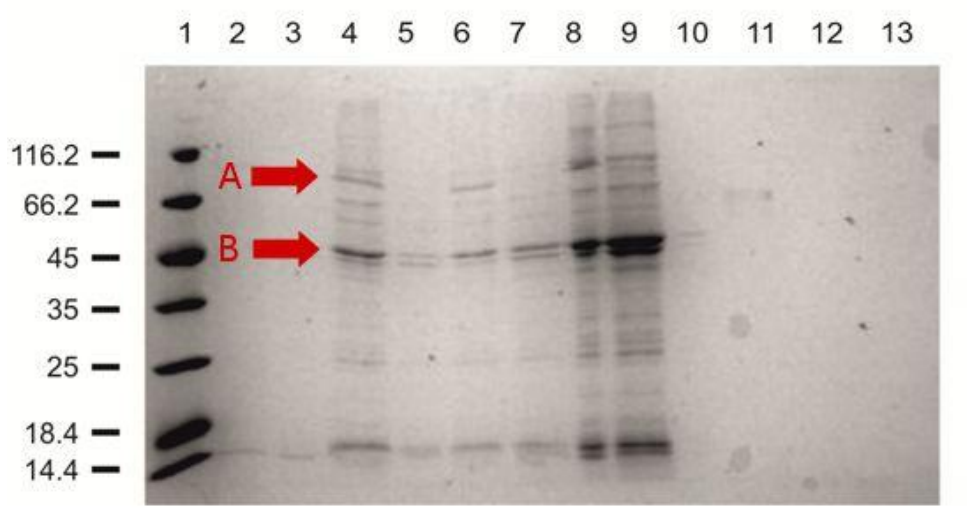


Figure 2-5 SDS PAGE of omp8 (lanes 2-9) cells and omp9 (lanes 10-13) cells with expression plasmid pSAB2.2-M5 after different periods of time, which shows over expression of a protein at ~45 kDa (A) after 4h for the omp8 cells. Lane 1 – Protein marker; Lane 2 –0 h grow up, unboiled; Lane 3 –0 h grow up, boiled; Lane 4 –2 h grow up, unboiled; Lane 5 –2 h grow up, boiled; Lane 6 –3 h grow up, unboiled; Lane 7 –3 h grow up, boiled; Lane 8 –4 h grow up, unboiled; Lane 9 –4 h grow up, boiled; Lane 10 –0 h grow up, unboiled; Lane 11 –0 h grow up, boiled; Lane 12 –2 h grow up, unboiled; Lane 13 –2 h grow up, boiled.

The cells were subsequently passed through a Constant Systems cell disrupter and an SDS PAGE of these samples showed omp8 cells over-expressed protein at 45 kDa which is the mass of the unfolded maltoporin monomer (Figure 2-5, B). It is known that maltoporin runs as the folded trimer on SDS PAGE due to its high stability. Here, both boiled and unboiled samples have over-expression at 45 kDa suggesting that the majority of maltoporin being over-expressed in the cell was unfolded. Although there is also a smaller band at 97 kDa (Figure 2-5, A), where the folded trimer runs, which disappears on boiling so a proportion of the protein is likely to be folded.[99]

Samples for omp9 were only taken after 0 and 2 h as it took 5 hours to reach the optimum OD_{600} = 0.6. Neither of these samples showed any protein (Figure 2-5). As the omp9 cells take a significantly longer time to grow and the omp8 cells had been shown to over-express protein it was decided to carry on with only the omp8 cells.

Omp8 cells without the maltoporin expression plasmid were cultured using the same method as used for the plasmid-containing cells (Figure 2-6). As no over-expression of protein was observed, this control experiment demonstrated that the extra protein bands seen in Figure 2-5 resulted from the maltoporin expression plasmid and were thus likely to be maltoporin.

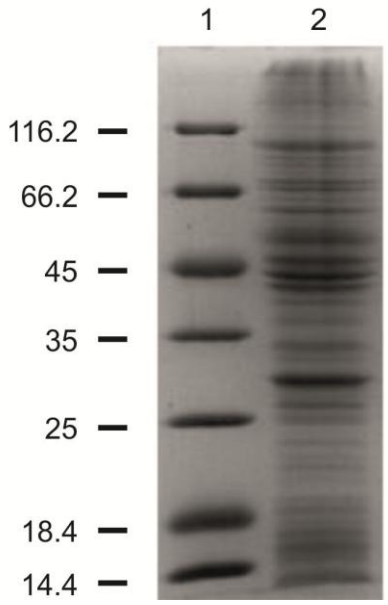


Figure 2-6 SDS PAGE of omp8 cell control, induced without maltoporin expressing plasmid. No over expression was found after a 4 h incubation. Lane 1 – Protein marker; Lane 2 – Cell pellet.

Maltoporin expression was thus carried out on a larger scale of 8 L. Here the cells were harvested by centrifugation and passed through a Constant Systems cell disrupter before centrifugation to remove soluble proteins. The remaining pellet was then resuspended in Triton X-100, a non-ionic surfactant, and shaken overnight at 37 °C to solubilise any detergent-soluble protein in the pellet. The suspension was pelleted at 48,000 x g for 30 min. The supernatant was retained and the pellet was resuspended in TEN buffer containing urea (8 M) before pelleting again at 48,000 x g for 30 min. This procedure produced an initial supernatant of Triton X-100-solubilised protein and a secondary supernatant containing urea-solubilised proteins.

SDS PAGE was performed on the Triton X-100-solubilised and urea-solubilised fractions, as well as the remaining pellet (Figure 2-7). This gel showed a large band at 45 kDa in the urea-solubilised fraction. It also showed a smaller band at 97 kDa in the Triton X-100 band which dropped to 45 kDa on boiling the sample before analysis. This suggests that only the folded maltoporin was solubilised by Triton X-100, while the unfolded maltoporin was only solubilised by urea.

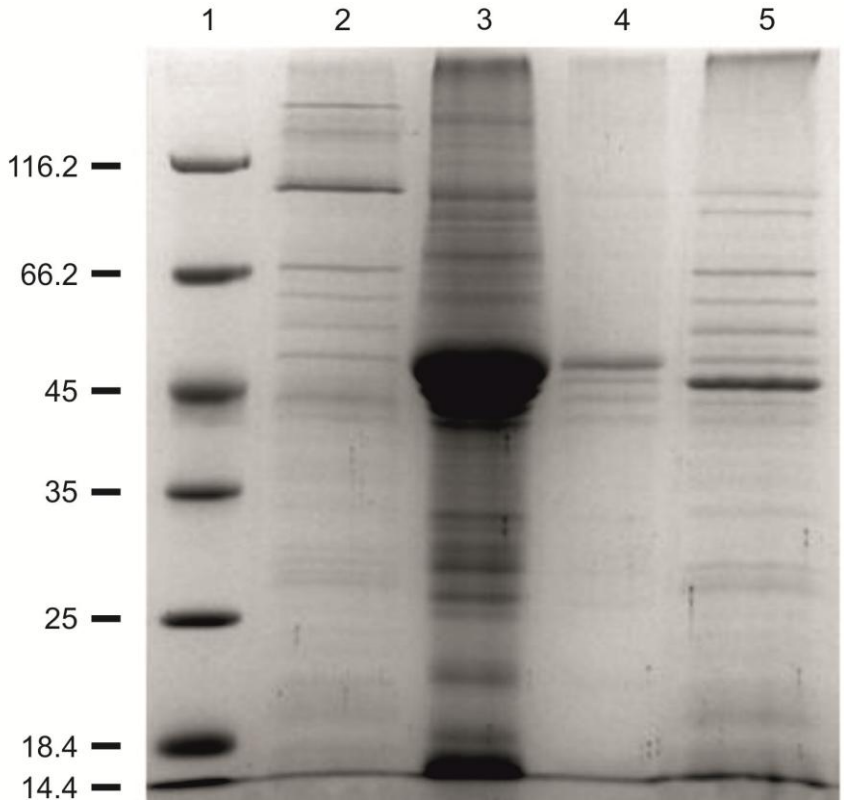


Figure 2-7 SDS PAGE of omp8 cells with maltoporin expression plasmid. The gel shows protein which is soluble in Triton X-100 running at 97 kDa (lane 2) and at 45 kDa (lane 5) after boiling which is consistent with the expected behaviour of maltoporin. Lane 1 – Protein marker; Lane 2 – Triton X-100-solubilised protein, unboiled; Lane 3 – Urea-solubilised protein; Lane 4 – Final pellet; Lane 5 – Triton X-100-solubilised protein, boiled.

The Triton X-100 fraction was purified using an amylose column as maltoporin would be able to bind to the maltooligosaccharides at the surface of the resin. At this point the protein was eluted in buffer containing Octyl-POE instead of Triton X-100. This was because Octyl-POE is not UV active allowing for the concentration of the sample

to be easily measured. It also has a critical micelle concentration (CMC) of 6.6 mM compared to 0.24 mM for Triton X-100. This higher CMC allows for easier diffusion of the detergent. The protein at 97 kDa was shown to bind the amylose column indicating that this protein was indeed maltoporin (Figure 2-8). As the amylose column did not bind any other proteins from the fraction, the fractions eluted with maltose provided a pure sample of maltoporin.

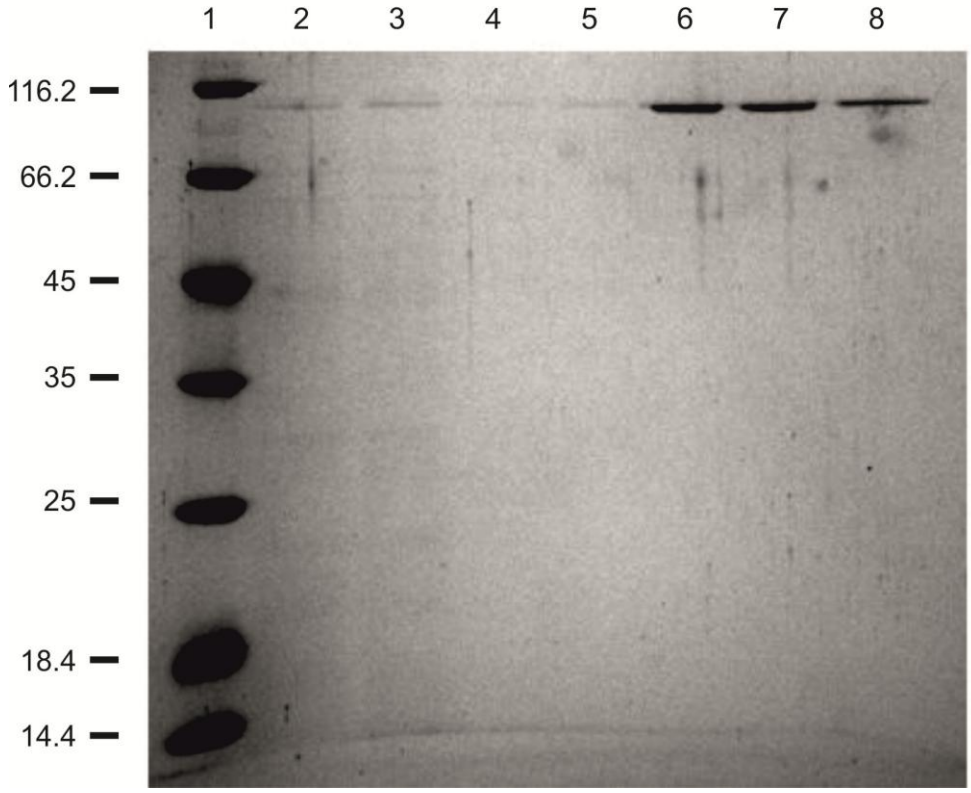


Figure 2-8 SDS PAGE showing amylose column purification of Triton X-100 soluble proteins. Lane 1 – Protein marker; Lane 2 – Flow through; Lane 3 – Wash fraction; Lane 4 – 1st elution fraction; Lane 5 – 2nd elution fraction; Lane 6 – 3rd elution fraction; Lane 74th elution fraction; Lane 8 – 5th elution fraction.

Quantification of the concentration of protein collected from the membrane was made difficult by the presence of an unknown concentration of Triton X-100 which absorbs light at 280 nm. Following removal of Triton X-100 through dilution and dialyses, a concentration of 2.2 mg per L incubation was obtained. These results are consistent with expression of membrane proteins.[98]

2.2.3 Optimisation of the expression of maltoporin

The current method of expression for maltoporin had resulted in 2.2 mg / L grow-up. It was estimated that this project would require more than 50 mg of maltoporin, so optimisation of this method was sought. As this initial procedure also yielded a much larger amount of unfolded maltoporin, the refolding and purification of the urea-solubilised fraction was desirable. Gradual dilution of this solution should allow the refolding of the protein. The urea supernatant was therefore diluted two-fold with urea (8 M) in TEN buffer and then four-fold with Triton X-100 (3% v/v) in TEN buffer. The solution was also subsequently diluted to 1 M and then 0.1 M urea although SDS PAGE showed no refolding of maltoporin.

It was considered that the inability of maltoporin to refold may be a result of much of the protein still having its leader sequence attached and that this additional strand of peptide interferes with refolding. This hypothesis is supported as when boiled, maltoporin runs lower than the urea-soluble unfolded protein on SDS PAGE (Figure 2-7). This shows the unfolded protein is of a higher mass than maltoporin and by approximately the size of the leader sequence.

The leader sequence could still be attached because the over-expression of maltoporin occurred at such a rate that the chaperone proteins that export maltoporin to the periplasm were overloaded. As the leader sequence is removed in the periplasm, the failure of the cell to export maltoporin out of the cytoplasm may have resulted in the leader sequence not being cut from a majority of maltoporin.

In order to overcome this issue the rate at which maltoporin was expressed needed to be more closely matched to the rate at which it is exported to the periplasm. This could be achieved by slowing the expression rate through a reduction in the incubation temperature to 16 °C and inducing with a lower concentration of IPTG. A longer period of time was also allowed for expression and as such a more supportive SOC media was used. This media contains a higher concentration and variety of salts increasing the cells' survivability.

A SOC media expression was performed with the omp8 cells containing the pSAB2.2-M5 plasmid in 8 x 1 L cultures. The flasks were inoculated with cells at differing stages of growth allowing them to be induced at different optical densities and an analysis of the most advantageous stage to induce them could be carried out. Five of the flasks at induction OD_{600}s of 0.4, 0.6, 1.4, 2.2, and >2.5 were selected to be purified using the maltoporin purification procedure. SDS PAGE of the supernatants and pellets of each one showed variations in line with the density of cells when they were induced (Figure 2-9).

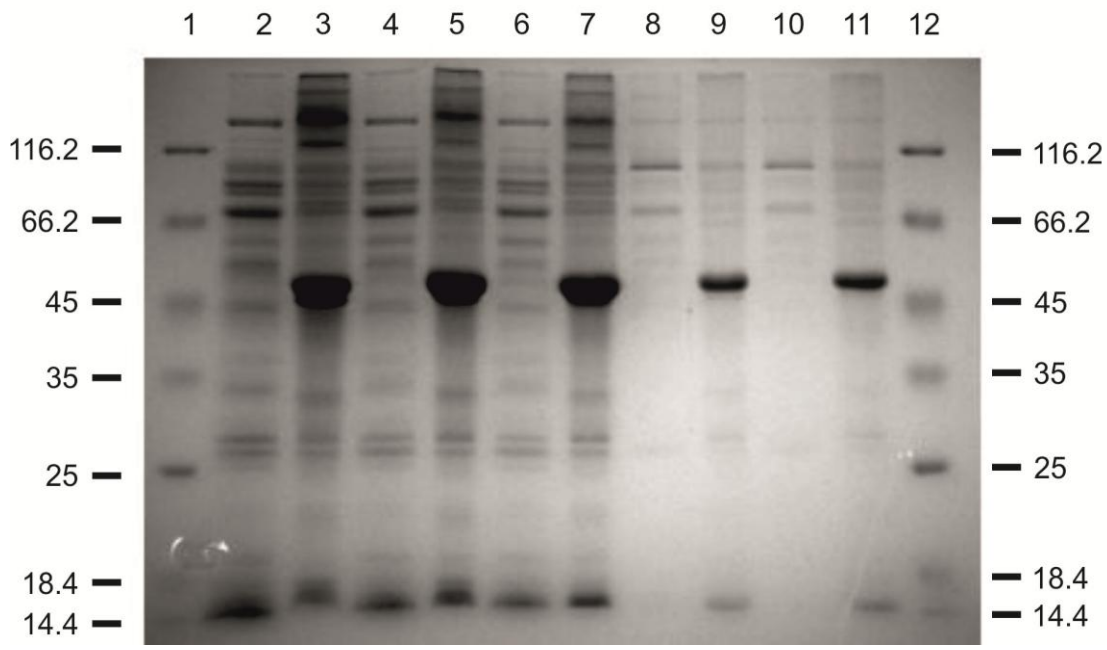


Figure 2-9 SDS PAGE showing 12 h incubation of omp8 cells with expression plasmid at 16 °C in SOC media induced at different ODs. Both the membrane fraction and pellet are shown., At OD600 = 0.4 and 0.6 a single band can be seen at 46 kDa in the pellet fraction and 97 kDa in the membrane fraction. These bands are lost at higher OD. Lane 1 – Protein marker; Lane 2 – OD_{600} = >2.5, membrane fraction; Lane 3 – OD_{600} = >2.5, pellet; Lane 4 – OD_{600} = 2.2, membrane fraction; Lane 5 – OD_{600} = 2.2, pellet; Lane 6 – OD_{600} = 1.4, membrane fraction; Lane 7 – OD_{600} = 1.4, pellet; Lane 8 – OD_{600} = 0.6, membrane fraction; Lane 9 – OD_{600} = 0.6, pellet; Lane 10 – OD_{600} = 0.4, membrane fraction; Lane 11 – OD_{600} = 0.4, pellet; Lane 12 – Protein marker.

Induction at OD_{600} of 0.4 and 0.6 led to expression of maltoporin as the folded trimer. Induction at OD_{600} of 1.4 and higher led to no over expression of folded maltoporin which is in contrast to the amount of maltoporin produced as the unfolded monomer. While induction at 0.4 and 0.6 do show over-expression, there is significantly less protein produced in the cells than those induced at 1.4 or higher (Figure 2-10).

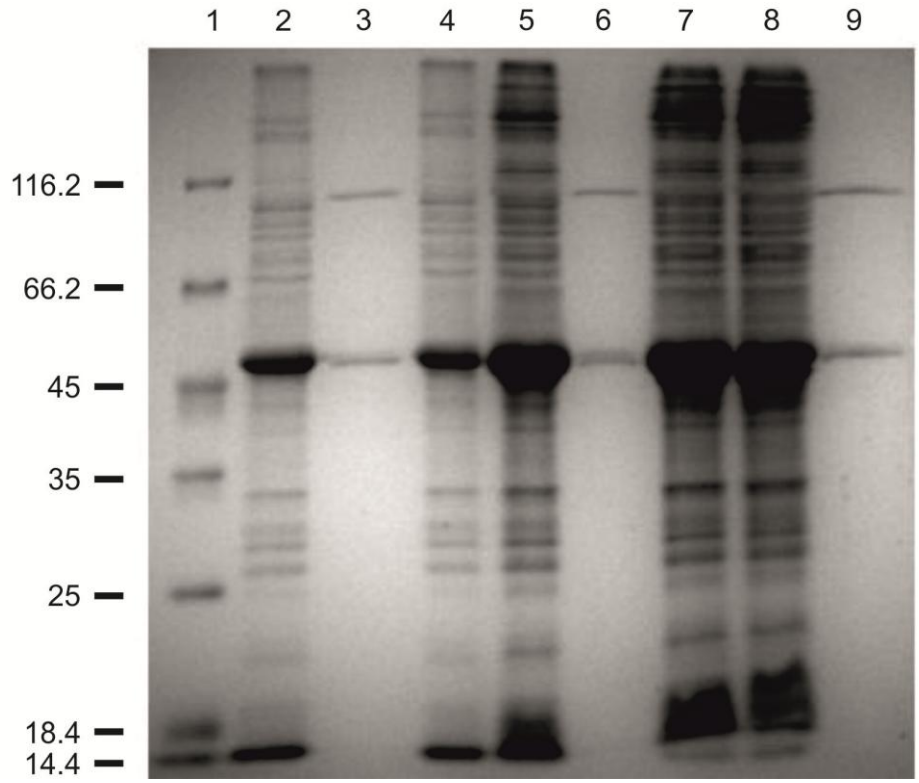


Figure 2-10 SDS PAGE showing pellets of 12 h incubation of omp8 cells with expression plasmid at 16 °C in SOC media induced at different ODs compared to boiled and unboiled maltoporin. The single bands at 46 kDa in the OD600 = 0.4 and 0.6 lanes run at the same position as boiled maltoporin indicating that it is maltoporin. Lane 1 – Protein marker; Lane 2 – OD_{600} = 0.4; Lane 3 – OD_{600} = maltoporin, boiled (100 °C, 10 min); Lane 4 – OD_{600} = 0.6; Lane 5 – OD_{600} = 1.4; Lane 6 – OD_{600} = maltoporin, boiled (100 °C, 10 min); Lane 7 – OD_{600} = 2.2; Lane 8 – OD_{600} = >2.5; Lane 9 – maltoporin, boiled (100 °C, 10 min).

More importantly it can be seen that when induction is performed at an OD_{600} of 0.4 and 0.6, the unfolded monomer expressed in the detergent-insoluble fractions appears as a single band compared to the double bands shown in 1.4 and higher (Figure 2-10). SDS PAGE of the insoluble fractions maltoporin shows the band resulting from

induction at 0.4 and 0.6 runs at the same weight as the maltoporin unfolded monomer. This result contrasts with the previous incubation in LB media at 37 °C which showed a marked difference in the weight of the dominant band and the unfolded monomer (Figure 2-7). From this, it appears that the slower expression has allowed the cell time to remove the leader sequence from maltoporin.

As it appeared that the leader sequence had been successfully removed from maltoporin the refolding of the unfolded maltoporin could be re-attempted.

2.2.4 Refolding of maltoporin

Baldin et al. had shown that maltoporin could be refolded when diluted into detergent from a denaturant.[99] Once diluted below 1 M of denaturant, the protein should spontaneously refold, with octyl-POE being the preferred detergent for this method. As maltoporin had been now been expressed without the leader sequence a more rigorous attempt at its refolding was embarked upon.

The pellets from a 1 L incubation for maltoporin expressed in SOC media at an OD_{600} of 0.6 and >2.5 were dissolved in 7 M guanidine hydrochloride (GdmCl) in 30 ml TEN buffer. The samples were left shaking overnight at 37 °C to solubilise the unfolded protein. Centrifugation at 48,000 x g removed any insoluble protein and the supernatant was diluted with TEN buffer and octyl-POE to a final concentration of 1.3 M GdmCl and 2.5% octyl-POE. A concentration below 1.5 M of GdmCl had been shown to cause refolding of maltoporin.[99] The solution was left stirring overnight at room temperature to refold. In both samples the protein was found to have aggregated as the unfolded monomer and precipitated out of solution.

The refolding experiment was carried out again but with the protein diluted by a factor of 3. The greater dilution should hinder aggregation of the protein. In this case, no precipitation was observed but SDS PAGE did not show any refolding of the protein either.

As the refolding experiment had shown no refolding a larger systematic testing of refolding conditions was undertaken. The cells induced in SOC media at OD_{600} = 0.6 were used in all experiments as these were judged by SDS PAGE to have the least contamination by other proteins (Figure 2-10).

Table 2-1 Different unfolding and refolding buffers, reagents and detergents

No.	Buffer	Unfolding agent (conc.)	Refolding Detergent	Other reagents	Dilution Factor*
1a	20 mM HEPES, pH 7.4	GdmCl (7.3 M)	0.5% Octyl-POE	0.02% NaN_3	6
1b	20 mM HEPES, pH 7.4	GdmCl (7.3 M)	0.5% Octyl-POE	0.02% NaN_3	10
2	20 mM HEPES, pH 7.4	GdmCl (7.3 M)	0.5% Octyl-POE	0.02% NaN_3	6
3a	20 mM HEPES, pH 7.4	Urea (6 M)	0.5% Octyl-POE	0.02% NaN_3	6
3b	20 mM HEPES, pH 7.4	Urea (6 M)	0.5% Octyl-POE	0.02% NaN_3	10
4a	50 mM Tris, pH 8.0	Urea (6 M)	0.7% DDM	0.1 mM EDTA	6
4b	50 mM Tris, pH 8.0	Urea (6 M)	0.7% DDM	0.1 mM EDTA	10
5	10 mM Borate, pH 10.0	Urea (8 M)	0.7% DDM	2 mM EDTA	4
6a	50 mM Tris, pH 8.0	Urea (4 M)	0.7% DDM	10% Glycerol, 0.1 M NaCl	6
6b	50 mM Tris, pH 8.0	Urea (4 M)	0.7% DDM	10% Glycerol, 0.1 M NaCl	10
7	50 mM Tris, pH 8.0	Urea (6 M)	0.1% Deoxycholic acid	20% Isopropanol	4
8a	50 mM Tris, pH 8.0	Urea (6 M)	0.5% SDS		4
8b	50 mM Tris, pH 8.0	Urea (6 M)	0.5% SDS		10
9a	50 mM Tris, pH 8.0	Urea (6 M)	0.5% Tween		4
9b	50 mM Tris, pH 8.0	Urea (6 M)	0.5% Tween		10
10a	50 mM Tris, pH 8.0	Urea (6 M)	0.5% Triton X-100		4
10b	50 mM Tris, pH 8.0	Urea (6 M)	0.5% Triton X-100		10

*The cells from 50 ml cell of cultures were pelleted and re-suspended in 5 ml of denaturing buffer. These were incubated at 37 °C o.n. before dilution and addition of detergent as indicated in the table.

A large variety of different buffers and reagents were used in the folding of maltoporin to increase the chances of finding successful conditions (Table 2-1). Experiments 1-6 had been previously shown to refold maltoporin or similar pore proteins[109,110] while 7-10 used four untested detergents. In each experiment, the protein was first unfolded in either urea or guanidinium hydrochloride by overnight incubation at 37 °C before

dilution in buffer to a concentration at which maltoporin has been shown to refold.[99] After dilution of the samples SDS PAGE showed none of the conditions were successful in folding maltoporin (Figure 2-1). During these procedures it was found that the use of guanidinium hydrochloride caused the protein to precipitate out of the denaturant solution while at least 8 M urea was needed to completely solubilise the protein, hence the more intense band for sample 5 (Figure 2-11).

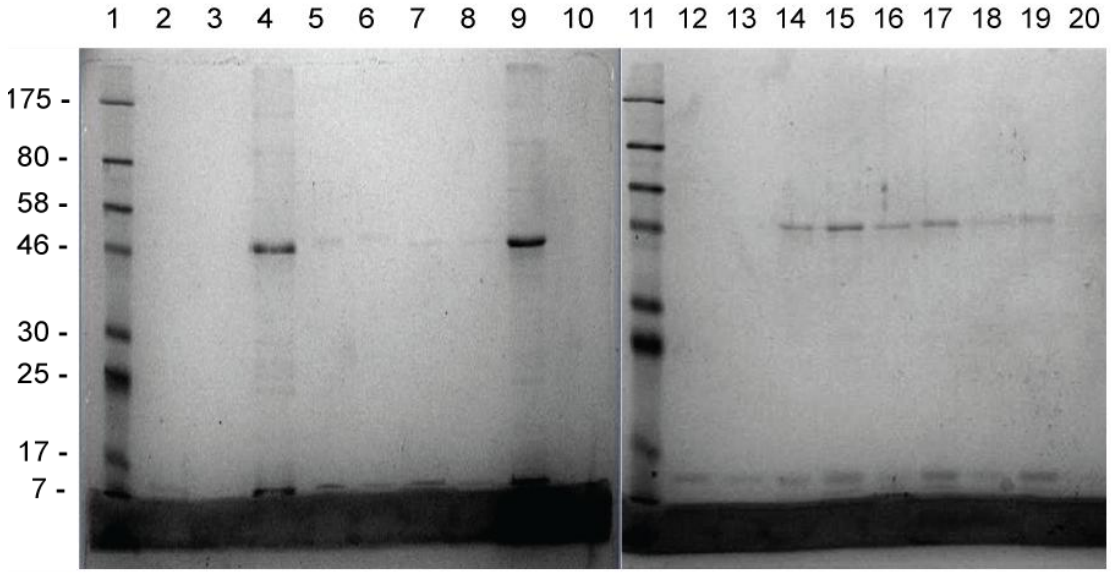


Figure 2-11 SDS PAGE of refolding conditions from Table 2-1 Different unfolding and refolding buffers, reagents and detergents. Lane 1 – Protein marker; Lane 2 – 1a; Lane 3 – 1b; Lane 4 – 2; Lane 5 – 3a; Lane 6 – 3b; Lane 7 – 4a; Lane 8 – 4b; Lane 9 – 5; Lane 10 – blank; Lane 11- Protein marker; Lane 12 – 6a; Lane 13 – 6b; Lane 14 – 7; Lane 15 – 8a; Lane 16 – 8b; Lane 17 – 9a; Lane 18 – 9b; Lane 19 – 10a; Lane 20 – 10b.

Maltoporin is known to have at least one disulfide linkage in its structure[90] which could have cross-linked between proteins forming larger aggregates. The degree of cross-linking was investigated by removing DTT from the SDS PAGE loading buffer which should leave all disulfide linkages intact. This experiment showed the existence of aggregates that were too large to pass into the resolving and stacking gels (Figure 2-12). As these aggregates could hinder protein folding, DTT would now be added to all refolding buffers as it had also been shown that maltoporin can refold without disulfide linkages.[99] This may also explain the higher intensity of sample 2 in Figure

2-11 (lane 4), as this sample had 1 mM of DTT added which should have disrupted the larger aggregates resulting in less protein precipitating out of the GdmCl denaturant solution.

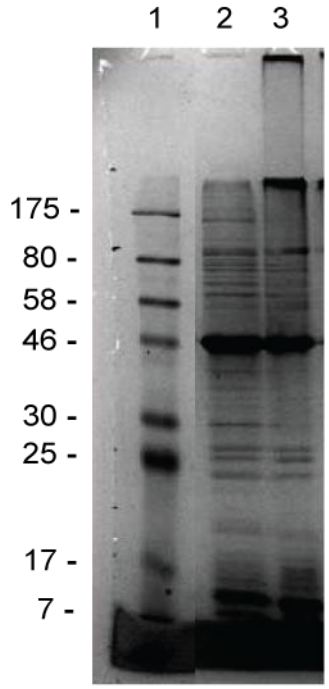


Figure 2-12 SDS PAGE of omp8 urea-soluble proteins with and without DTT in the loading buffer showing large aggregates. Lane 1 – Protein marker; Lane 2 – Omp8 urea soluble protein with DTT; Lane 3 – Omp8 urea soluble protein without DTT.

Another set of refolding experiments was carried out using DTT to reduce disulfide linkages and implementing dialysis instead of dilution to reduce the concentration of the denaturant (Table 2-1). Dialysis should change the concentration of urea or GdmCl more slowly than dilution, thus minimising the chance of protein precipitating from solution. Octyl-POE was used as it is the preferred detergent for refolding maltoporin in the literature[99] while Triton X-100 was used as it is a much cheaper, but comparable detergent.

Table 2-2 Initial conditions used for the refolding of maltoporin before dialysis.

No.	Buffer	Unfolding agent (conc.)	Other reagents	Refolding Detergent
11a	10 mM Borate, pH 10.0	GdmCl (7.3 M)	100 mM DTT	0.5% Octyl-POE
11b	10 mM Borate, pH 10.0	GdmCl (7.3 M)	100 mM DTT	0.5% Triton X-100
12a	50 mM Tris, pH 8.0	GdmCl (7.3 M)	100 mM DTT	0.5% Octyl-POE
12b	50 mM Tris, pH 8.0	GdmCl (7.3 M)	100 mM DTT	0.5% Triton X-100
13a	10 mM Borate, pH 10.0	Urea (8 M)	100 mM DTT	0.5% Octyl-POE
13b	10 mM Borate, pH 10.0	Urea (8 M)	100 mM DTT	0.5% Triton X-100
14a	50 mM Tris, pH 8.0	Urea (8 M)	100 mM DTT	0.5% Octyl-POE
14b	50 mM Tris, pH 8.0	Urea (8 M)	100 mM DTT	0.5% Triton X-100

*The cells from 50 ml cell of cultures were pelleted and re-suspended in 5 ml of denaturing buffer. These were incubated at 37 °C o.n. before a 10x dilution and addition of detergent as indicated in the table.

All of the refolding solutions were dialysed to 0 M urea/GdmCl and 0 M DTT, although again no folding of the protein was seen. Due to the time involved in running the folding studies and the availability of folded maltoporin in the membrane, this route for purification of the protein was abandoned.

2.2.5 The large scale expression and purification of maltoporin

To produce enough folded maltoporin the expression would need to be scaled up beyond 1 L flasks. To do this omp8(DE3)BL21 cells with the plasmid expressing maltoporin and media were supplied to Prof Peter Henderson's lab where a 30 L fermenter run was carried out generating a 50 g cell pellet. The pellet was purified in the same manner as before but with the use of a 5 ml MBPTrap column (GE Healthcare) which was found to have an increased binding capacity compared to the 20 mL column of amylose resin (NEB) used previously (Figure 2-13). Purification still required multiple runs as the total amount of maltoporin considerably exceeded the capacity of the MBPTrap column, therefore an AKTA FPLC machine was used to automate and speed up the process. The yield of maltoporin was 1.2 mg of protein per

litre, or 35.8 mg of protein in total. The fermenter run was repeated producing 70 mg of protein in total which was sufficient for the rest of the project.

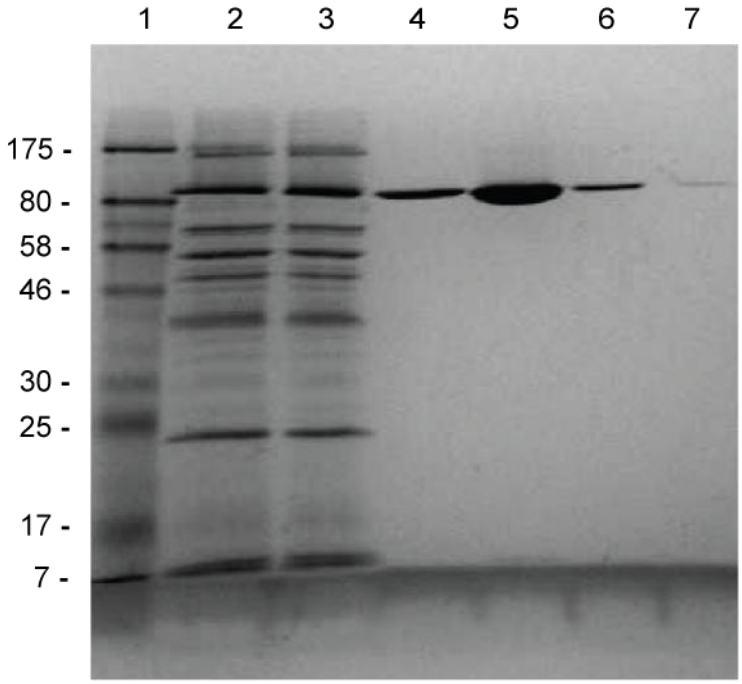


Figure 2-13 SDS PAGE of maltoporin purification with a 5 ml MBPTrap column from a 30 L fermenter run. Lane 1 – Protein marker; Lane 2 – Flow through; Lane 3 – Flow through; Lane 4 – Elution fraction 1; Lane 5 – Elution fraction 2; Lane 6 – Elution fraction 3; Lane 4 – Elution fraction 1; Lane 5 – Elution fraction 2; Lane 6 – Elution fraction 3; Lane 7 – Elution fraction 4.

2.2.6 Circular dichroism (CD) of maltoporin

It is difficult to ascertain the mass of maltoporin using mass spectrometry due to its large size and hydrophobicity. Therefore other methods were employed to determine that the protein in question definitely was maltoporin. It has been demonstrated that the protein can bind to an amylose column and runs on SDS PAGE at a mass of 97 kDa if the sample is not boiled before loading onto the gel; whereas it has an apparent mass of 45 kDa if the sample is boiled. All these observations agree with literature examples.[99,111]

CD was performed on the protein to establish whether it contained the high proportion of beta sheets consistent with a beta-barrel found in maltoporin. This was found to be the case with CD showing a characteristic minimum at 217 nm that typifies β-structures (Figure 2-14).[112]

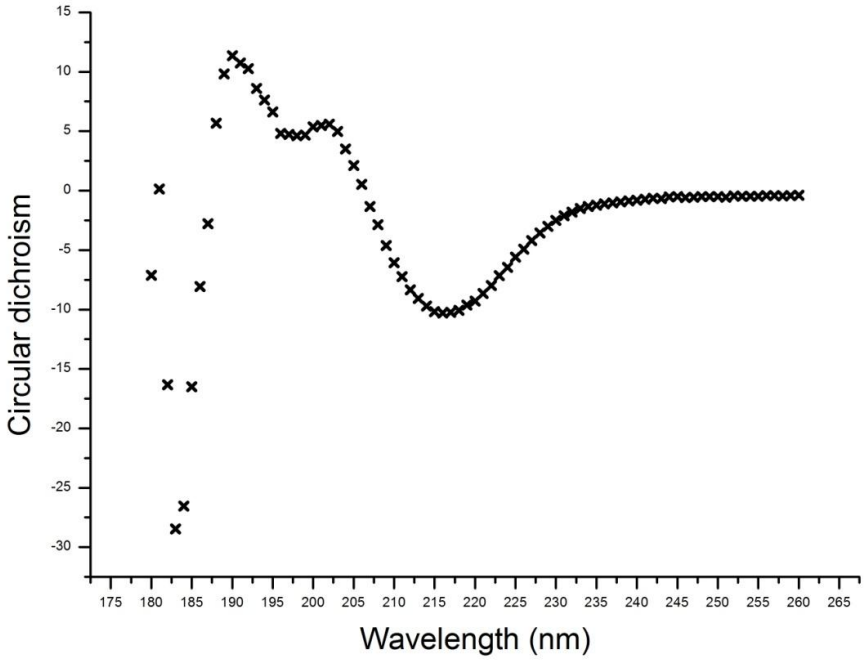


Figure 2-14 A CD spectrum of the purified protein showing a characteristic line in keeping with a beta-barrel protein.

2.2.7 Investigations into the binding of maltoporin using microscale thermophoresis (MST)

The binding of maltoporin has previously been tested with electrochemical conductance measurements in black lipid membranes. In the Turnbull lab this

equipment was not readily available and so the implementation of a more commonly used biophysical technique was sought.

One of these techniques is Microscale thermophoresis (MST). MST works by measuring the movement of molecules along a temperature gradient at different concentrations. Any change in the hydration shell of a protein due to ligand binding will result in a relative change in movement along the temperature gradient and thus binding affinities can be determined. MST has been shown to be an effective method to measure binding using nanomolar quantities of protein.[113]

To allow maltoporin's movement to be tracked by the instrument it was non-specifically labelled with FITC. This was performed by adding FITC in DMSO to the protein in sodium carbonate buffer at pH 8.5. Use of the nanodrop showed a labelling efficiency of 0.2 molecules of FITC per monomer. Both maltoheptaose and maltose were tested against the FITC-labelled maltoporin. Starting at 0.5 M a 15-fold serial dilution of the sugars was performed and the binding studied by MST.

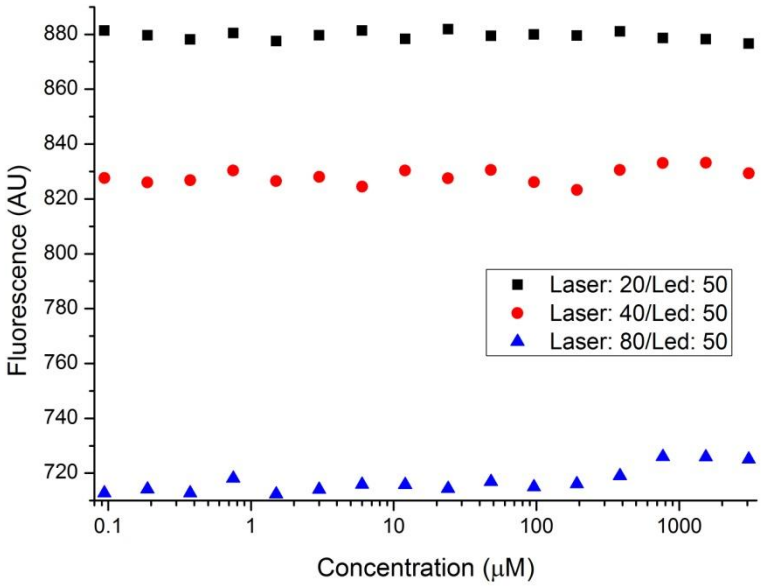


Figure 2-15 MST results for maltoheptaose (3 mM) against maltoporin (570 nM).

No change in the thermophoretic motion of maltoporin was found when the concentration of ligand was varied. It was thought that as changes in movement along

the temperature gradient were based on changes in the hydration shell of the protein this may be an inefficient method for the measurement of binding within the maltoporin channel (Figure Figure 2-15). A different method was therefore sought to measure the binding of maltoporin.

2.2.8 Investigations into the binding of maltoporin using Isothermal titration calorimetry (ITC)

ITC was selected as a method to measure binding to maltoporin as it required no further derivatisation of the protein and it relied on the heat changes on binding as opposed to any change in the hydration shell of the protein. As a method it also gives comprehensive thermodynamic data in addition to binding affinities. There is no literature precedent for the use of ITC with maltoporin and the first challenge lay in accurately matching the buffers for the protein and ligand. This matter was complicated by the need to also match the detergent concentration which cannot easily be achieved by dialysis because of micelle formation.

Therefore, the detergent was initially removed from the maltoporin solution by precipitation of the protein with acetone. The resulting pellet was redissolved in buffer containing a known amount of octyl-POE and dialysed against the same buffer-detergent solution. To test how well this method was at matching the detergent concentration, the maltoporin sample was subject to an ITC experiment which involved titrating the buffer against the protein solution. This experiment showed only a small heat of dilution which indicated that the buffer was sufficiently matched to allow further titrations (Figure 2-16).

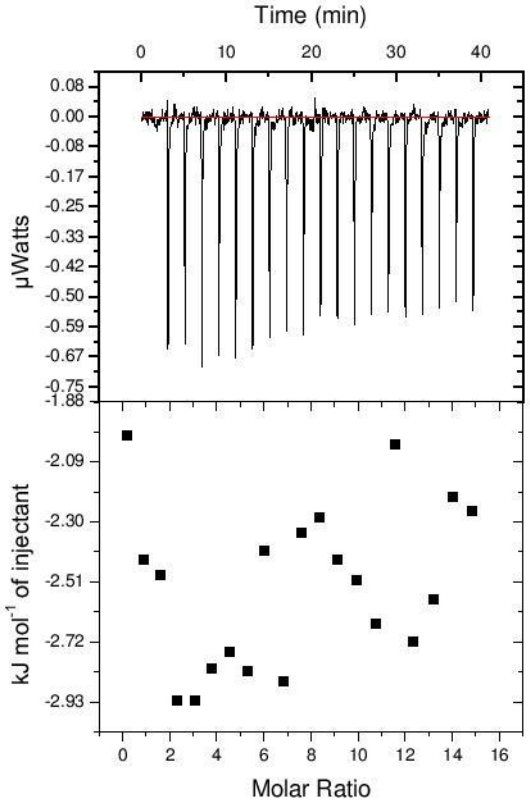


Figure 2-16 Buffer titrated into maltoporin in Octyl-POE

Binding to maltoporin was subsequently tested using maltose, maltotetraose and maltoheptaose. First maltoheptaose was tested although only very weak binding was seen by ITC (Figure 2-17). As the theoretical binding affinity for maltoheptaose would be less than 60 µM, at a ligand concentration of 7.5 mM, a large proportion of maltoporin should be bound within the first few injections.[68] This was not seen in the data with the point at which binding started to level off at a molar ratio of over 30. There is also a small heat of dilution when with the large number of water molecules displaced and hydrogen bonds formed it would be thought that the heat of binding would be high. This is not reflected in the data where only a small amount of heat is shown to be released.

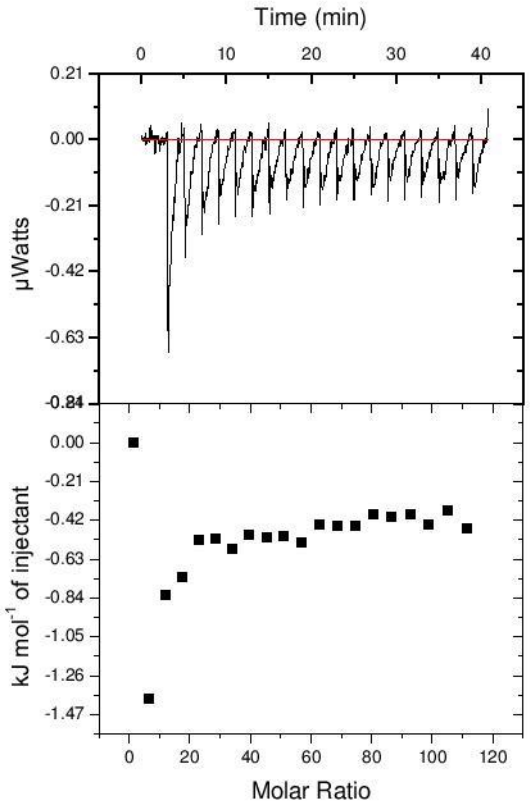


Figure 2-17 Titration of maltoheptaose (7.5 mM) into maltoporin (14 μM) in Octyl-POE

It was thought possible that the octyl-POE may have been occupying the binding site of maltoporin leading to a much weaker apparent affinity in the ITC experiment. If octyl-POE was occupying the binding site then its replacement by a maltooligosaccharide would also create a much smaller change in heat than if the sugar was displacing water.

To overcome this potential problem, it would be necessary to remove detergent from the buffer without maltoporin precipitating from solution. ITC of membrane proteins has previously been achieved with the use of Amphipol A8-35 in place of detergent. This large amphiphilic polymer can wrap around membrane proteins keeping them in solution without the use of detergent.[114]

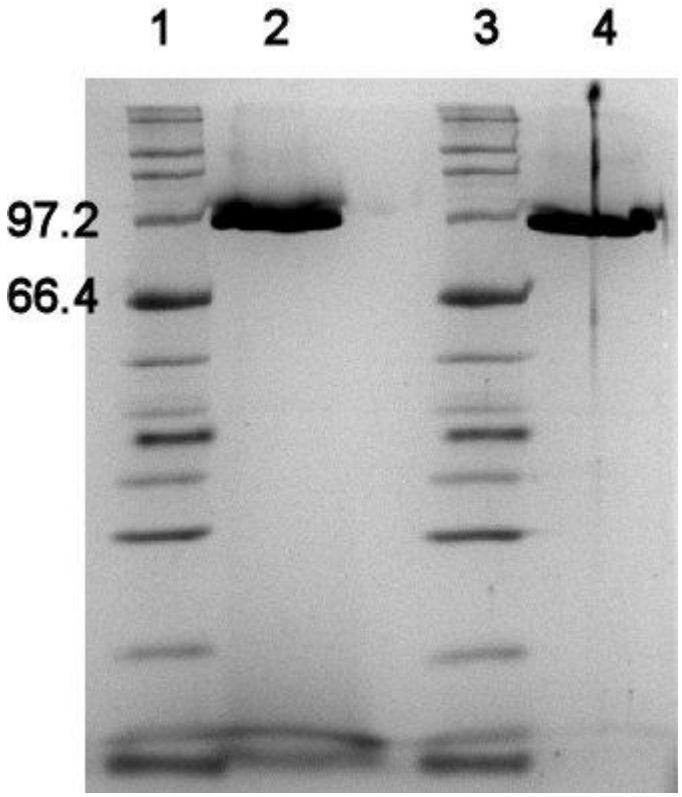


Figure 2-18 Comparison of Amphipol A8-35 solubilised protein and octyl-POE solubilised protein. Lane 1 + 3 – protein marker, Lane 2 –maltoporin solubilised with octyl-POEmaltoporin; Lane 4 – maltoporin solubilised with amphipol A8-35

The detergent in a 1 ml sample of maltoporin (27 μM) was replaced with Amphipol A8-35 by adding five equivalents of the polymer. The solution was left for 15 minutes before pre-washed SM2 biobeads (Bio-Rad) were added to remove detergent. The solution was agitated for 2 hours before the buffer containing maltoporin was decanted from the solution. SDS PAGE showed maltoporin as the folded trimer suggesting the amphipol had successfully kept the protein in solution (Figure 2-18).

ITC experiments were repeated to show that the buffers were again matched. Maltoheptaose was tested against the amphipol-solubilised protein as this was the sugar with the highest expected binding affinity. These ITC experiments again showed a curve consistent with much weaker binding than was expected. For maltoheptaose the baseline had shifted a large amount (Figure 2-19). This is generally due to the system not having enough time to return to equilibrium between injections. The

experiment was therefore repeated with a 5 minute interval between injections instead of 2 minutes, thus giving the system longer to equilibrate.

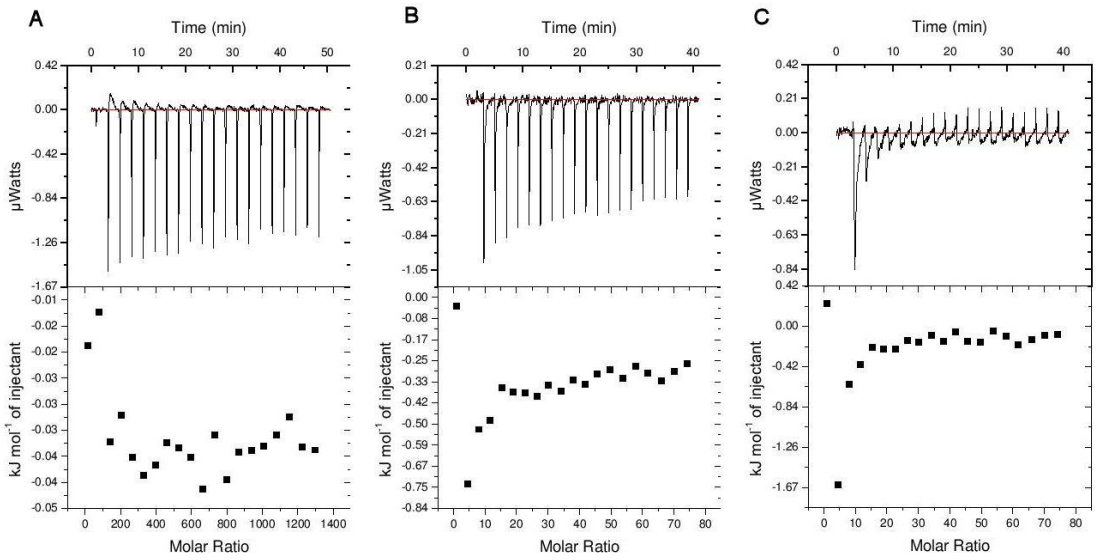


Figure 2-19 ITC with amphipol solubilised maltoporin. A - Maltose (100 mM) into maltoporin (16 μM); B – Maltotetraose (7.5 mM) into maltoporin (21 μM); C – Maltoheptaose (7.5 mM) into maltoporin (21 μM).

With the solution having longer to return to equilibrium it became clearer that the observed heat changes were unlikely to be due to maltoheptaose interacting with the pore of maltoporin. The point at which the base line was reached was still at a molar ratio of over 20. This value gives a binding affinity that is much larger and therefore incompatible with the previous binding affinities for MOSs. This concluded that the peaks seen could not be due to the binding of MOSs in the pore of maltoporin.

The peaks could either be due to heat of dilution, or to complexation at a much weaker binding site, perhaps in the loops of maltoporin. There is also a much broader secondary peak seen in the ITC data which could be due to maltoheptaose binding in the pore (Figure 2-20). If so, then it suggests that the threading into and binding of maltoheptaose in the pore of maltoporin has very slow kinetics.

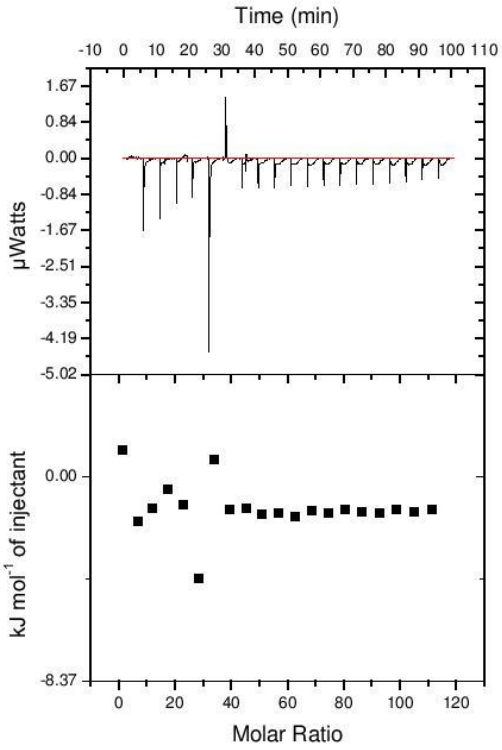


Figure 2-20 ITC of maltoheptaose (7.5 mM) into maltoporin (14 μM) with 4 min between injections.

As accurate ITC data relies on a very fast binding event it appears that the binding of maltoporin to maltooligosaccharides occurs on too long a timescale for ITC to be an effective technique.

2.2.9 The expression and modification of maltoporin mutant G382C

As it had been concluded that binding between maltoporin and maltooligosaccharides occured too slowly to be measured by ITC, a different approach was needed. Methods using fluorescently-labelled maltoporin could give information on binding and would not be adversely affected by the binding kinetics. The solutions could be pre-mixed and allowed to equilibrate before being measured.

To fluorescently label maltoporin a cysteine residue would need to be added to the surface of the protein. Maltoporin already contains two cysteine residues at positions 22 and 38, but these are buried deep within the protein and are found not to be reactive to labelling when the protein is folded.[111,115] Position 382 was picked to be mutated as this was in the loop region of the protein and therefore would have the greatest exposure to ligands binding in the pore of maltoporin (Figure 2-21). [22]

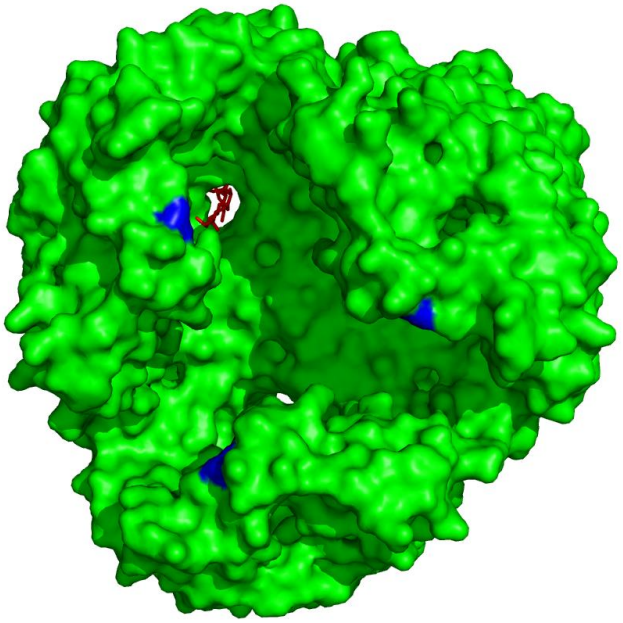


Figure 2-21 Maltoporin pictured from above its extracellular face with position 382 highlighted in blue.

Site directed mutagenesis was employed to introduce the mutation G382C. A PCR reaction was carried out with the pSAB2.2-M5 plasmid containing the gene for *LamB* and primers encoding for the G382C mutation. After the reaction the plasmid was digested with Dpn1 which showed no loss of the plasmid at ~5700 bp. Dpn1 only cleaves at methylated sites therefore it cleaves the parent plasmid but not the PCR product. This is because bacteria methylate DNA but the PCR product has been produced synthetically so it will not have been methylated. As an agarose gel of the reaction showed a strong band after Dpn1 digestion it suggests there is a large amount of the synthetic product produced (Figure 2-22).

As the mutatgenesis appeared to be successful, the plasmid was transformed into XL10 cells, grown on an agar plate and three colonies were extracted and sequenced which showed the correct mutant had been made.

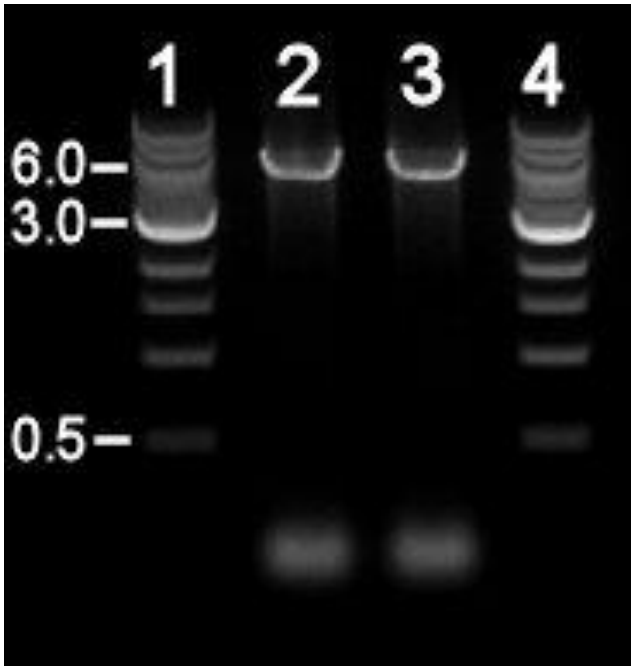


Figure 2-22 A Dpn1 digest of the mutagenesis product showing no loss of plasmid. Lane 1 – DNA ladder; Lane 2 – Undigested plasmid; Lane 3 – Dpn1 digested plasmid.

The pSAB2.2-M5 plasmid containing the *LamB* G382C gene was transformed into omp8 cells for expression. These were then expressed following the same procedure as the for wild type maltoporin except for the introduction of DTT into any buffers used. This was to ensure no intramolecular sulfide bridges were formed resulting in aggregation of the protein.

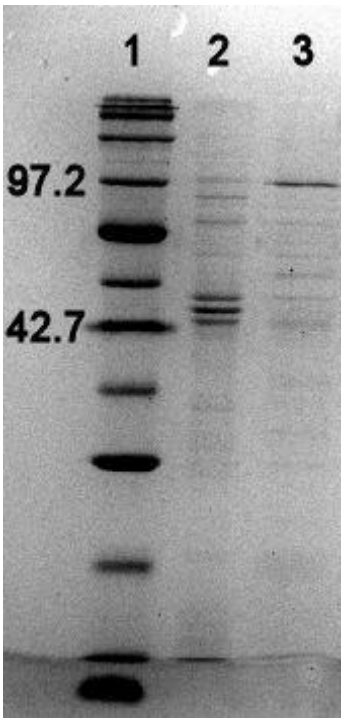
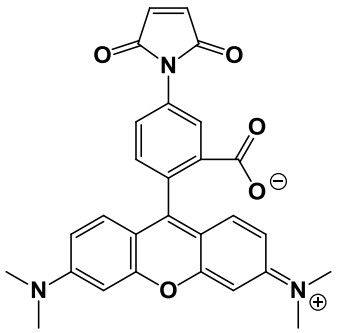


Figure 2-23 Gel demonstrating the successful expression of maltoporin G382C mutant. Lane 1 – Protein ladder; Lane 2 – Remaining cell pellet; Lane 3 – Triton X-100-solubilised protein

SDS PAGE demonstrated a strong band at ~97 kDa in the Triton X-100-solubilised fraction (Figure 2-23) showing the mutant had successfully been expressed.

2.2.10 Labelling of the G382C mutant

As the mutant has been successfully expressed it could now be labelled with a fluorescent molecule containing a maleimide group. Tetramethylrhodamine-5-maleimide **2.1** (Santa Cruz Biotechnology) was chosen as the fluorescent molecule as it could act as a FRET pair with both fluorescein and dansyl groups that were being used in the ligands to be evaluated (Figure 2-24).



2.1

Figure 2-24 Tetramethylrhodamine-5-maleimide

T. Ferenci and co-workers had reported a greater degree of labelling of a cysteine mutant at pH 8.4, while labelling still occurred at pH 7.4.[111] Both of these methods were investigated with the use of TCEP to ensure that the cysteines were reduced before labelling. The reaction at pH 8.4 was shown to be more effective but also labelled the wild-type maltoporin which suggested that at the higher pH either the internal cysteines could be labelled or, more likely, that non-specific labelling at free amines on the protein was also occurring. Labelling at pH 7.4 was shown to be more selective with the wild-type maltoporin showing no labelling while the G382C mutant fluoresced under UV light (Figure 2-25)

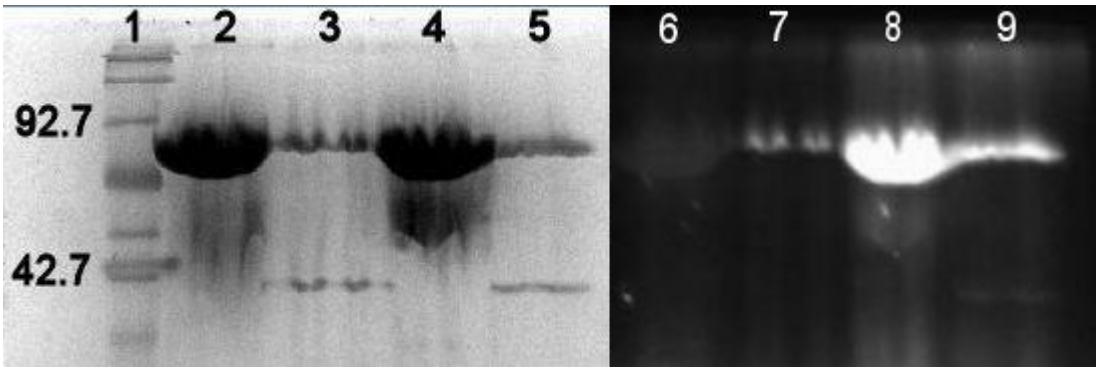


Figure 2-25 Labelling of maltoporin G382C with tetramethylrhodamine maleimide. The image on the left is shown under UV light. Lane 2 + 6 – WT maltoporin pH 7.4; Lane 3 + 7 – G382C maltoporin pH 7.4; Lane 4 + 8 – WT maltoporin pH8.4; Lane 5 + 9 – G382C maltoporin pH 8.4

2.3 Expression and purification of MBP

It was also necessary to express MBP for use in the project. As it is commonly used as a fusion protein to aid solubility and purification, it is available in a variety of vectors. An empty pMal-c5X plasmid encoding MBP was transformed into *E. coli* C43 cells. The cells were then grown to the exponential phase before induction with IPTG.

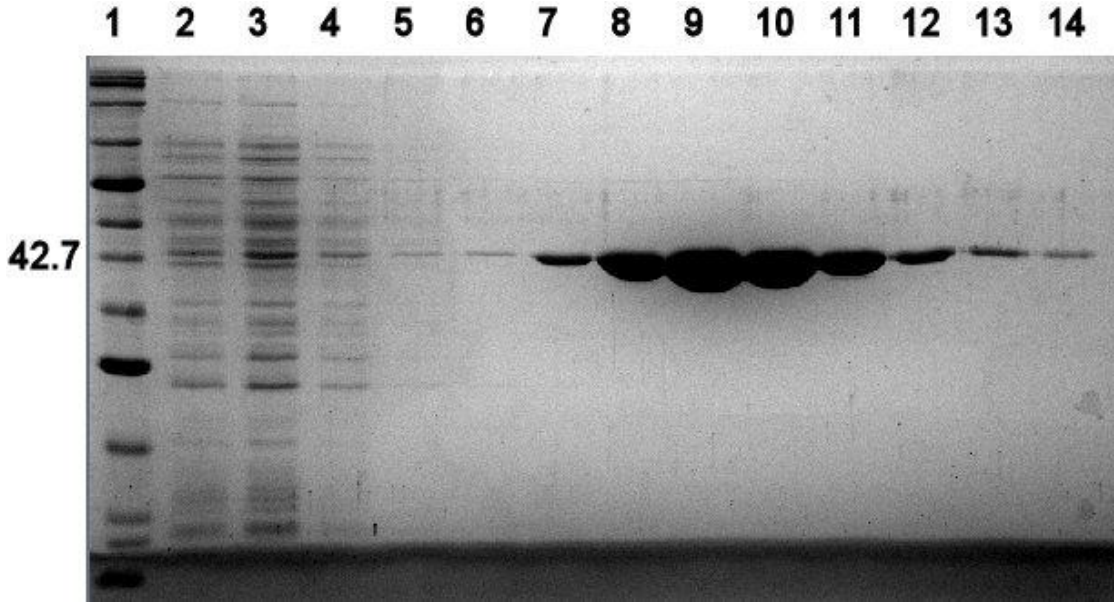


Figure 2-26 Amylose column purification of MBP with 20% glucose. Lanes 2 + 3 flow through; Lanes 4 + 5 washes; Lanes 6 – 14 glucose elutions.

MBP fusion proteins are usually purified by use of an amylose column and elution with maltose. This strategy would not be ideal for the current project as MBP has a μM binding affinity for maltose and so if eluted with this ligand, the MBP would be inactivated for further binding. Therefore, it was decided to elute with a high concentration of glucose as it has a much lower binding affinity and could thus be easily removed by dialysis. This method proved to be successful with a strong band at ~45,000 Da in the elution fractions producing 40 mg / L of MBP (Figure 2-26).

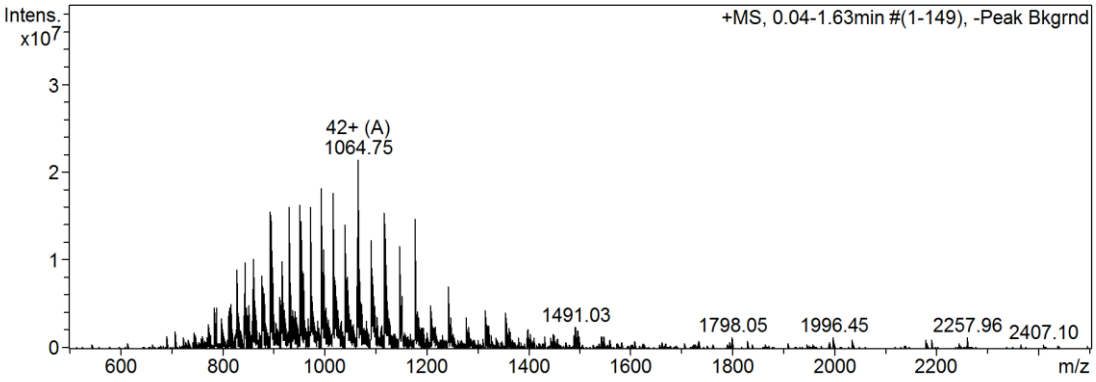


Figure 2-27 Mass spectrometry trace of MBP

Mass spectrometry gave a mass of 44670 Da compared to the expected mass of MBP being 44669 Da, confirming it was the correct product (Figure 2-27).

ITC was performed on MBP to confirm it had not been inactivated during its purification. Titration with maltose gave a good binding curve with a dissociation constant of 9 μM confirming its binding site was free and it could still interact with ligands (Figure 2-28).

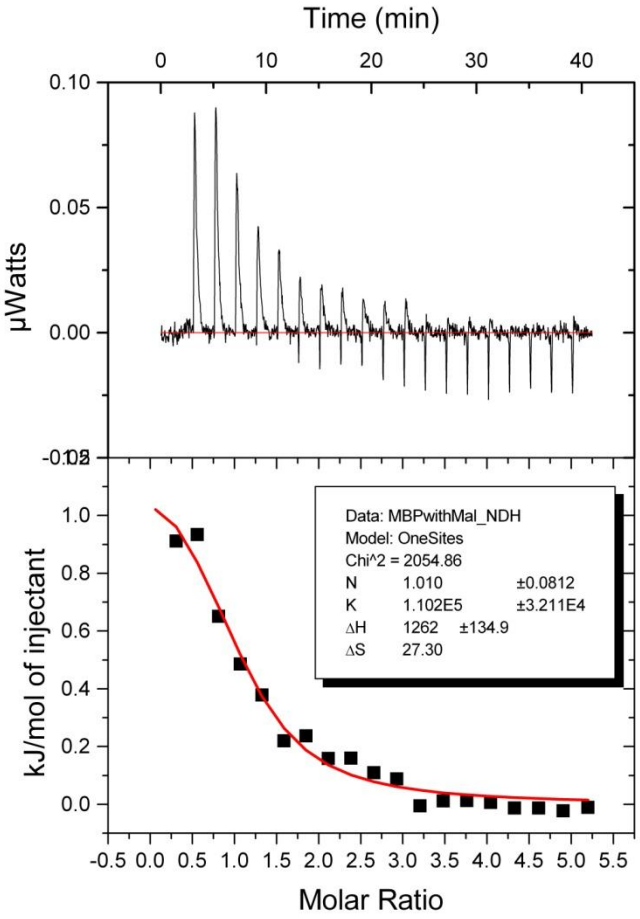


Figure 2-28 ITC with maltose (1 mM) and MBP (40 μM)

2.4 Conclusion

The two principal proteins required for this project were successfully expressed, purified and analysed. Maltoporin was successfully expressed on a workable scale using *E. coli* BL21(DE3)omp8 cells and purified with the use of an amylose column. MBP was expressed from an empty pMal c5x plasmid in *E. coli* C43 cells and purified with an amylose column and eluted with glucose.

Although literature procedures for the expression of maltoporin were available these did not prove reliable and a significant amount of optimisation was required. During

this process information about maltoporin was discovered. It was found that the leader sequence is integral to successful expression of the protein. It was also demonstrated that an OD_{600} of 0.6 is optimum for maltoporin expression and above this value the amount of folded maltoporin expressed is significantly reduced. A slower growth rate is also desirable for efficient removal of the leader sequence from maltoporin. Refolding of maltoporin has previously been shown to be possible from purified maltoporin but after testing a wide range of conditions it was shown not to be possible directly from inclusion bodies.

MST was found to be unsuitable for measuring binding of maltoporin to maltooligosaccarides, presumably as there was little change to the hydration shell of maltoporin on ligand binding. ITC was also evaluated as a binding assay. However, the threading of maltooligosaccharides into the pore of maltoporin was found to occur on a much longer timescale than previously thought, thus making ITC unsuitable for use with this protein.

A maltoporin mutant of G382C was successfully produced and this mutant was derivatised using a rhodamine maleimide compound.

Chapter III: Preliminary studies into the synthesis of the maltose fragment

3.1 Central maltose fragment design

Central to the design of the switch is maltose, a known substrate for maltoporin. This maltose moiety will need to be derivatised at both the reducing and non-reducing end to allow its insertion into the centre of the axle. This central maltose fragment was the crux of the synthesis and its design was based on the known permeability of maltoporin as well as the ease of its integration into the rest of the switch.

To assure the ability of the maltose fragment to pass through maltoporin, its design was kept as close to the natural ligand as possible. An α-maltoside was selected for the initial design as this is the type of linkage found within maltooligosaccharides. The fragment would also be derivatised at the 4'-position and the anomeric centre, again mimicking the natural construct (Figure 3-1). These design criteria provide challenges for synthesis of the compounds as α-glycosides are notoriously more difficult to synthesise than their β-linked cousins and the 4'-position would need to be derivatised regioselectively.

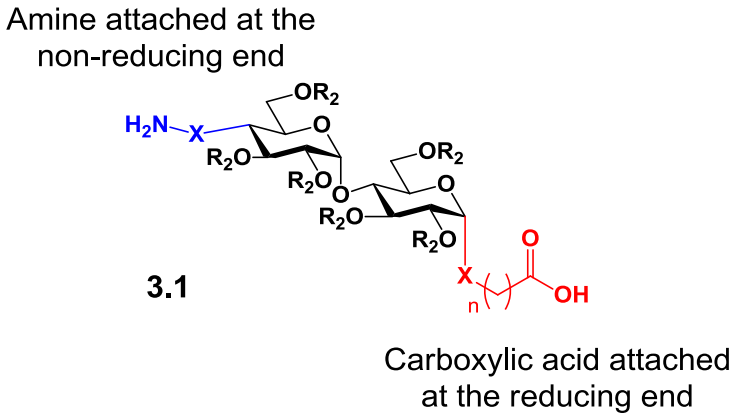


Figure 3-1 Maltose fragment **3.1** derivatised at either end with an amine at the non-reducing end and a carboxylic acid at the reducing end.

To allow the efficient synthesis of the switch a maltose fragment **3.1** derivatised at one end with an amine and a carboxylic acid at the other, for its use in solid phase peptide synthesis (SPPS) (Figure 3-1). This synthetic strategy would allow the length and type of linker to be varied at will.

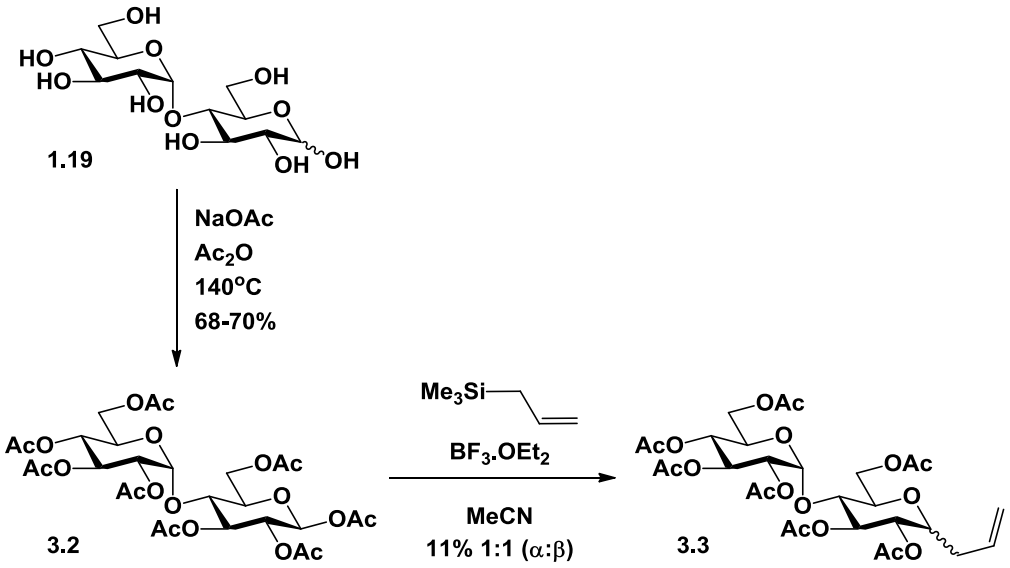
Two parallel synthetic routes were pursued towards production of the maltose mimic. One route involved the introduction of a carboxylic acid at the reducing end of the disaccharide while the other aimed to introduce an amine at the non-reducing end. It was envisaged that once both syntheses had been optimised they could be combined into a single procedure leading to a maltose fragment with both the carboxylic acid and amine attached.

3.2 Introduction of a carboxylic acid at the reducing terminus of maltose

As previously discussed maltoporin is only permeable to α(1→4)–linked sugars therefore in the production of the switch the linkages between maltosyl groups should ideally also be α–configured. α-Anomers are often formed using glycosyl donors with a non-participating protecting group in the 2-position. However, other work previously performed by members of the group had shown that acetylated sugars perform better in SPPS. Therefore, an acetylated α-linked maltose would be the ideal substrate. However, as acetates are participating groups and therefore glycosylation with them will selectively give the β-product, a standard glycosylation procedure could not be followed without the need for extensive remodelling of the protecting groups.

3.2.1 Preliminary studies into the formation of an α-*C*-glycoside

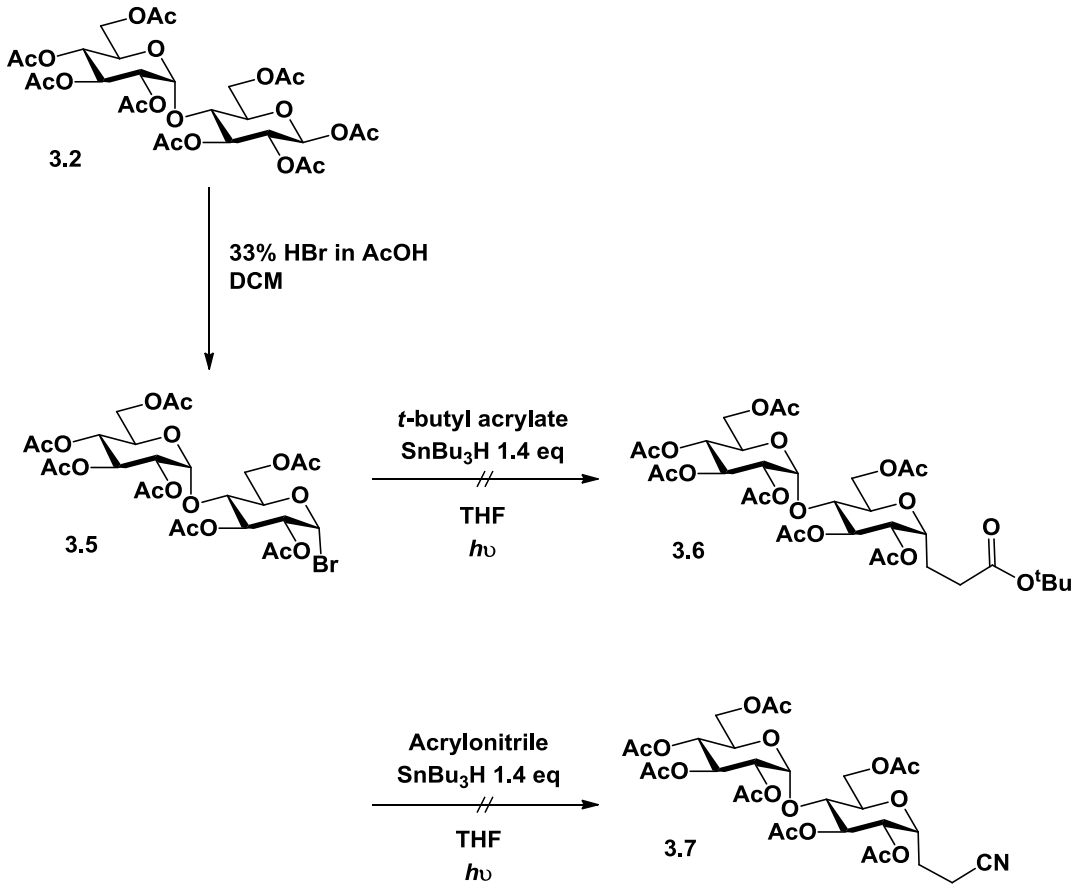
Giannis and Sandhoff have reported a procedure for making α-configured *C*-glycosides from sugar peracetates.[116] As C-glycosides are resistant to glycosidase degradation they are an attractive target for use in the synthesis of glycopolymers. Therefore, initial work focussed around the formation of a *C*-glycoside. This required the formation of acetylated maltose which would act as the donor for *C*-glycosylation.



Scheme 3-1 The C-glycosylation of per-acetylated maltose **3.2** which was found to give a mixture of isomers.

With the peracetate **3.2** in hand the *C*-glycoside **3.3** was synthesised following the general procedure of Giannis and Sandhoff (Scheme 3-1). [116] Although they reported yields in excess of 50% for glucosides and cellubiosides, in my hands a yield of only 11% was achieved for the maltose substrate. This result was attributed to an incomplete reaction as well as the formation of a hemiacetal byproduct. Due to a lack of stereoselectivity and the poor yields this route was abandoned.

Angelo Alberti and co-workers have demonstrated previously that glycosyl halides, including acetobromomaltose, can be converted into α-*C*-glycosides[117] when treated with acrylonitrile and tributyltin hydride under UV irradiation. These reactions are reported to proceed in near quantitative yields and with complete stereoselectivity. Acetobromomaltose **3.5** was formed from acetylated maltose **3.2** using a standard procedure with the use of hydrogen bromide in acetic acid (Scheme 3-2).[118]



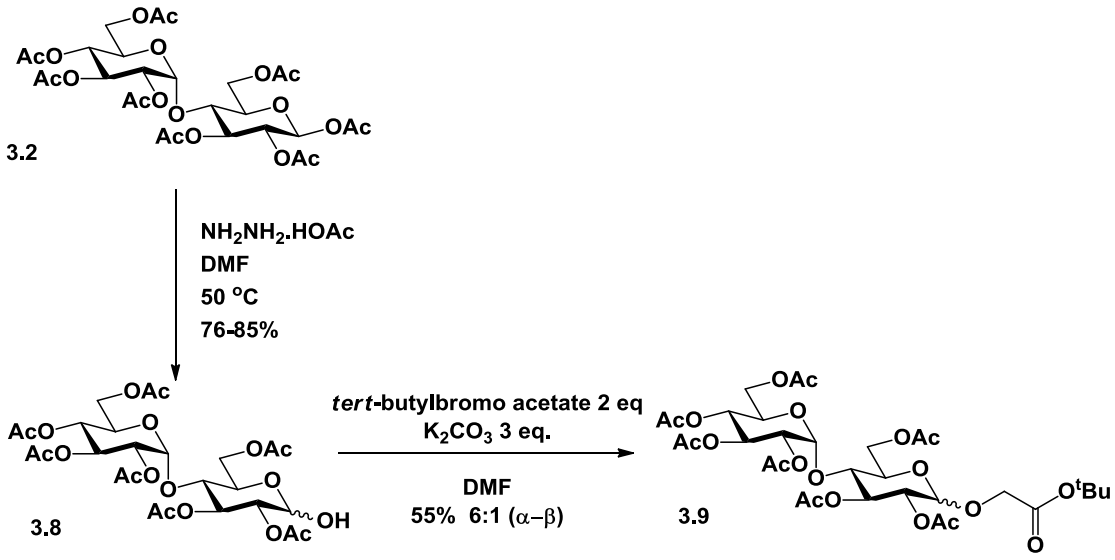
Scheme 3-2 Formation of C-glycosides **3.6** and **3.7** via acetobromomaltoside **3.5** using a UV catalysed reaction.

The radical chemistry was performed under UV light but no product was seen with acrylonitrile or *t*-butyl acrylate. Mass spectrometry showed evidence of polymerisation of *t*-butyl acrylate but even with the use of acrylonitrile, that was shown not to polymerise, no product was seen. Due to the difficulties in the formation of a *C*-glycoside it was decided to focus on the formation of an *O*-glycoside as a model system.

3.2.2 Preliminary studies into the formation of an α-O-glycoside

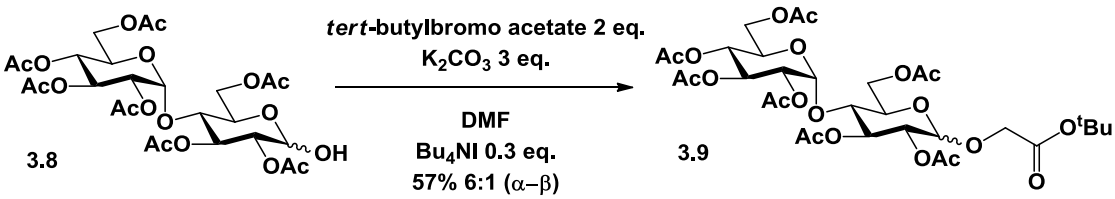
O-Glycosides are much more common, would be simpler to make and could be used as models for studies with maltoporin and MBP *in vitro*. The usual strategy for making

α-glycosides involves using glycosyl donors with non-participating groups. However, the glycosylation reactions rarely proceed with complete stereoselectivity.[119,120] An alternative strategy for making *O*-glycosides is anomeric alkylation. This approach can provide simple α-glycosides in comparable selectivity to traditional methods,[121] and is compatible with the ester protecting groups that are desirable for SPPS. First, peracetate **3.2** was selectively deprotected at the anomeric position using hydrazine acetate in DMF.



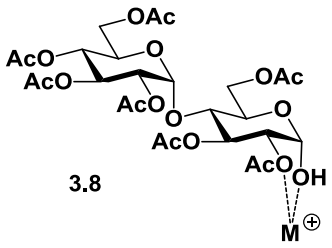
Scheme 3-3 Deprotection and alkylation of the anomeric hydroxyl group.

Alkylation of hemiacetal **3.8** was achieved using tert-butyl bromoacetate and potassium carbonate (Scheme 3-3). This reaction gave a 6:1 (α:β) mixture of anomers **3.9** which were not separable by column chromatography. Some cleavage of the disaccharide glycosidic linkage also occurred which contributed to a decreased yield. The reaction, although successful to a certain extent, had taken 11 days to reach completion.



Scheme 3-4 Alkylation of the anomeric hydroxyl group with the addition of Bu4N.I.

As the alkylation step was slow it was repeated in the presence of tetrabutylammonium iodide (TBAI) to replace the bromine with the more reactive iodide leaving group which should reduce the reaction time (Scheme 3-4). It was found that this additive allowed the reaction to proceed to completion in 24 h with no effect on yield or stereoselectivity.

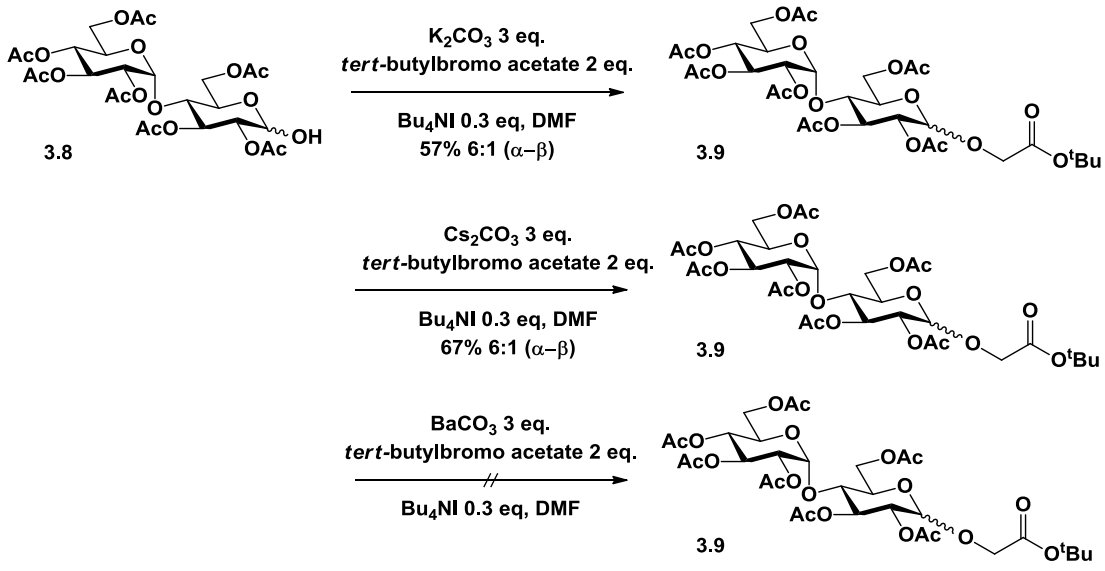


3.8

Figure 3-2 Proposed origins of the stereoselectivity shown in the alkylation reaction.

The speed of the reaction had been increased so now the carbonate base was varied to attempt to improve the stereoselectivity and/or yield of the reaction (Scheme 3-4). This was done as research by Y. Queneau and co-workers from which the original reaction was taken suggested that the stereoselectivity arose from the metal of the carbonate chelating to the acetate in position 2 and the anomeric hydroxyl group, thus holding it in the α-configuration (Figure 3-1).[121] It was thought that a metal with a higher charge may give higher selectivity.

Alkylation did not occur with the use of $BaCO_3$ while Cs_2CO_3 gave a 10% increase in yield (Scheme 3-5). No change in the stereoselectivity of the reactions was found. No reaction was seen with $BaCO_3$ as it is very insoluble and so likely could not participate in the reaction. Why Cs_2CO_3 would increase the yield over K_2CO_3 is unclear.

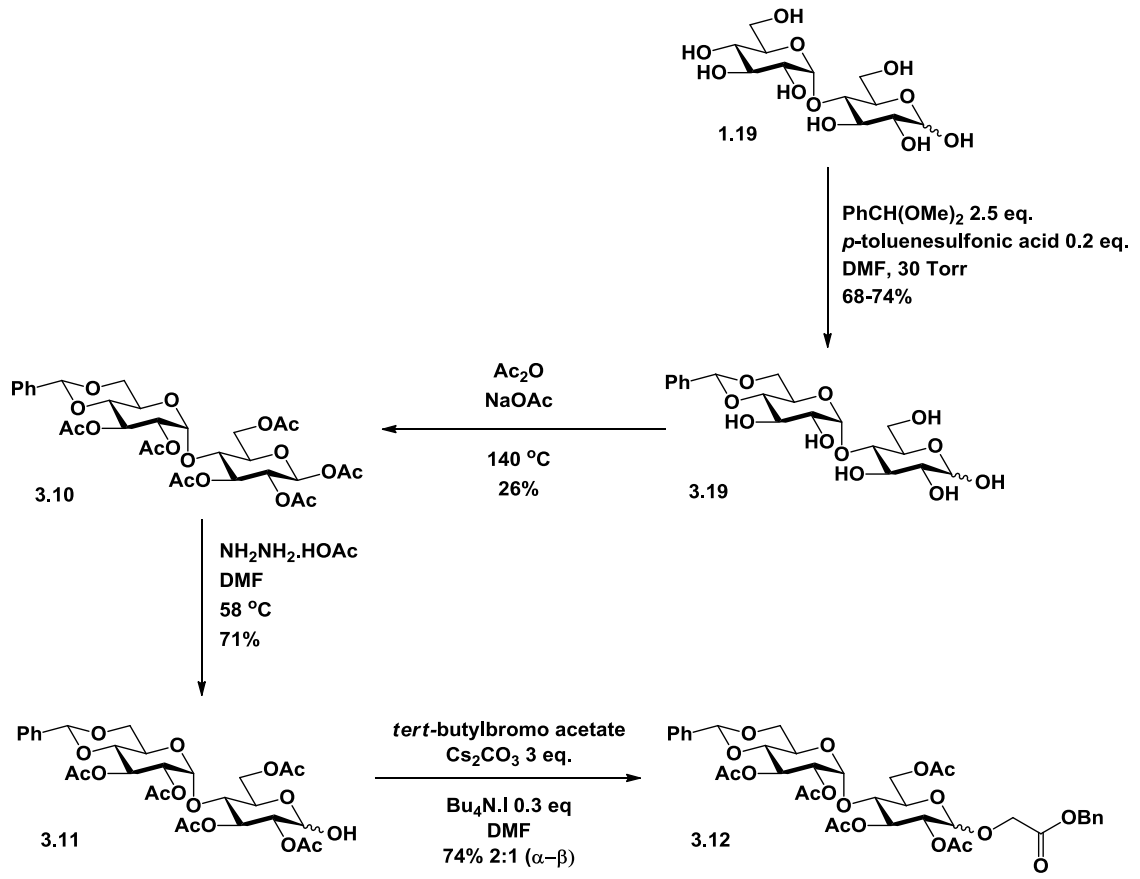


Scheme 3-5 Anomeric alkylation as carried out with a variety of carbonates

Although the yields and rate of reaction had been improved it was still impossible to separate the anomers. So the reaction was repeated using benzylidene acetal derivative **3.10** as it was thought that the added rigidity may allow selective crystallisation of a single anomer.

Removal of the anomeric acetate forming 1-hydroxy product **3.11** was successful (Scheme 3-6). Benzyl bromoacetate was chosen for the alkylation step instead of a *tert*-butyl bromoacetate as the benzyl group would be stable under the acidic conditions needed for removal of the benzylidene acetal.

The reaction gave a better yield than had previously been obtained for the anomeric alkylation reaction; however the stereoselectivity was poorer with a ratio of 2:1 for the α:β anomers (Scheme 3-6). The bulk of a benzyl group compared to a *tert*-butyl group is less so this may account for the loss in stereoselectivity. Although this would suggest a smaller role for the metal ion in the stereoselectivity of the reaction. Crystallisation of the product **3.12** was not possible and the anomers were still found to be inseparable by column chromatography. Partial deprotection of the product **3.12** (either removal of the benzylidene acetal, acetate groups or benzyl group) was attempted but no separation could be achieved.



Scheme 3-6 Removal of the anomeric acetate with hydrazine acetate and alkylation with benzyl bromoacetate.

The selective formation of an α-glycoside had proved difficult although the use of acetate protecting groups was still desired due to their proven success in SPPS. The conventional methods for α-glycoside formation were not investigated due to the added steps involved in changing protecting groups early on in the synthesis of the switch. It was therefore decided to investigate the potential use of β-glycosides.

3.2.3 Is maltoporin permeable to β-maltosides?

The work so far has illustrated the difficulty in achieving formation of the α-glycoside selectively. This led to research into whether maltoporin could be permeable to the much more easily synthesised β-glycoside. Crystal structures have shown two maltose

molecules end on in the binding site arranged in a configuration analogous to a β-glycosidic linkage (Figure 3-3). This observation does not necessitate that a β-linked molecule could pass through the pore, but it does suggest that it could bind to maltoporin.

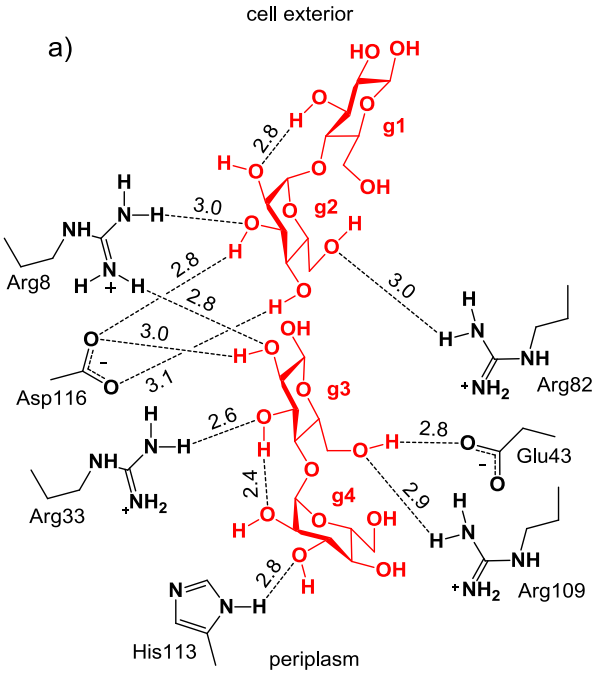
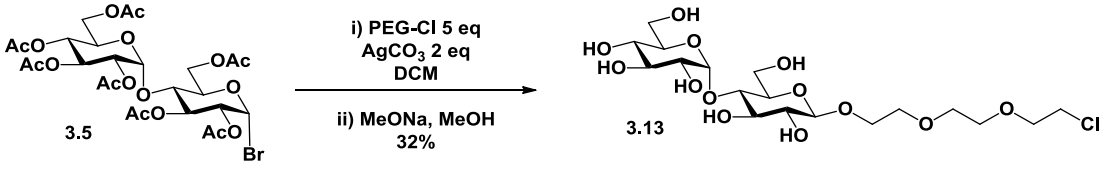


Figure 3-3 Binding site of maltoporin demonstrating the orientation of sugars into a β-linkage like arrangement.

To investigate the permeability of maltoporin, a β-glycoside was synthesised with a PEG group as the aglycone as this is similar to the linking groups in the final polymer. The β-glycoside **3.13** was produced stereoselectively from acetobromomaltose **3.5** (Scheme 3-7).



Scheme 3-7 Synthesis of β-glycoside 7 from acetobromomaltose 5.

A liposome swelling assay was used to measure permeability as it is a simple procedure which has been used previously for maltoporin.[99,122] In a liposome swelling assay giant multilamellar vesicles are formed in the presence of dextran and punctuated with maltoporin. This allows suitable ligands to pass into the vesicles and due to osmotic pressure causes them to swell (Figure 3-4). The swelling of these vesicles can be followed by light scattering and these values can then be compared to a negative control in the absence of maltoporin. As maltose and sucrose have previously been studied using this method, they can be used as positive and negative controls, respectively.

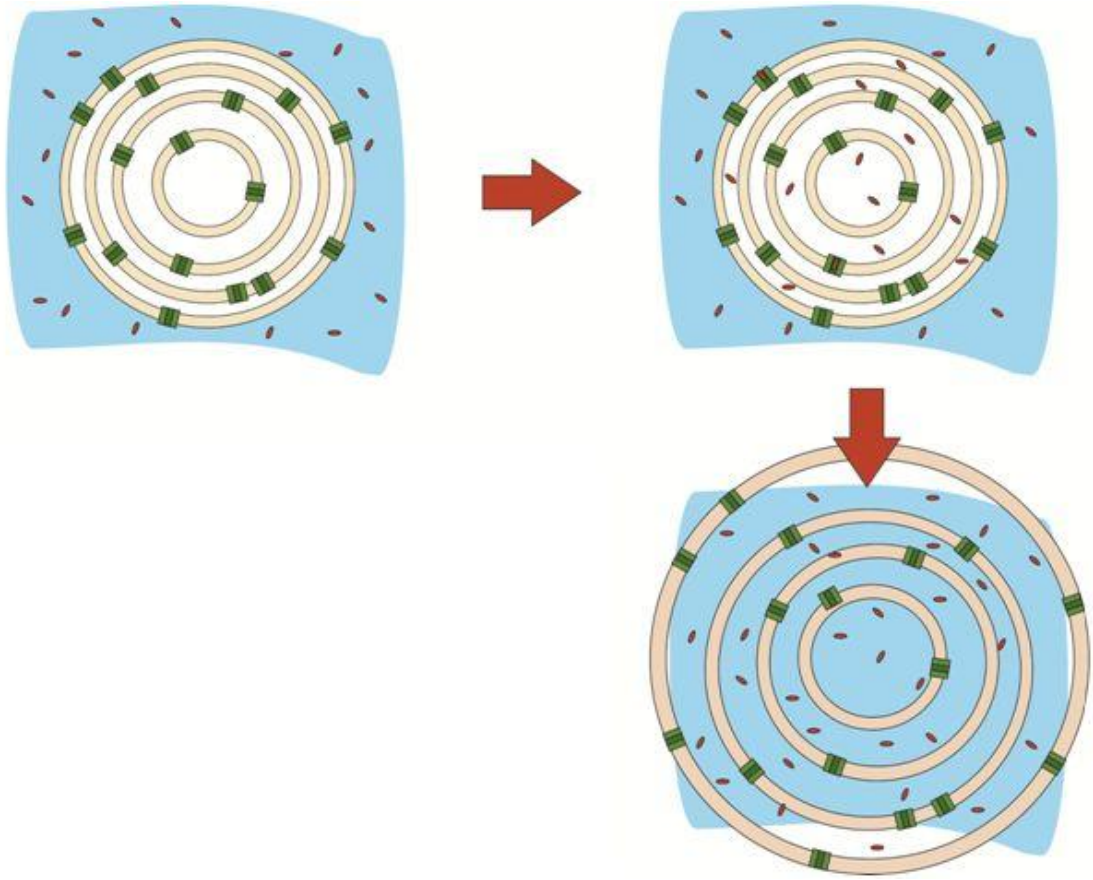


Figure 3-4 The liposome swelling assay

Following the liposome swelling assay procedure,[123] 20 μl of 20 mM sugar solutions were added to 600 μl of liposomes containing 0.2 μM maltoporin. The absorption was measured every 5 seconds for 1 minute after addition and the gradients of the sugars compared. A bigger loss in absorption means a greater degree of swelling of the liposomes and therefore maltoporin has a greater permeability to that ligand.

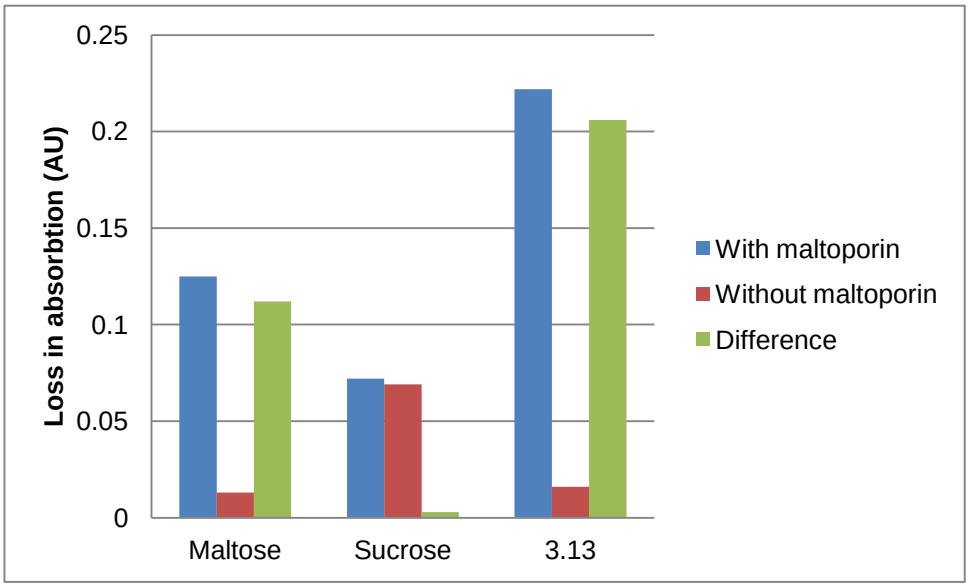


Figure 3-5 Liposome swelling assay results showing loss in absorbtion of vesicles with maltoporin (blue), without maltoporin (red) and the difference between these two results (green).

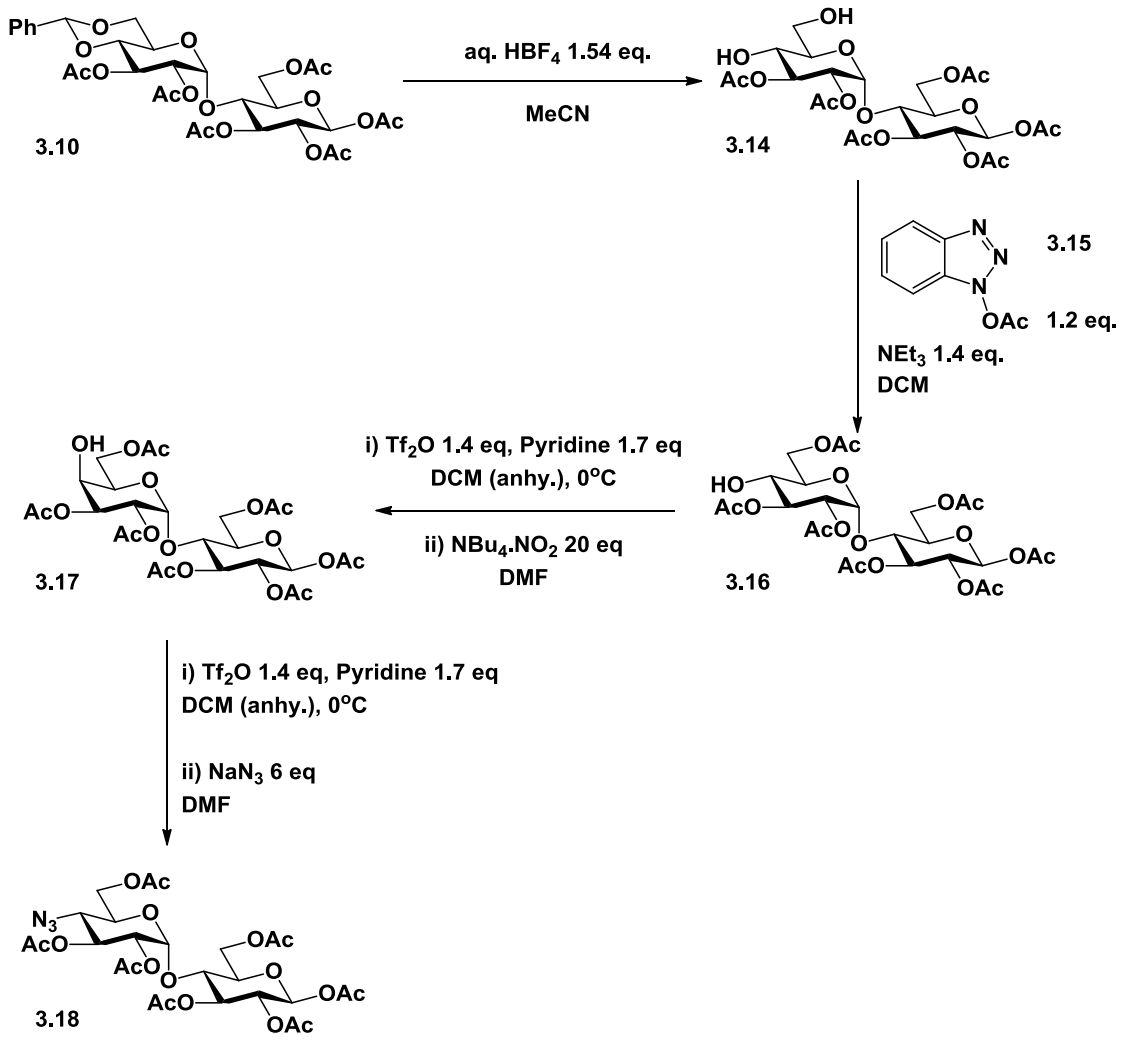
The liposome swelling assay results (Figure 3-5) show maltoporin to be permeable to maltose but not to sucrose. This is in agreement with T. Schirmer and co-workers.[124] The β-maltoside **3.13** is shown to have a higher degree of permeability than maltose. This could be explained by the β-maltoside having a lower affinity for maltoporin. It has previously been demonstrated that maltooligosaccharides with a higher affinity for maltoporin reside in the pore for longer.[68] If the binding of the β-maltoside is weaker than maltose it would spend less time in the binding site and so pass through the pore faster. As only its ability to pass through the pore of maltoporin is desired the synthesis of a maltose mimic could be continued with a β- instead of an α-glycosidic linkage.

3.3 Preliminary studies into the selective derivatisation of the 4'-position

This section will outline investigations into derivatisation of the 4'-position and its optimisation. The chemistry at the non-reducing end comprised two distinct challenges: the first being selective deprotection of the 4'-position; the second being attachment of a functional group to the unprotected hydroxyl group. Sugar chemistry is notorious for

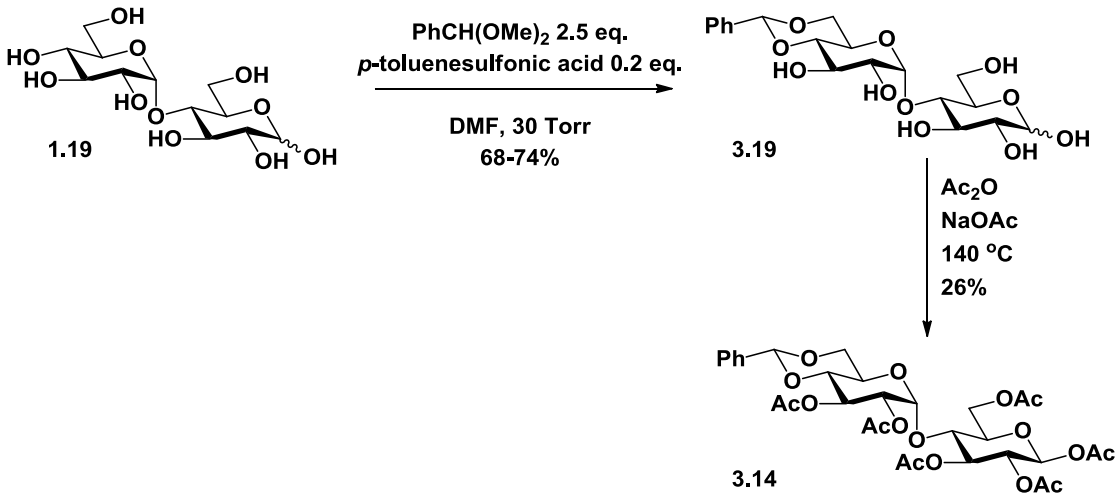
its dependence on protecting group chemistry and, as such, several methods exist for the selective deprotection of the 4-position of glycosides based on the regioselectivity of protecting group manipulations.

3.3.1 Initial plan for introduction of an amine at the non-reducing terminus of maltose



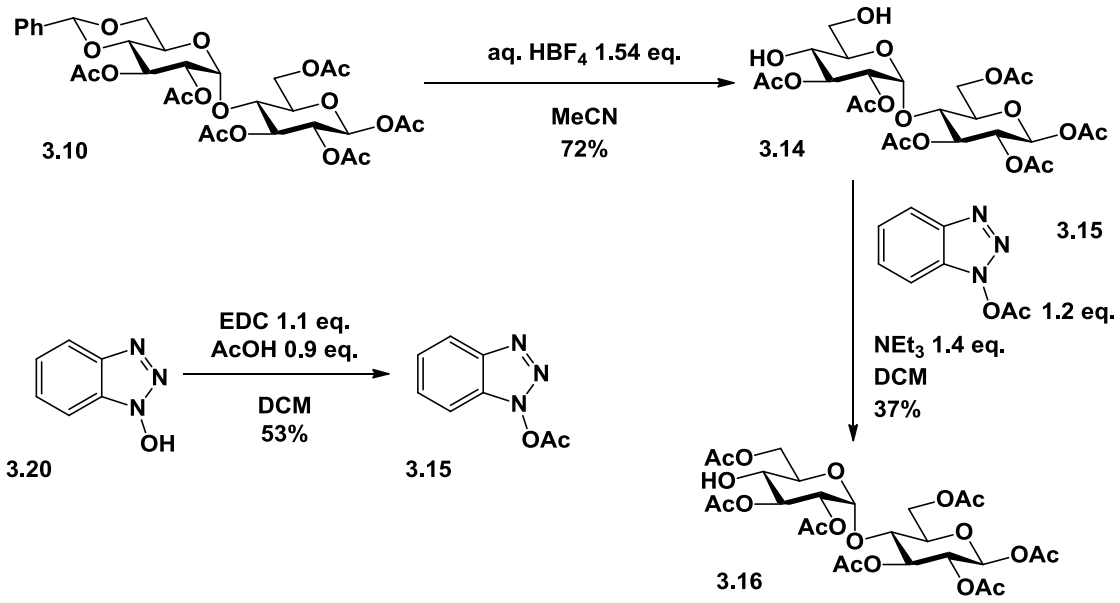
Scheme 3-8 The patented synthesis of amino sugar 15.[86]

A patented route exists for the selective deprotection of the 4'-position followed by formation of a 4'-amine starting from the benzylidene acetal protected sugar **3.10** (Scheme 3-8).[86] Benzylidene acetals have been shown to selectively protect the 4- and 6-positions of sugars as they are the only positions able to form a stable 6-membered ring. This molecule could be selectively synthesised from maltose.



Scheme 3-9 Protection of the 4' and 6' positions on maltose with a benzylidene acetal.

The 4' and 6' positions of maltose **1.19** were protected using dimethoxy toluene in the presence of acid (Scheme 3-9) to give benzylidene acetal **3.19**. After the reaction was complete, the product was stirred with TFA to remove any acyclic acetals that had formed with other hydroxyl groups. Flash column chromatography gave the product in good yields allowing for the protection of the remaining hydroxyl groups. This was done using a hot acetylation protocol as procedures in the presence of amine bases usually lead to a mixture of anomers. The hot acetylation protocol is selective for the β-anomer (Scheme 3-9),[125] removing the additional complication of needing to characterise a mixture of diastereoisomers. The hot acetylation was stereoselective with ~9:1 formation of the β:α glycoside, giving exclusively the β-product **11** following recrystallization. As the reaction could be scaled up easily, the low yield could be accepted given its stereoselectivity. With the regioselectively protected maltoside **3.10** in hand, the 4'-position could be deprotected.



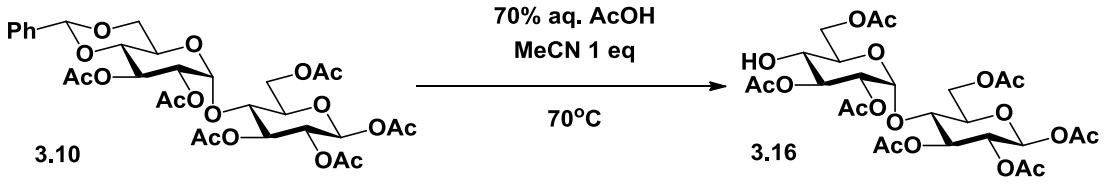
Scheme 3-10 Removal of the benzylidene acetal with fluoroboric acid before acetylation of the 6' hydroxyl group using acetylated HOBt.

Following the method of P. Deshong and co-workers the benzylidene acetal was removed with the use of fluoroboric acid in acetonitrile, to give the diol **3.14**. To selectively protect the 6'-hydroxyl group a weak acetylating agent was needed. Acetylated HOBt **3.15** had been reported to enable this reaction,[86] however in my hands the reaction resulted in moderate yields (Scheme 3-10). The acetylating agent was shown to not to be very selective, as the 4'-position was substantially acetylated before all the 6'-position had reacted. As benzylidene acetal removal followed by acetylation gave an overall yield of 27%, a new more selective method was sought.

3.3.2 The testing of acetic acid as an efficient reagent for the selective acetylation at the 6'-position

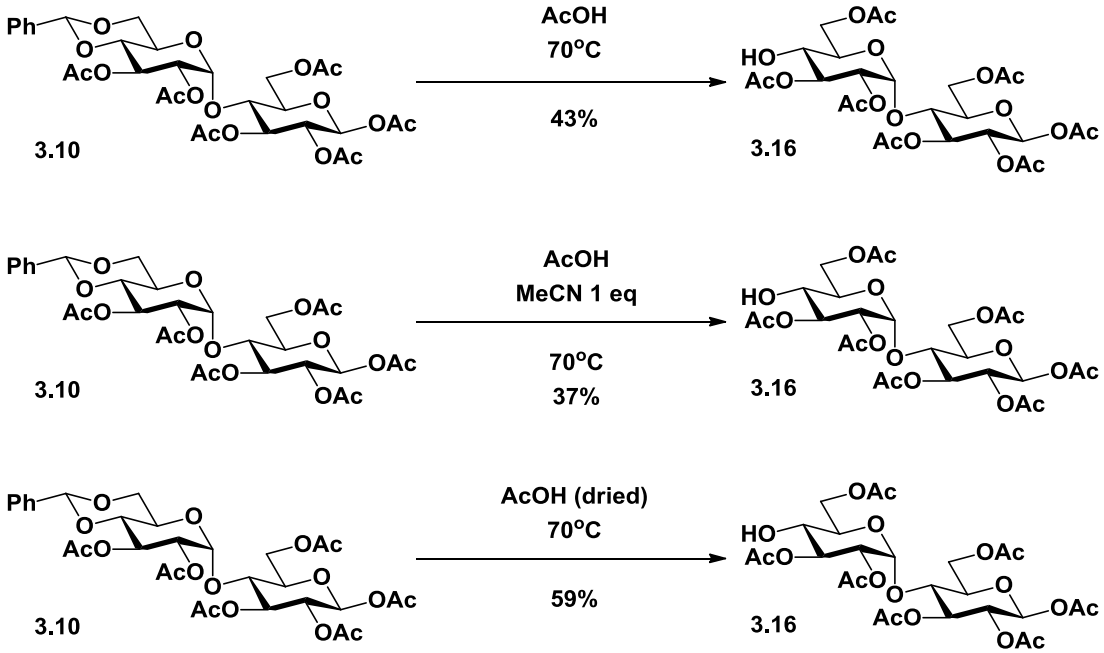
A novel one step synthesis based on a personal communication with Stefan Oscarson was investigated as it combined deprotection and acetylation into a single step. The Oscarson group had observed 6'-acetylation in near quantitive yields while attempting to hydrolyse a benzylidene acetal using aqueous acetic acid in acetonitrile. In my

hands 4'-hydroxyl **3.16** was produced in ~10% yields (Scheme 3-11) with deacetylation shown to occur after 4 h.



Scheme 3-11 First attempt at the combined benzylidene acetal removal and 6' acetylation.

The reaction appeared to be promising as the product had been produced even though it was also consumed after a short period of time. Which of the reagents were important in the reaction was unclear, but deacetylation would be unlikely to occur if H_2O was excluded, so the reaction was repeated under dry conditions.

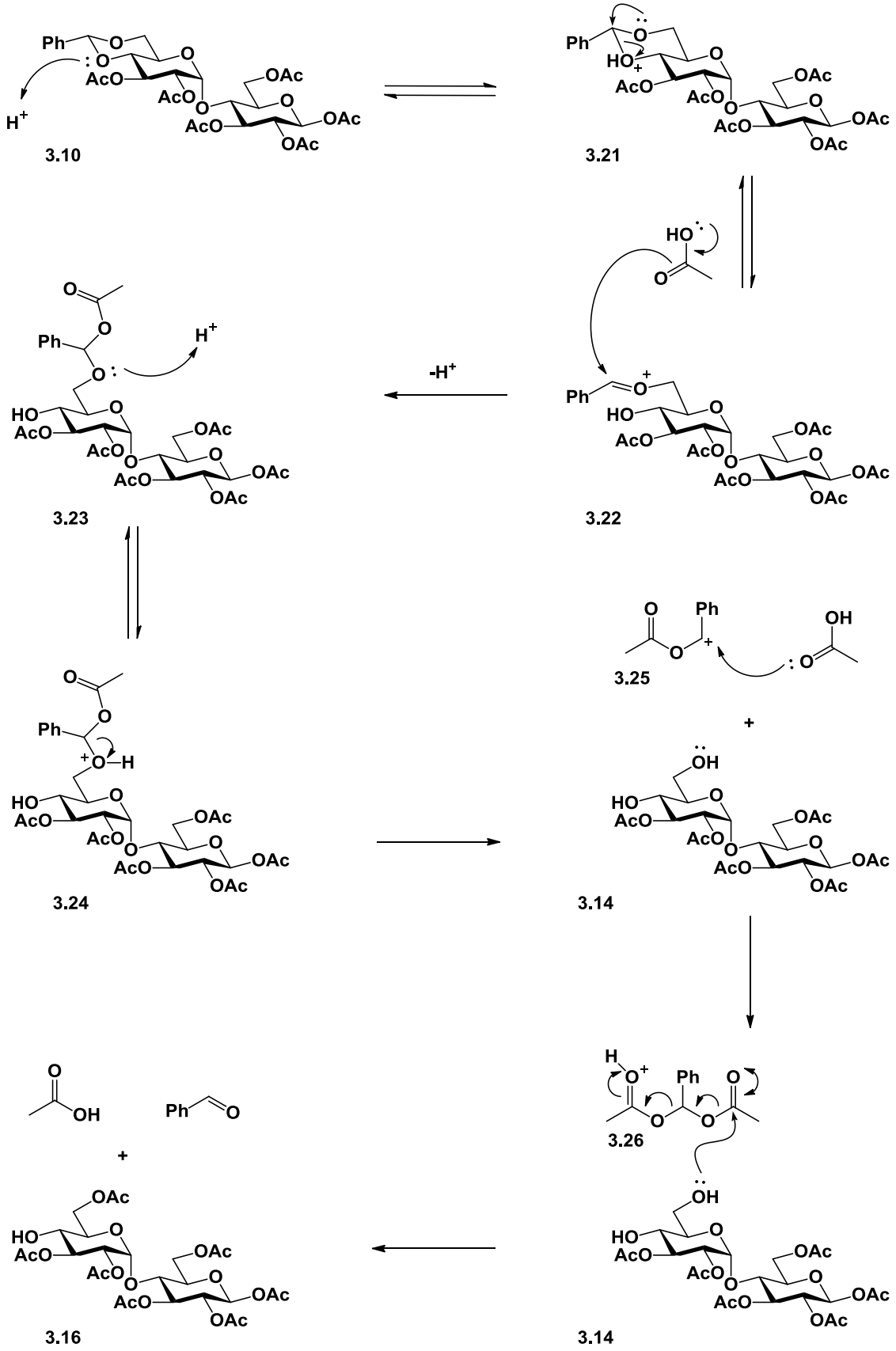


Scheme 3-12 The various methods attempted for the combined benzylidene acetal removal and subsequent 6' acetylation.

It was found that excluding water from the reaction increased the yield to 43% and this increased again to 59% if the acetic acid was dried via crystallisation prior to use. It

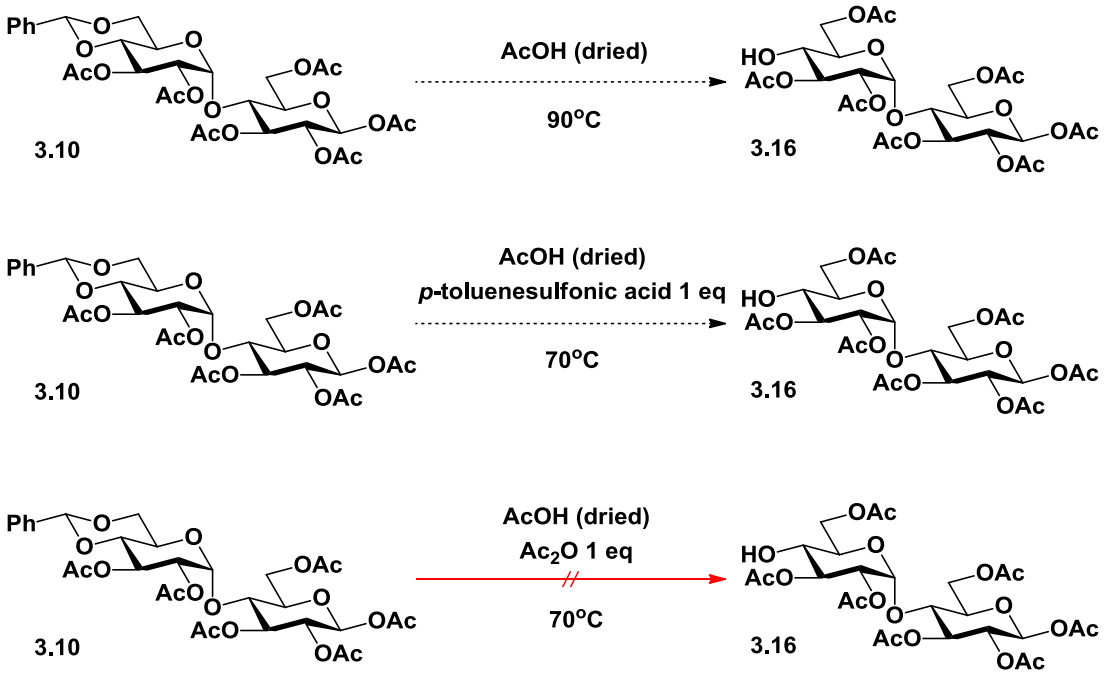
was also discovered that MeCN was not essential for the reaction. Now that the conditions to improve the yield of reaction were known, a possible reaction mechanism was proposed (Scheme 3-12).

It was thought that the benzylidene acetal could be protonated allowing ring opening to produce an oxacarbenium ion **3.22**. Acetic acid may then act as a nucleophile attacking the benzylic position to produce acyclic acetal **3.23**. The benzylidene acetal could then be lost through an acid catalysed S_N1 reaction with another molecule of acetic acid to give the benzylidene diacetate **3.26**. It is this molecule **3.26** that could then act as an acetylating agent, reacting with the more reactive 6'-position to give the target molecule **3.16**. This hypothesis seems plausible as the positive charge would be stabilised by delocalisation on carbocation **3.22** and acetic acid would seem to be the only species in the reaction mixture able to act as a nucleophile. The final step yields the product plus acetic acid and benzaldehyde (Scheme 3-13).



Scheme 3-13 Reaction scheme for the putative mechanism of the removal of the benzylidene acetal and subsequent acetylation of the primary alcohol.

Although the yield had been increased significantly, the reaction had taken 8 days to reach completion. Therefore, with a hypothesis for the mechanism in hand, three different methods were investigated with the aim of increasing the reaction rate. A higher temperature, stronger acid and drying agents were all tested (Scheme 3-14) with the reactions followed by TLC.

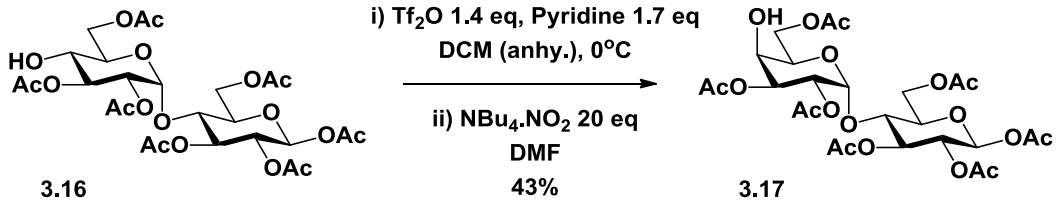


Scheme 3-14 Deprotection/acetylation steps carried out at higher temperature, with p-toluenesulfonic acid added and with acetic anhydride added. These reactions were not purified but followed by TLC.

Addition of acetic anhydride gave no product within 24 h. This could mean that water is needed in some catalytic amount during the reaction for example during the initial removal of the benzylidene acetal. From TLC anaylsis heating the reaction and the use of a stronger acid resulted in faster product formation but also caused cleavage of the glycosidic bond and formation of the per-acetylated products leading to a reduced yield. As these changes in method had produced no increase in efficiency of the reaction and a successful method was now available for the deprotection of the 4'-position the optimisation was stopped and derivatisation at the 4'-position pursued.

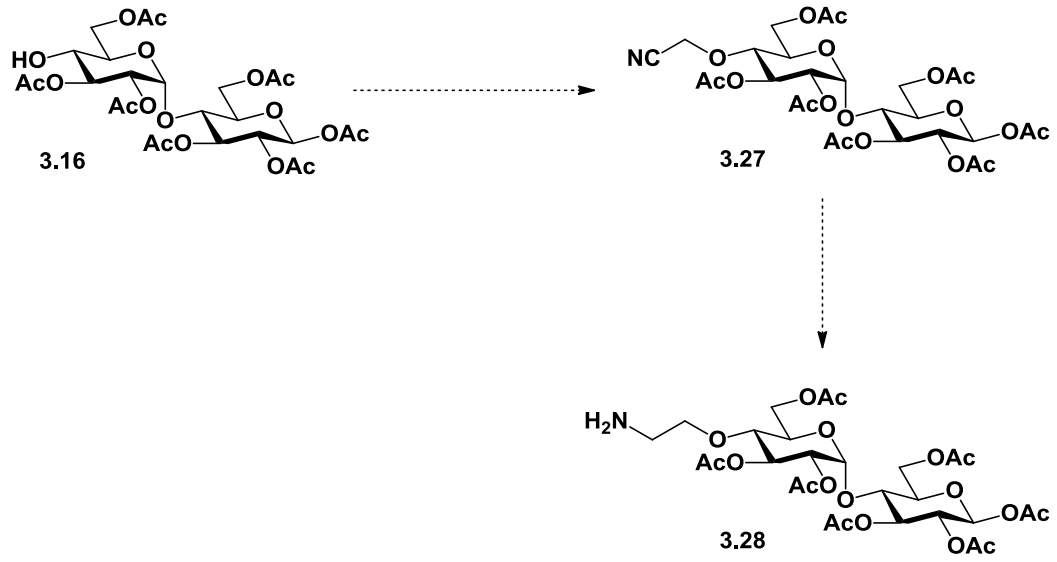
3.3.3 Alkylation at the 4'-position of an acetylated sugar

Following the work of P. Deshong and co-workers (Scheme 3-8), the 4'-position would be converted to an amine allowing its use in SPPS. The configuration at the 4'-position was inverted by conversion of the 4'-alcohol to a triflate followed by an S_N2 reaction with tetrabutylammonium nitrite. The intermediate nitrite ester hydrolysed on aqueous work-up to give galactoside **3.17** in 43% yield (Scheme 3-15). However, NMR spectroscopy showed the product comprised of a 10:1 ratio of axial and equatorial isomers.



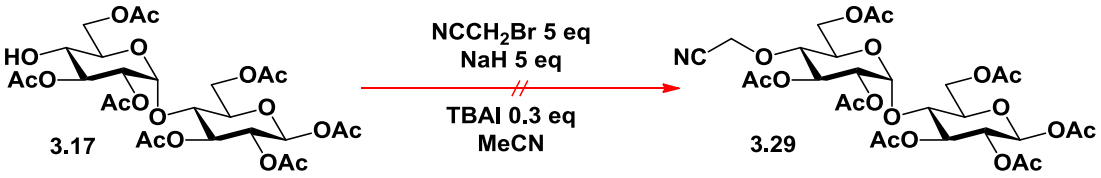
Scheme 3-15 Inversion of the 4' position.

As a mixture of diastereoisomers was formed in a low yield a different strategy was considered in which the 4'-hydroxyl group would be alkylated with bromoacetonitrile. The nitrile group could then be reduced to an amine (Scheme 3-16).



Scheme 3-16 Alkylation strategy for introduction of an amine at the non-reducing terminus.

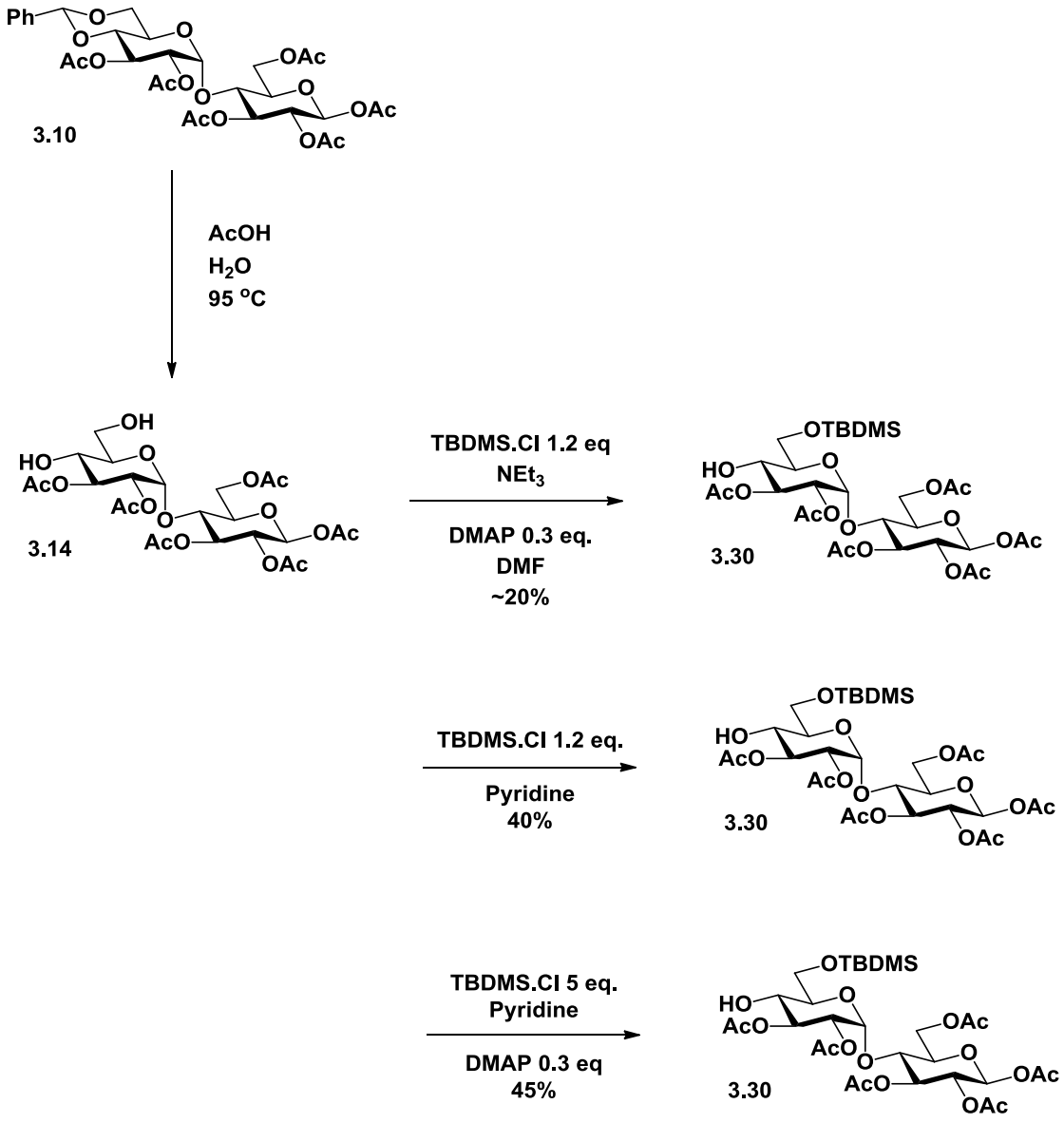
Alkylation at the 4'-position was tested with bromoacetonitrile (Scheme 3-17). Alkylation was found to occur but a mixture of products was produced due to the formed 4'-alkoxide also picking up acetate groups resulting in non-specific alkylation. Acetate groups are also known to migrate between adjacent hydroxyl groups especially under conditions where the alkoxide is formed. Although alkylation was shown to be incompatible with acetate protecting groups, it did present itself as a promising route due to the reduced number of steps compared to inversion and the variety of functional groups that could be attached using this method. It was therefore decided to pursue the alkylation reaction and search for a different protecting group strategy.



Scheme 3-17 Attempted alkylation at the 4' position of the heptaacetate protected sugar.

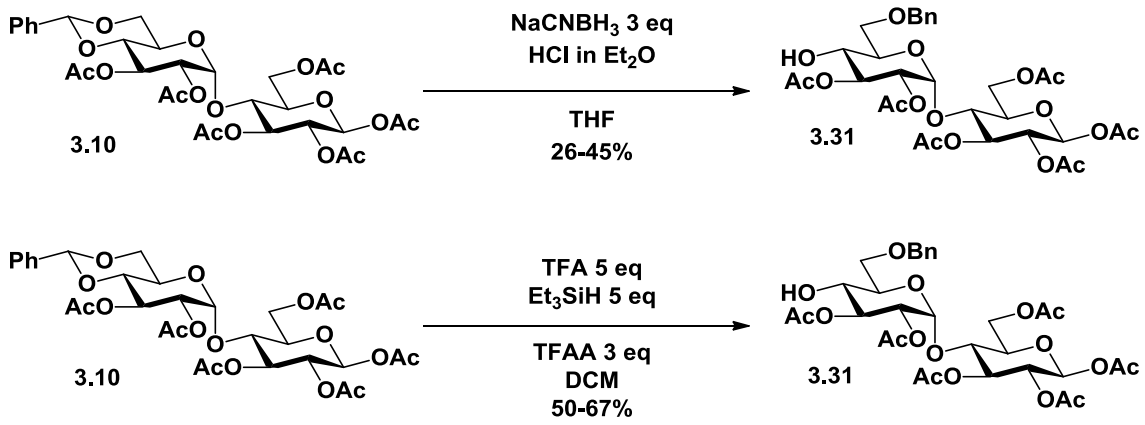
3.3.4 New protecting group strategies

As acetate groups were found to be incompatible with alkylation new methods to replace these protecting groups were investigated. First a new strategy for the protection of the 6'-position was investigated. Aqueous AcOH was used to remove the benzylidene acetal and the 6'-position was protected with a silyl-protecting group (Scheme 3-18). Three different conditions were used for silyl-protection with the use of pyridine as a solvent and DMAP as the catalyst found to give the best yields.



Scheme 3-18 Reaction schemes for the removal of the benzylidene acetal and protection of the 6' position with a TBDMS group.

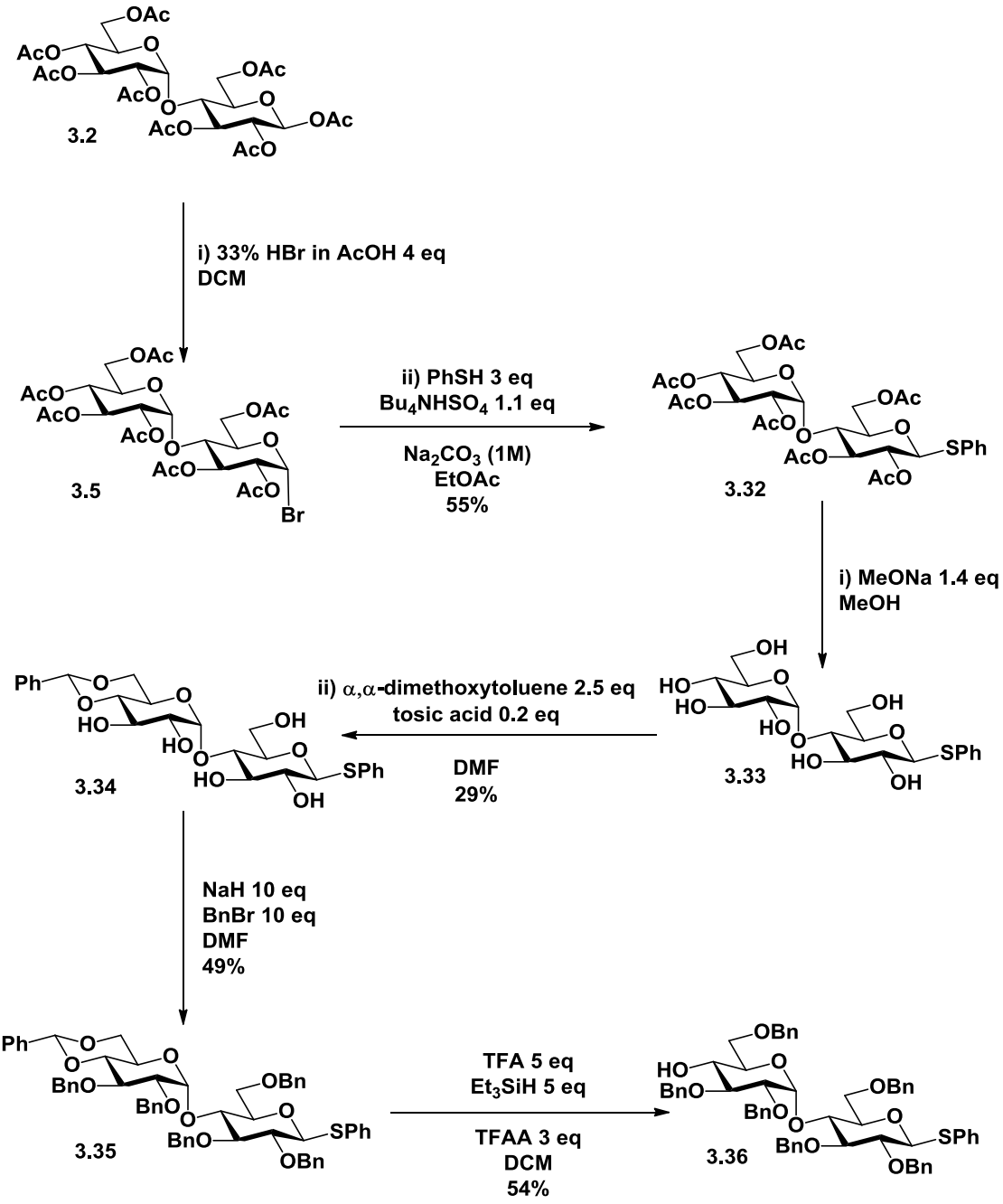
The yields for protection with a silyl protecting group were not as high as would be desired so the reductive opening of the benzylidene acetal was investigated. Sodium cyanoborohydride reductive opening of the benzylidene acetal gave yields similar to the use of a silyl protecting group. Another method using triethylsilane, known to be an effective reducing reagent with benzylidene acetals[126], and TFA in the presence of the trifluoroacetic acid anhydride was tested. This method gave the product **3.31** in one step and in higher yield (Scheme 3-19).



Scheme 3-19 Ring opening and reduction of the benzylidene acetal.

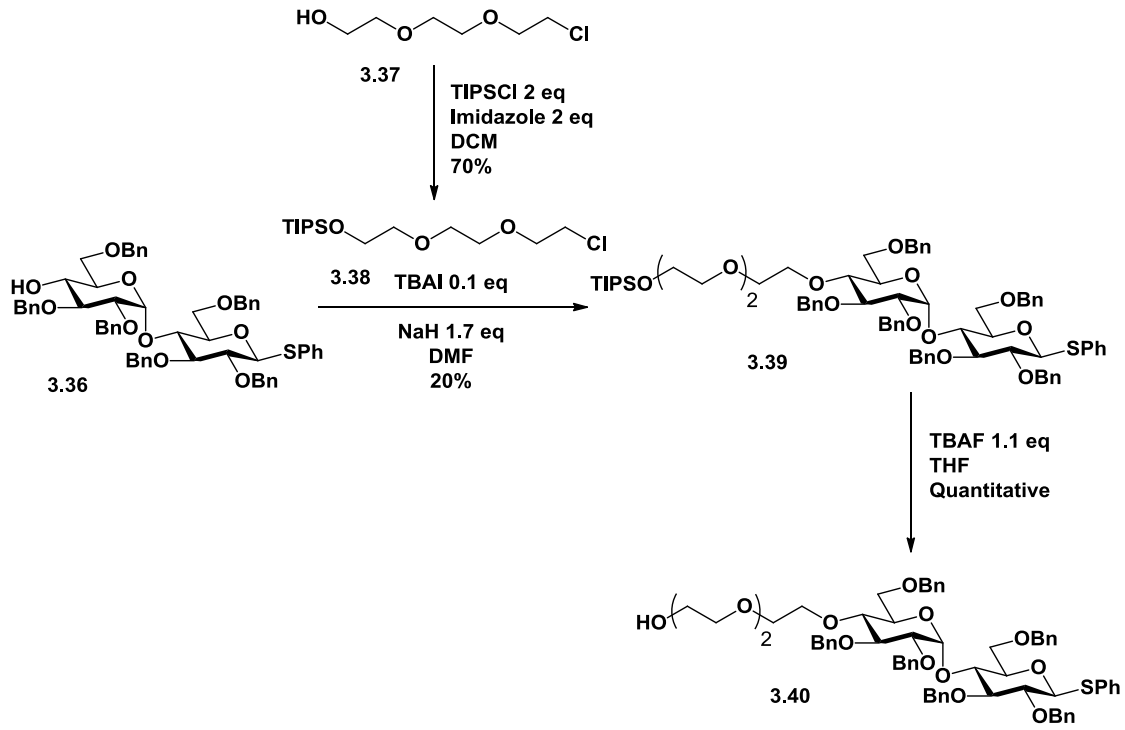
Now an effective method had been found for the deprotection and protection of the 4'- and 6'-positions respectively this method could be combined with a new protecting group strategy. As reducing the benzylidene acetal results in the formation of a benzyl group it made sense to use benzyl groups to protect the rest of the molecule as they could all be removed in one step.

To test the alkylation reaction with benzyl protecting groups a single anomer was desired to allow for ease of characterisation. A β-thioglycoside was chosen as it would be stable to all the conditions of the synthesis and could be orthogonally reacted to allow derivatisation of the reducing end of the sugar. From acetobromomaltose **3.5** a thiophenolglycoside **3.32** was formed, this was then deprotected with sodium methoxide in methanol. Once derivatised with a benzylidene acetal it was also protected with benzyl groups and the 4'-position deprotected using triethylsilane showing the reaction to be robust to different protecting groups. The synthesis of the 4'-deprotected maltoside **3.36** was a success producing 1 g of the product **3.36** over 8 steps in a 8% overall yield (Scheme 3-20).



Scheme 3-20 The synthesis of 4'-deprotected thioglycoside **3.36** from per-acetylated maltose.

With a benzyl-protected maltoside in hand alkylation of the 4'-position could be tested. This was carried out using TIPS-protected 2-(2-(2-chloroethoxy)ethoxy)ethanol **3.38**. The PEG group was chosen as it gave the option of later derivatisation with a number of different functional groups as well as putting the position of connection distal from the sugar to hopefully avoid perturbing binding to the protein. TIPS was selected as it is one of the most base stable silyl ethers which could be cleaved orthogonally to the benzyl ethers (Scheme 3-21).



Scheme 3-21 Protection of 2-(2-(2-chloroethoxy)ethoxy)ethanol **3.37** with TIPS ether and subsequent derivatisation of thioglycoside **3.36**

Alkylation at the 4'-position was successful but low yielding likely in part due to the reaction proceeding via an unfavourable S_N2 reaction β to an oxygen (Scheme 3-21). The TIPS group was removed using TBAF in quantitative yield leaving a lone hydroxyl group to be derivatised as needed. The preliminary studies on the non-reducing end of the sugar were now complete. It has been shown that the 4'-position can be selectively deprotected and functionalised.

3.4 Preliminary studies conclusion

This chapter has covered the preliminary studies into forming the maltose fragment derivatised at the 4'-position and anomeric position needed for the synthesis of the switch.

Investigations into formation of the desired α-glycoside showed this synthetic route to be problematic. Therefore a β-glycoside's ability to pass through maltoporin was tested with a liposome swelling assay. This test found that maltoporin is permeable to β-maltosides therefore the switch axle could contain β-linked maltosides.

The work at the non-reducing end showed the use of triethylsilane was the most convenient route to selective 4'-deprotection. It also demonstrated that alkylation was the most promising method to derivatise the 4'-position and benzyl protecting groups were the best type of protecting group to achieve this. A sugar was also successfully alkylated at the 4'-position allowing these methods to be taken through into the synthesis of the switch axle.

Chapter IV: The synthesis of molecules designed to probe the binding of maltoporin

4.1 Introduction

During this project a variety of compounds have been synthesised alongside the development of the switch. These compounds have all been designed to probe the working of maltoporin through binding studies. The first part of this chapter deals with the synthesis of three molecules which will help in understanding the binding of maltoporin in very different ways. While the second part of this chapter investigates the methods to test the binding of these molecules.

4.2 The synthesis of a pseudotetrasaccharide to probe the bind-and-slide mechanism of maltoporin

Maltoporin operates a bind-and-slide mechanism and it is this functionality that is being used to enable the operation of the switch. To understand this mechanism better and thus ensure the successful implementation of the switch, a pseudotetrasccharide was synthesised. This compound had many of the functional groups that would be present in the switch and its binding would give insights into how the final switch would operate in conjunction with maltoporin.

The pseudotetrasaccharide would consist of two maltose units connected by a PEG-linker. This would serve two purposes; it would demonstrate the permeability of maltoporin to this kind of structure and also deliver information on how a molecule like this would bind.

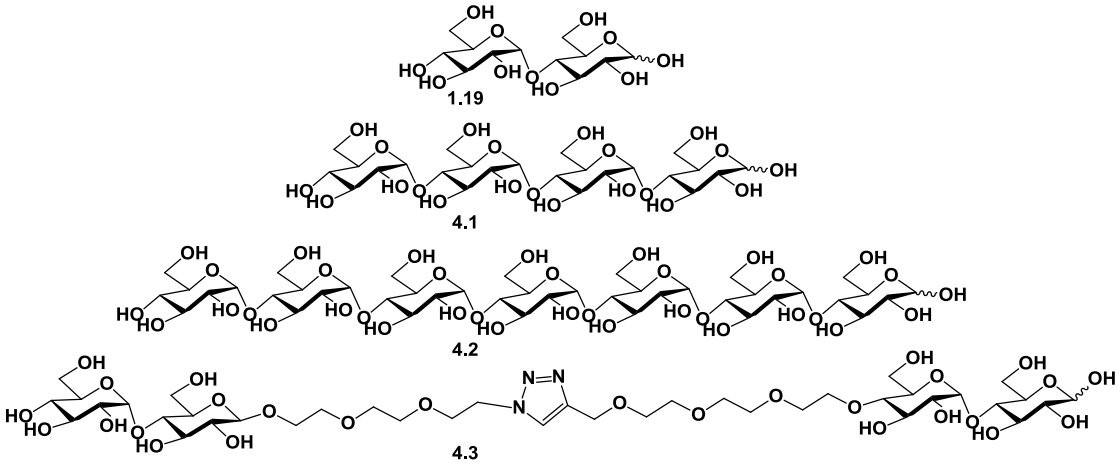
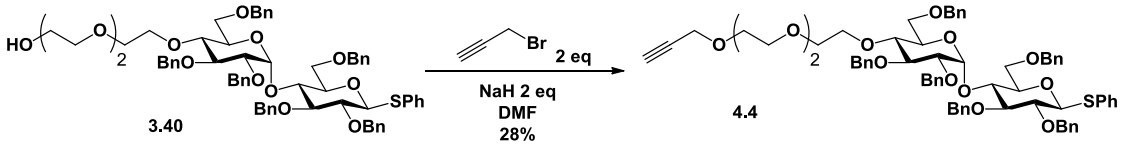


Figure 4-1 Maltooligosaccharides compared to the pseudomaltotetraose

A pseudomaltotetraose **4.3** (Figure 4-1) would be interesting to study as it is not known how important the distance between the binding sites on maltooligosaccharides is for their binding to maltoporin. A pseudomaltotetraose **4.3** could be thought to mimic two independent maltose units or it could bind with a similar affinity to maltotetraose **4.1** with the PEG-linker being of little importance. Maltoheptaose **4.2** will also be tested as the two molecules have a similar length and this could be a factor in binding.

4.2.1 Synthesis of the pseudotetrasaccharide

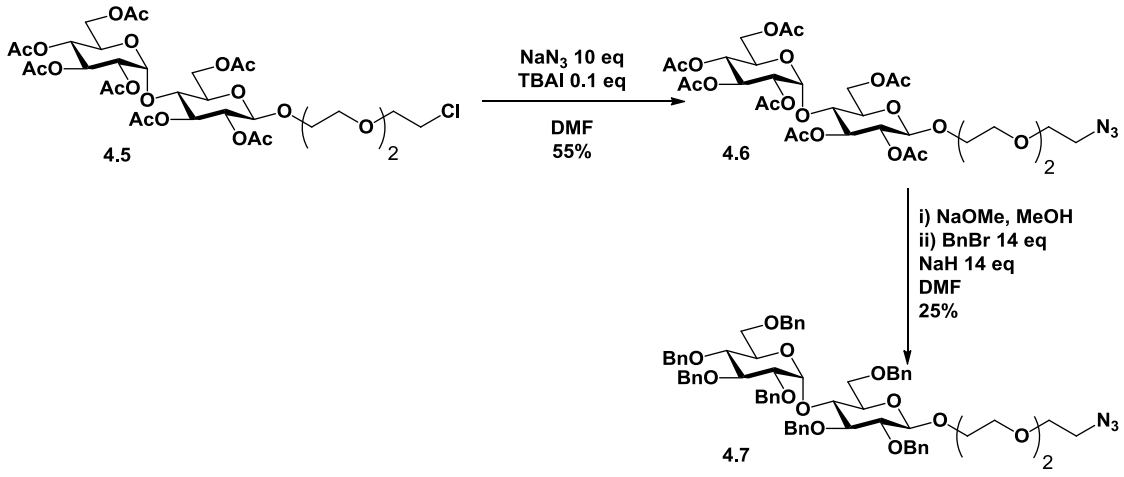
The thioglycoside **3.40** synthesised in the last chapter was reacted with propargylbromide to produce a maltoside **4.4** derivatised at the 4'-position with an alkyne (Scheme 4-1).



Scheme 4-1 Derivatisation of the free hydroxyl in donor sugar **3.40** with propargyl bromide.

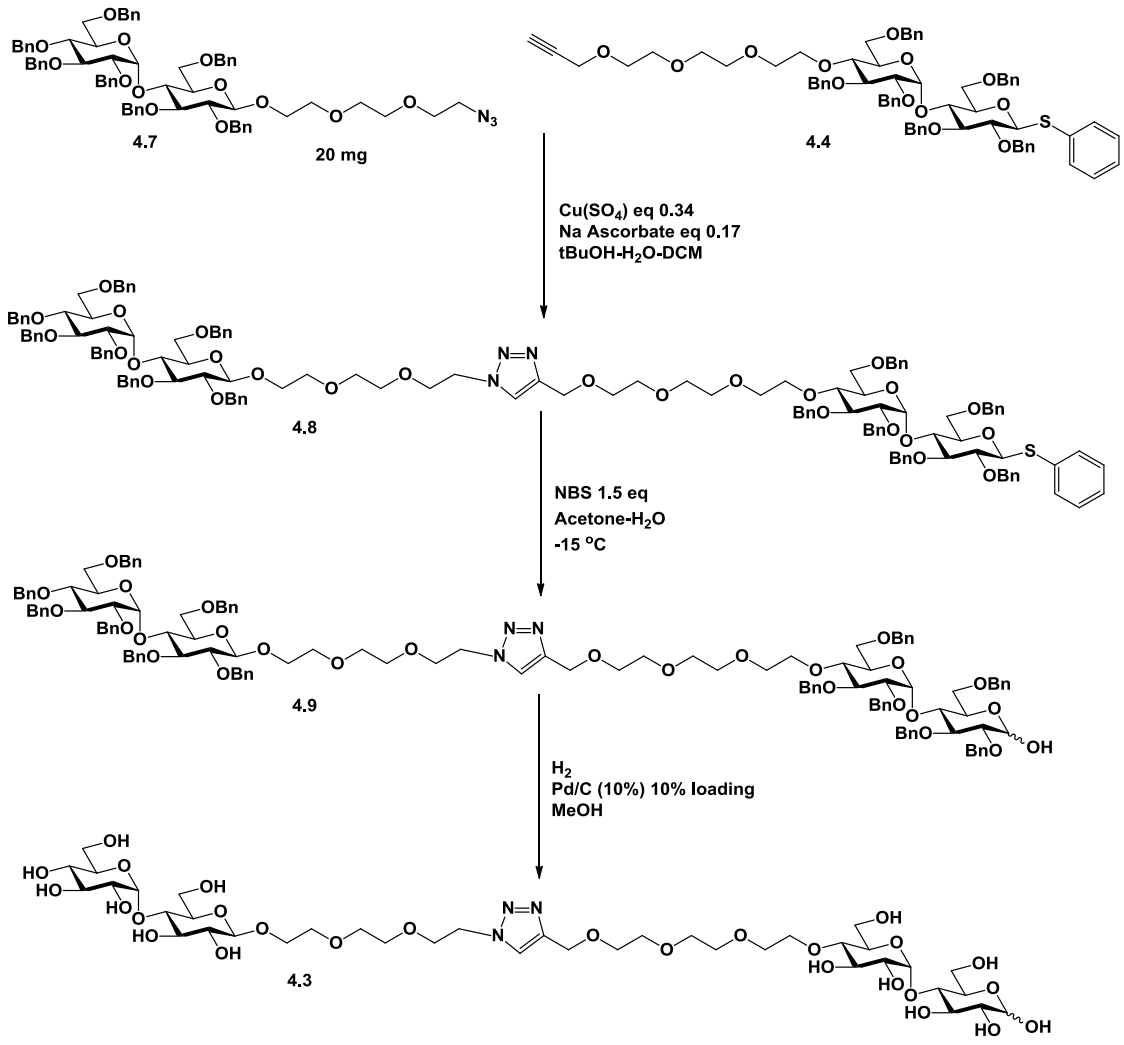
Another sugar **4.5** was functionalised with an azide to complement the alkyne at the 4'-position of the maltose fragment. Sodium azide was used to displace the primary

chloride in maltoside **4.5**. Disaccharide **4.6** was then deacetylated and protected with benzyl groups to complement those on the maltose fragment to which it would be connected (Scheme 4-2).



Scheme 4-2 Functionalisation of a PEG-linked maltoside with sodium azide

Now the two sides of the pseudotetrasaccharide **4.3** had been synthesised, they could be attached via a copper catalysed azide-alkyne cycloaddition (CuAAC) reaction. This reaction proceeded well, however the product could not be separated by column chromatography from residual starting materials. The products were therefore taken through the next two steps without purification (Scheme 4-3).



Scheme 4-3 Formation of the psuedotetrasaccharide.

After removal of the thiophenyl group, the benzyl protecting groups were removed by hydrogenation. Once deprotected, the product **4.3** could be purified by size exclusion chromatography giving **4.3** in a 27% yield over three steps.

4.3 Trivalent ligand synthesis

The synthesis of another, very different molecule to probe maltoporin's binding was also pursued. This followed an alternative method of adhering small molecules to the surface of bacteria via maltoporin. Utilising multivalency, a compound which could bind

all three of maltoporin's binding sites would be expected to have a very high affinity (Figure 4-2).

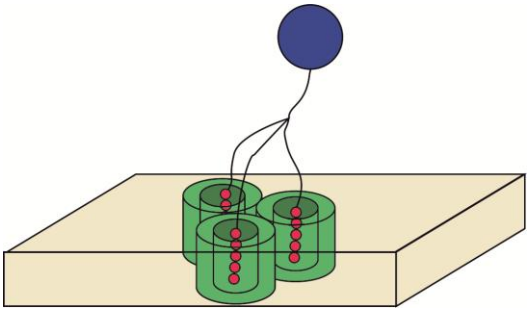


Figure 4-2 A cartoon of a multivalent molecule binding to maltoporin (green) via three maltooligosaccharides (red).

Previously in the lab, Dr Martin Fascione had developed a synthesis of multivalent ligands for maltoporin using SPPS (Figure 4-3). This synthesis utilised Dde-lysine **1.17** residues as they allow for introduction of the Boc-aminooxyacetic acid **4.17** groups at the final stage of the synthesis ensuring they do not interfere with any peptide coupling reactions. It also included a triglycine motif at the N-terminal of the peptide which would allow subsequent sortase ligation.[127] Once cleaved from the resin the Boc groups can be removed and the aminooxy groups reacted with maltoheptaose **4.2** to form mono-, di- and trivalent ligands **4.10** which should be able to bind one, two or all of maltoporin's monomers respectively. As Dr Fascione had successfully completed the synthesis of the mono- and di-valent compounds although purification of the trivalent analogue was found to be problematic. The aim of this project was to synthesise and purify the trivalent ligand.

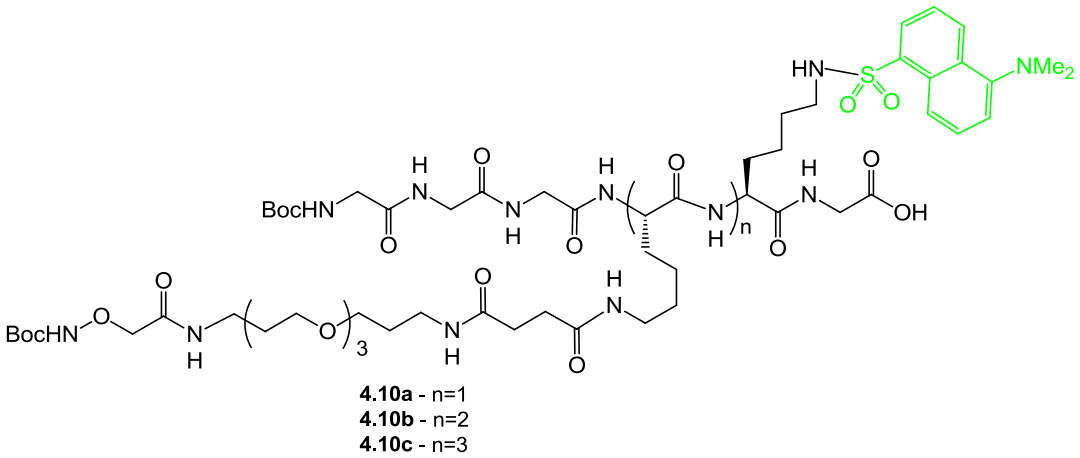
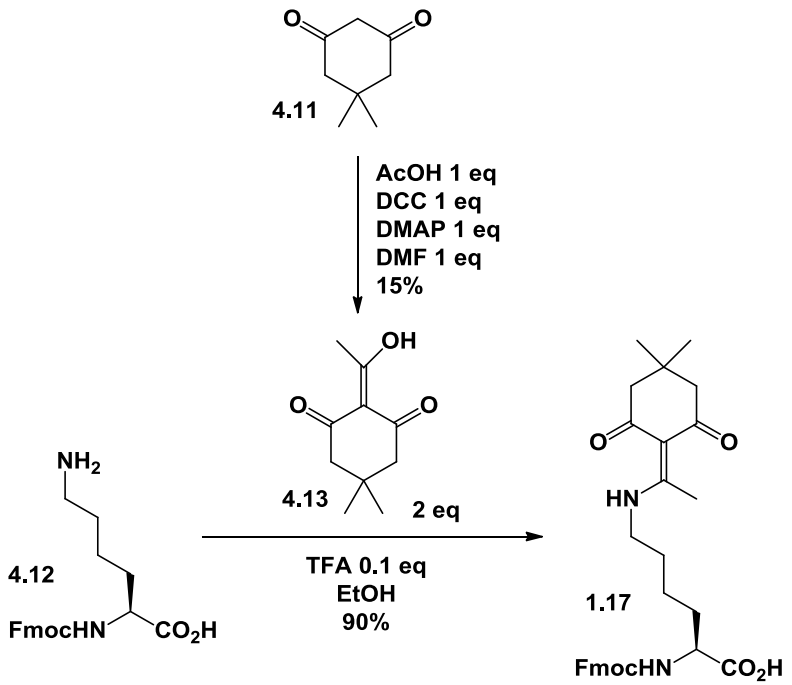


Figure 4-3 The mono-, di- and trivalent ligand before its connection to maltoheptaose via oxime ligation

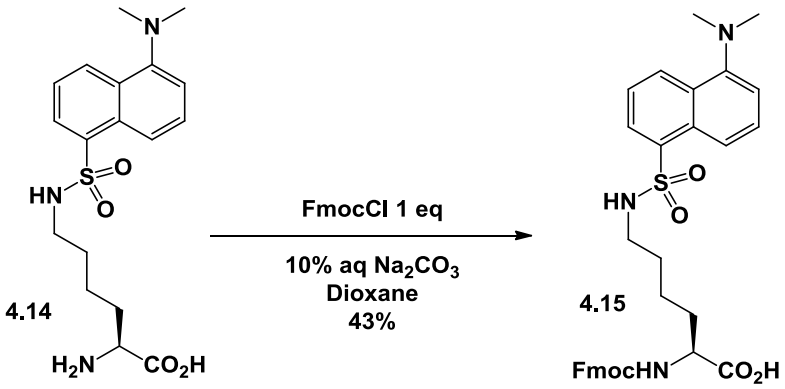
4.3.1 Building block synthesis for SPPS

Modified lysine residues were needed for the SPPS of the trivalent ligand, as this orthogonal protecting group could be removed in the presence of Boc-groups. A fluorescently-tagged lysine was also desired to allow easy detection of the products both during the purification of the compound and in fluorescence-based binding studies.



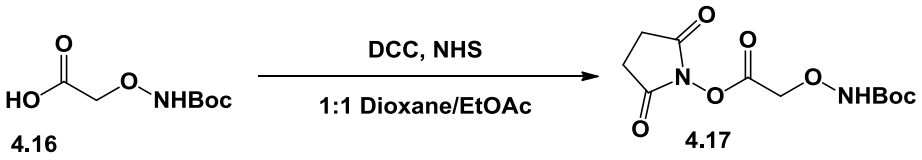
Scheme 4-4 Synthesis of Fmoc-lys(Dde)-OH **1.17** from lysine and dimedone.

Dde-OH **4.13** was used as a protecting group as it could be orthogonally removed by hydrazine leaving the terminal Boc-glycine protected. Dde-OH **4.13** was first synthesised from dimedone **4.11** before reaction with Fmoc-lys-OH **4.12** to produce Fmoc-lys(dde)-OH **1.17** (Scheme 4-4).



Scheme 4-5 Synthesis of Fmoc-lys(dansyl)-OH **4.15** from unprotected dansyl lysine **4.14**.

Previous attempts in the lab at synthesising Fmoc-lys(dansyl)-OH from dansyl chloride and Fmoc-lys-OH **4.14** had led to the formation of a side product that had proved difficult to remove; therefore a new route employing dansyl lysine and Fmoc chloride was followed. This produced Fmoc-lys(dansyl)-OH **4.15** in a 43% yield (Scheme 4-5).



Scheme 4-6 Protection of (Boc-aminooxy)acetic acid **4.16** with succinimide.

An aminooxy compound with an NHS ester to allowing for its use in SPPS was synthesised. (Boc-aminooxy)acetic acid **4.16** was activated with N-hydroxy succinimide to give **4.17** and kept crude as its purification had previously been shown to be unnecessary for SPPS (Scheme 4-6).

4.3.2 Assembly of the trivalent ligand

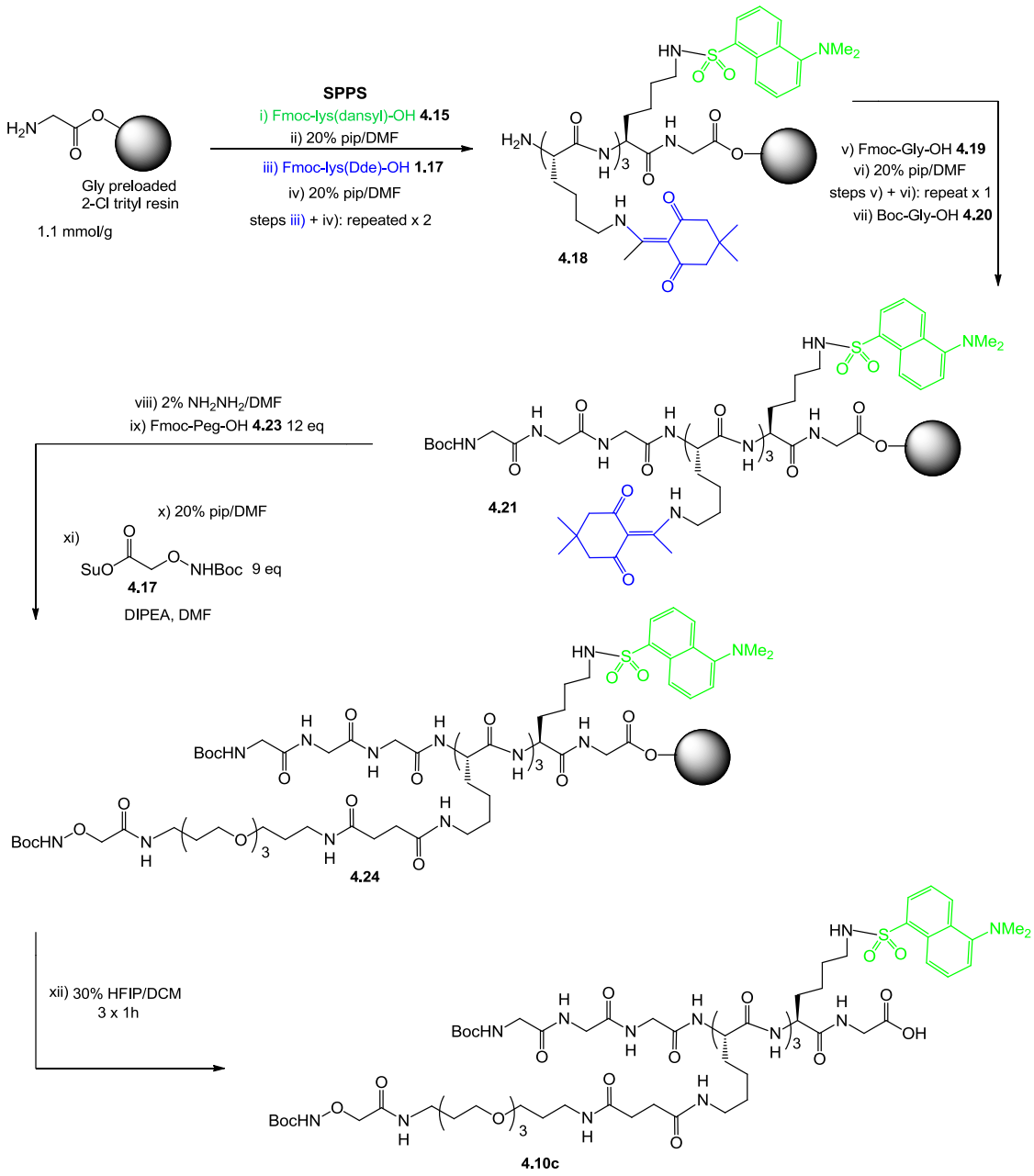
2-chlorotrityl resin was used for the assembly of the trivalent ligand **4.10c** as the product could be released without removing the Dde- and Boc-protecting groups. The

resin was already preloaded with a glycine residue. Dansyl lysine **4.15** was added followed by three Dde-lysines **1.17** and a further two glycines **4.19** (Scheme 4-7). The main chain was capped with a Boc-protected glycine **4.20** allowing for the selective removal of the Dde-groups while leaving the terminal amino acid protected.

Once the lysine residues were deprotected, the side chains could be attached; these consisted of PEG-groups **4.23** with an Fmoc-protected amine and carboxylic acid. Boc-aminooxyacetic acid NHS esters **4.17** were used to cap the PEG-groups to allow the attachment of sugars (Scheme 4-7).

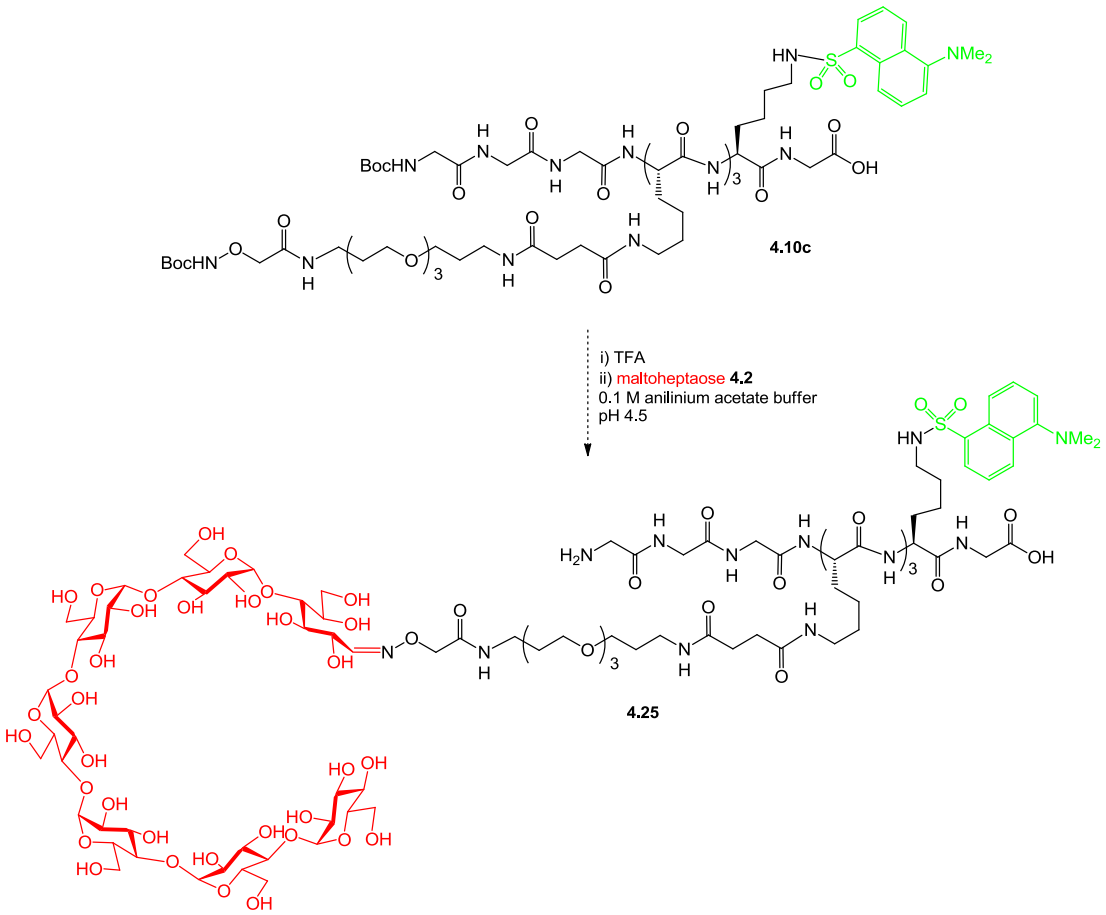
Once cleaved from the resin the ligand was isolated by size exclusion chromatography to give the main product **4.10c** with some impurities. Mass spectrometry showed these impurities to contain several products in which the aminooxy group, despite being Boc-protected, had reacted with additional Boc-aminooxyacetic acid NHS esters. The bis-Boc-protected reagent could be used instead to prevent this problem in future reactions.

The ligand was purified by HPLC to remove these side products. At this point analytical HPLC showed only one product **4.10c** which was confirmed to have the correct mass by HRMS.



Scheme 4-7 SPPS of trivalent maltoporin ligand **4.10c**.

The three ‘arms’ of the ligand **4.10c** were derivatised with maltoheptaose **4.2** through an oxime ligation. This involved removal of the Boc-groups with TFA before addition of maltoheptaose in an anilinium acetate buffer at pH 4.5 (Scheme 4-8). After 48 h the reaction mixture was passed down a size exclusion column to separate unreacted oligosaccharide.



Scheme 4-8 Oxime ligation of the trivalent ligand with maltoheptaose

NMR spectrometry showed a product containing signals for both the dansyl group and a sugar although analytical HPLC showed multiple products with similar retention times. It is likely that the oxime ligation did not go to completion and the multiple peaks were due to a mixture of mono-, di- and tri-valent ligands.

The multiple peaks were separated by HPLC but it was found not to be possible to analyse these products by mass spectrometry or NMR due to the small amount of material.

4.4 Fluorescent ligand

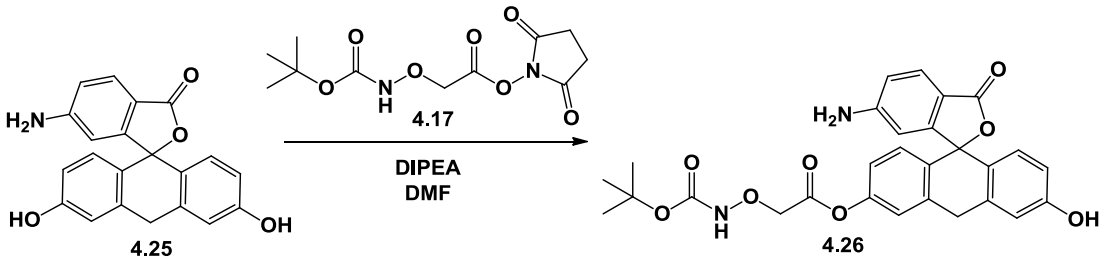
It had been demonstrated that ITC could not deliver the binding information needed for maltoporin, therefore other methods such as fluoresence anisotropy and FRET were investigated. For these experiments a fluorescent ligand that could be out competed during binding is needed this is because the molecules already synthesised for binding experiments do not have a fluorescent label which would be essential for these methods.

Maltoheptaose was selected as the molecule to be used as longer maltooligosaccharides are known to have higher affinities for maltoporin.[68] Fluorescent experiments are very sensitive and a low affinity ligand would need to be used in such high concentrations to fill the binding sites that it could potentially saturate the detectors. Fluorescein **4.25** was chosen as the fluorescent part of the molecule. This was because it can act as a FRET pair with tetramethylrhodamine **2.1**, the fluorescent group already attached to maltoporin (Chapter II).

A short linker was desired to attach the two molecules as this would hold the fluorescent group close to maltoporin on binding. This closeness would allow for more effective FRET and more pronounced anistropic effects due to the lack of movement around the linker.

4.5 Synthesis of the fluorescent ligand

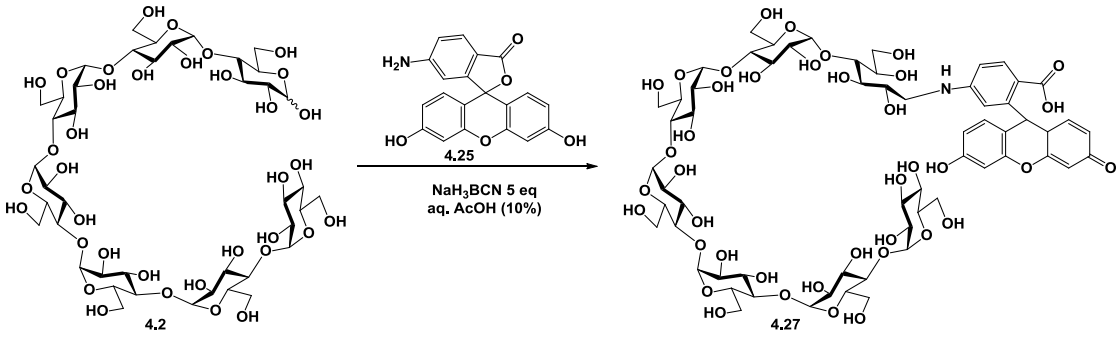
An aminooxy ligation was first selected as a method to attach fluorescein to maltoheptaose as this chemistry was already in use in other areas of the project and thus the reagents were already available. It also used a short linker in a minimum number of steps.



Scheme 4-9 Reaction of 6-aminofluorescein with Boc-aminooxyacetic acid NHS ester.

Reaction of 6-aminofluorescein **4.25** with Boc-aminooxyacetic acid NHS ester **4.17** (Scheme 4-9) produced a product **4.26**, which although having the correct mass, was found to hydrolyse in the presence of TFA. As an amide would be resistant to hydrolysis under these conditions it was postulated that an ester had instead been formed. This was supported by the amine in 6-aminofluorescein **4.10** being deactivated by the lactone, while the hydroxyl groups were both on activated rings.

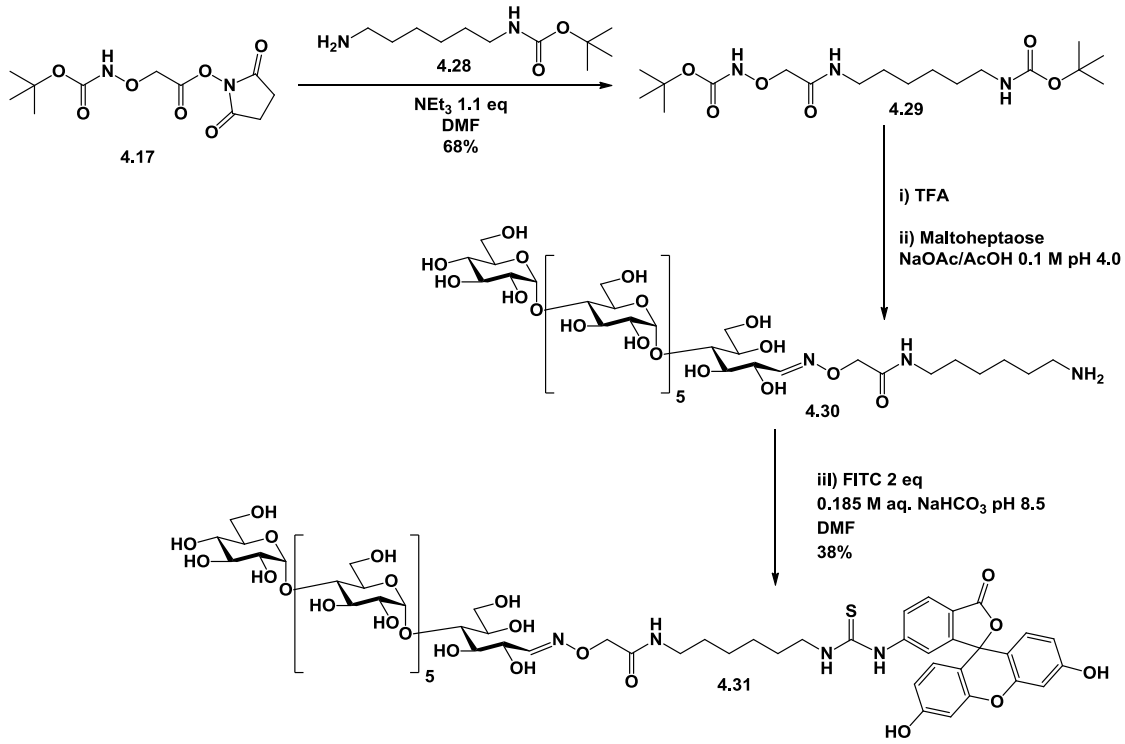
Reductive amination was attempted using maltoheptaose **4.2** and 6-aminofluorescein **4.25** (Scheme 4-10). Although the attachment of the two molecules was successful, the reaction also resulted in a loss of fluorescence. As the mass of the product **4.27** had also increased by two it was theorised that the fluorescein could also have been reduced during the reaction.



Scheme 4-10 Unsuccessful reductive amination of maltoheptaose and 6-aminofluorescein

6-Aminofluorescein **4.25** had proved to be a difficult reagent to work with so a new strategy for an oxime ligation was followed with the use of FITC. *N*-Boc-1,6-hexadiamine **4.28** was derivatised with Boc-aminooxyacetic acid **4.17** and

subsequently deprotected with TFA before an oxime ligation was performed with maltoheptaose **4.2** (Scheme 4-11). This left a free amine to which FITC could be attached. This method yielded the fluorescent ligand **4.31** in three steps.



Scheme 4-11 Formation of the fluorescent ligand via an oxime ligation

4.5.1 Fluorescent binding studies

The ability of the fluorescent ligand **4.31** to bind maltoporin was tested by fluorescence anisotropy. For this experiment a serial dilution of maltoporin from 110 - 0.2 μM was set up with a constant concentration (200 nM) of the fluorescent ligand.

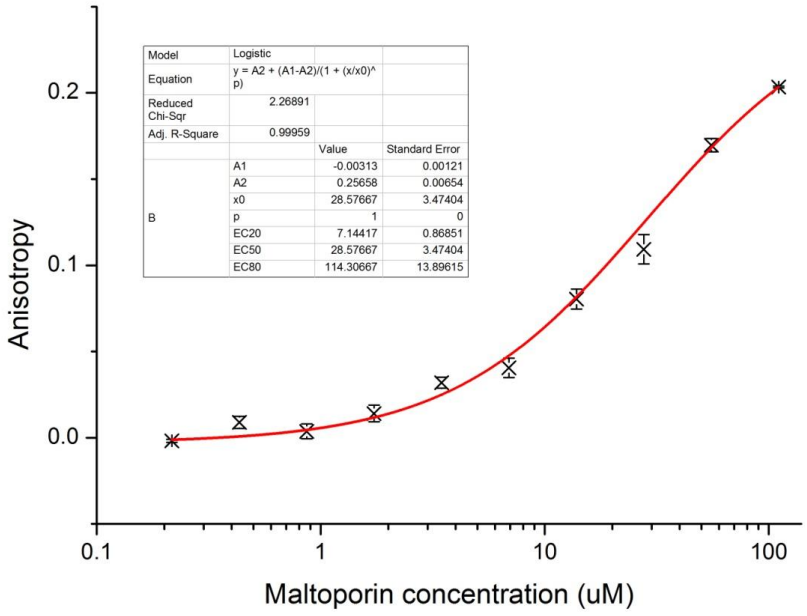


Figure 4-4 Fluorescence anisotropy experiment showing the fluorescent ligand **4.31** (200 nM) against a serial dilution of maltoporin.

The fluorescence anisotropy experiment showed increasing anisotropy as the concentration of maltoporin increased. This is to be expected as when bound the fluorescent ligand will tumble slower in solution and so the anisotropic effect will be greater. This experiment therefore demonstrated binding of the fluorescent ligand.

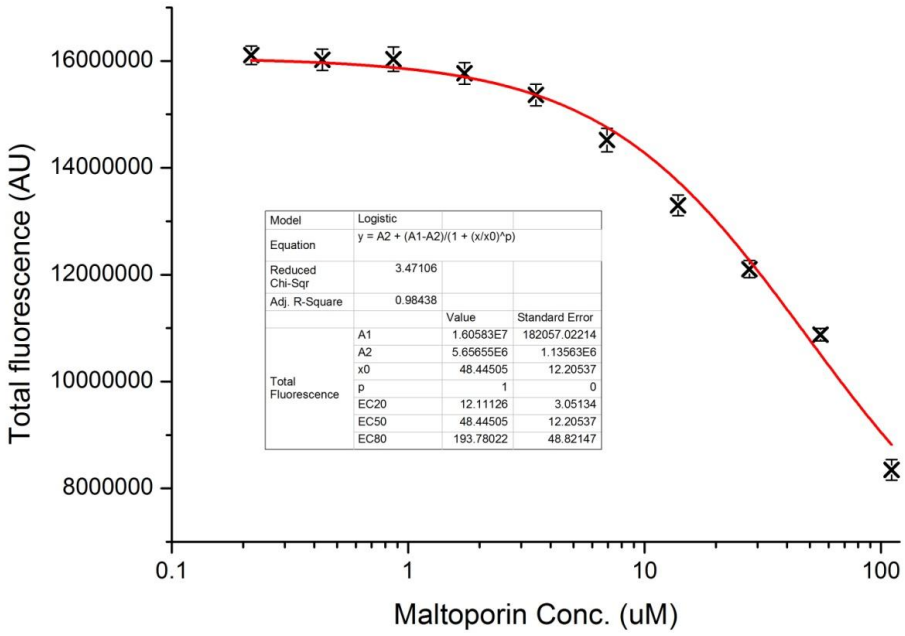


Figure 4-5 Total fluorescence against maltoporin concentration showing quenching of the fluorescent ligand

The fluorescence of the ligand **4.31** was also shown to be quenched on binding to maltoporin as a fall in total fluorescence was also measured (Figure 4-5). Although this was not a problem in itself the quenching of fluorescence would need to be taken into account for all binding measurements. To test the utility of the fluorescent ligand a competition assay was set up with maltoheptaose **4.2**. For this a concentration of maltoporin (15 μM) from the midpoint of the anisotropy curve was chosen. This concentration still had a large degree of anisotropy and would make best use of protein stocks. A serial dilution of maltoheptaose **4.2** was set up from 10 mM to 20 μM with each well also containing the fluorescent ligand **4.31** (200 nM) and maltoporin (15 μM).

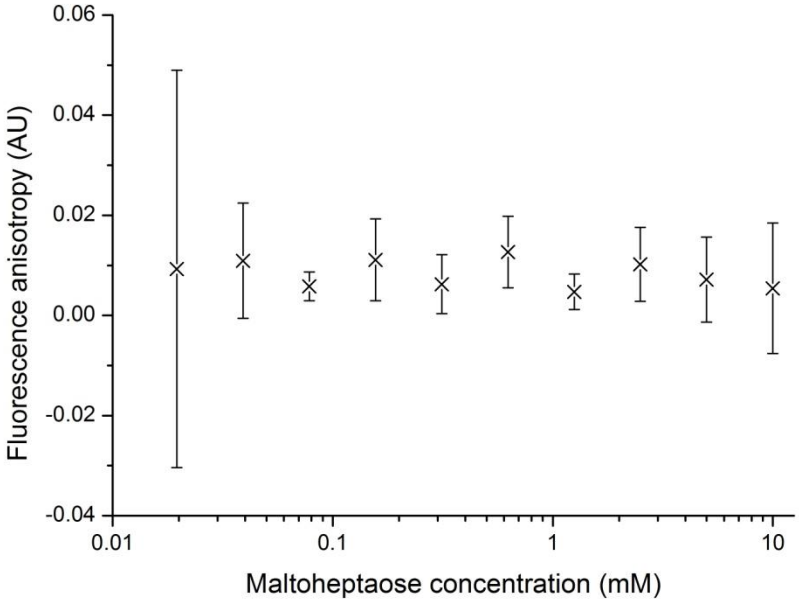


Figure 4-6 Fluorescence anisotropy experiment with a maltoheptaose concentration gradient with fluorescent ligand **4.31** (200 nM) and maltoporin (15 μM)

The competition assay showed no variation in anisotropy with maltoheptaose concentration which suggested the fluorescent ligand was not binding in the pore of maltoporin (Figure 4-6). To confirm this hypothesis a FRET experiment was carried out. If the fluorescent ligand was binding in the pore of maltoporin it should show some FRET activity with the tetramethylrhodamine attached to G382C maltoporin.

For the FRET assay both the fluorescent ligand **4.31** and TAMRA maltoporin were excited at 450 nm and their emissions measured. 450 nm was selected as fluorescein has the greatest absorption at this wavelength compared to TAMRA. A second measurement was taken of the fluorescent ligand and TAMRA maltoporin after they had been combined and allowed to sit for 30 min. A third measurement was also taken after the addition of maltoheptaose to the solution.

This method was carried out three times at different concentration of the fluorescent ligand while the protein concentration was set at 1.2 μM. If FRET occurs a reduction in fluorescein fluorescence should be seen coupled with an increase in TAMRA fluorescence as the fluorescein is quenched by TAMRA. The maximum emission for

the fluorescent ligand was seen to be 515 nm while it was 568 nm for TAMRA (Figure 4-7).

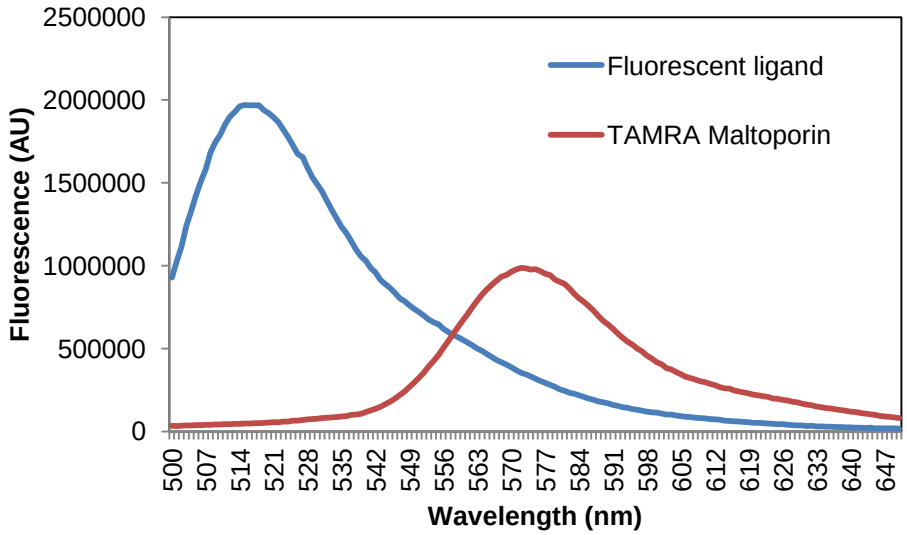


Figure 4-7 The emission spectra of the fluorescent ligand **4.31** (100 nm) and TAMRA maltoporin (1.2 μM).

At all three concentrations of the ligand **4.31** (100 nM, 2 μM and 20 μM) no change in fluorescence was seen (Figure 4-8). This supports the hypothesis that the fluorescent ligand **4.31** is not binding in the pore of maltoporin. As if the ligand was bound the distance between the two fluorophores should be within the 1 – 10 nm that FRET requires.[128] It was thought that the ligand **4.31** could potentially be binding to the micelles in solution instead of to maltoporin. This is because the ligand **4.31** has a hydrophobic head group consisting of fluorescein and an alkyl chain (Scheme 4-11). This binding would produce an anisotropic effect and could explain why no FRET occurred yet fluorescence anisotropy showed binding.

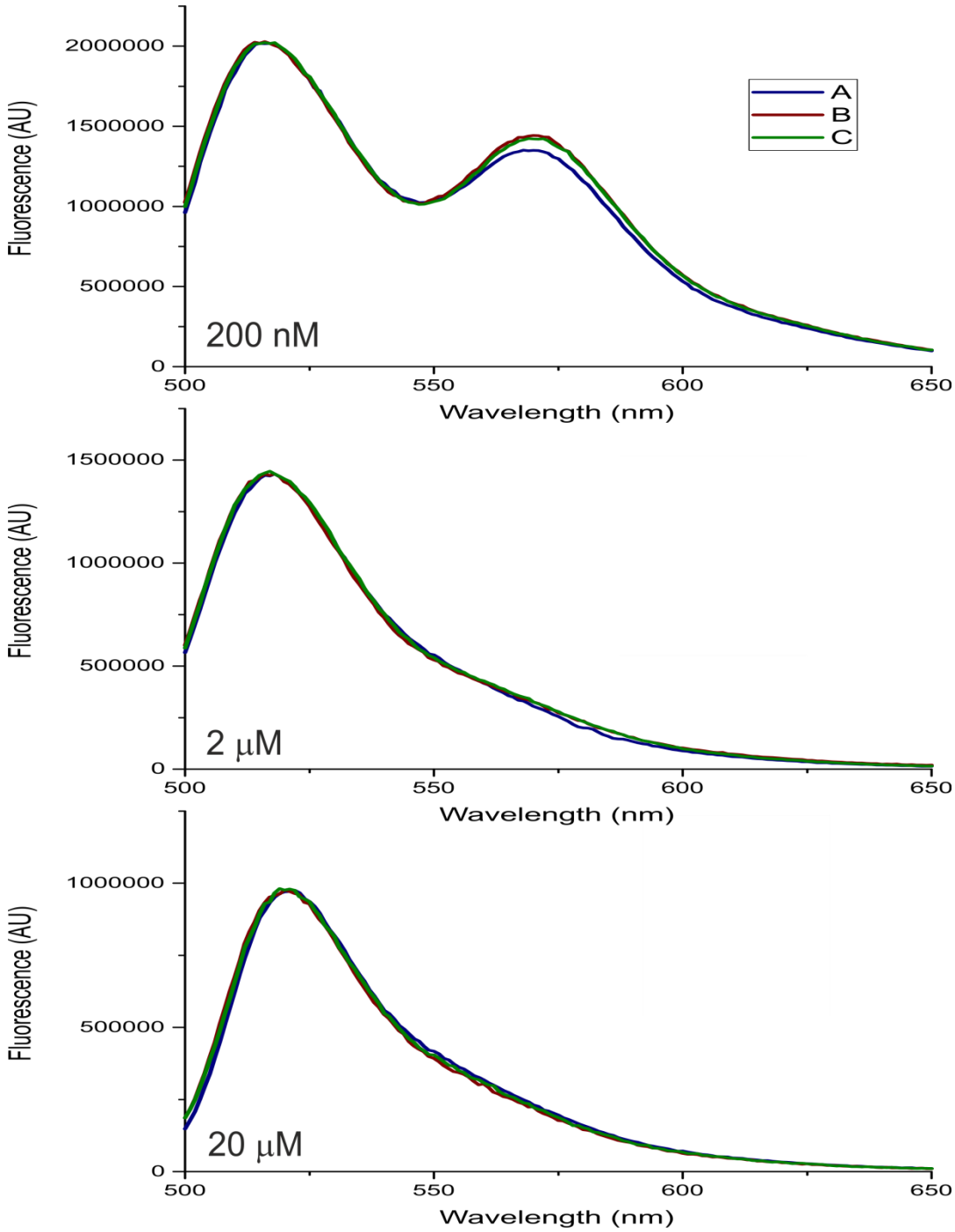


Figure 4-8 FRET assay. A – Fluorescent ligand **4.31** and TAMRA maltoporin (1.2 μM) assay; B - Fluorescent ligand and TAMRA maltoporin theoretical combined fluorescence; C - Fluorescent ligand X and TAMRA maltoporin (1.2 μM) and maltoheptaose (10 mM) assay

To test whether the fluorescent ligand was binding to micelles another fluorescence anisotropy experiment was run with samples in both water and detergent. 6-Aminofluorescein was also tested with fluorescent ligand **4.31**. The results of this test show a clear increase in anisotropy when the fluorescent ligand is in a 0.5% octyl-POE

solution (Figure 4-9). 6-Aminofluorescein is also shown to bind the micelles but to a lesser degree than the fluorescent ligand **4.31**. This could be due to the ligands structure being similar in nature to a detergent molecule with a hydrophobic head consisting of fluorescein and an alkyl chain with a hydrophilic tail of maltoheptaose (Scheme 4-11).

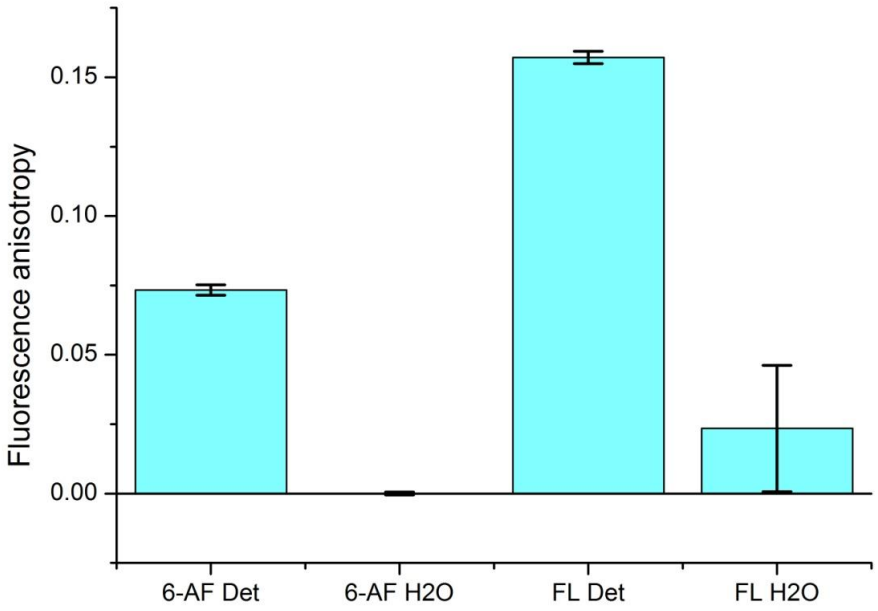


Figure 4-9 Relative fluorescence anisotropy between 6-aminofluorescein and Fluorescent ligand **4.31** in both water and detergent

The ligand **4.31** binding to the micelles would also explain the initial fluorescence anisotropy results (Figure 4-4). In concentrating the protein any micelles in solution would also be concentrated so their concentration would also be decreased when the protein was subjected to a serial dilution. As it had been found that the fluorescent ligand binds to the micelles of Octyl-POE a new method to solubilise maltoporin would be needed. The options include amphipol A8-35 which has been previously used in chapter II or alternatively lipids could be used in place of the detergent.

4.6 Conclusion

A pseudotetrasaccharide **4.3** was synthesised successfully connecting two maltose residues via a CuAAC. This novel molecule will allow for an investigation into the bind-and-slide mechanism to take place.

The synthesis of the trivalent ligand **4.10c** was successful. Its purification was also achieved by a combination of size exclusion chromatography and HPLC. The attachment of maltoheptaose residues via an oxime ligation still requires optimisation but can now begin from a pure starting material.

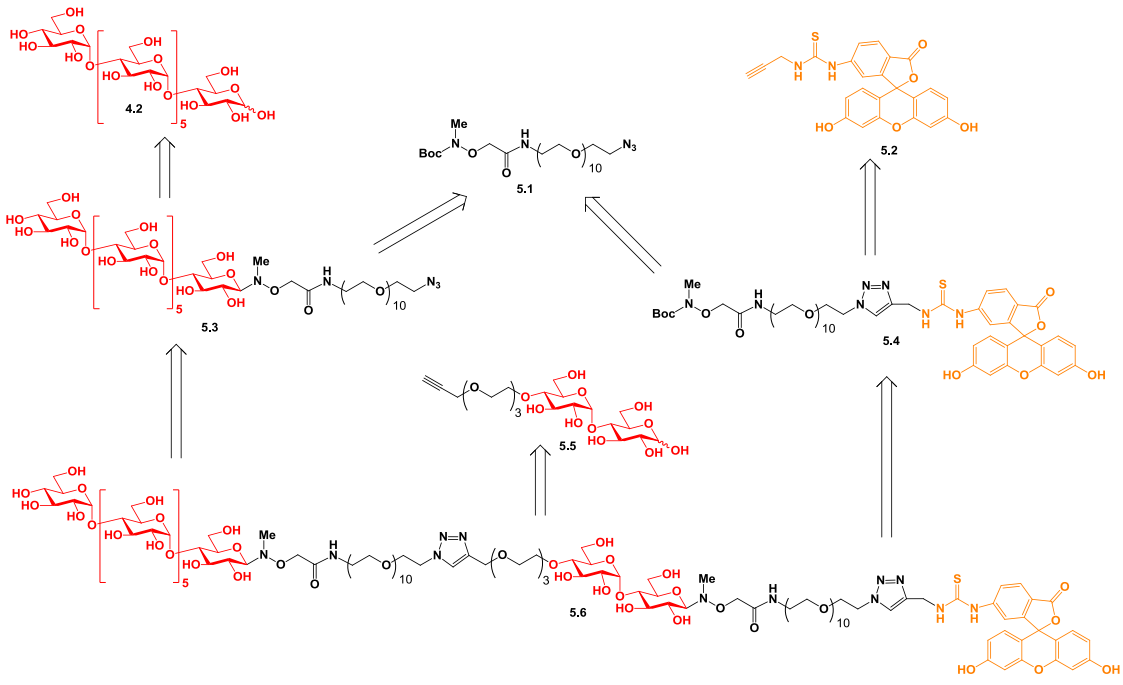
A fluorescent ligand **4.31** was successfully produced using oxime ligations. The binding of this molecule to maltoporin was investigated with fluorescence anisotropy which showed a good correlation between protein concentration and anisotropy. Further investigations with FRET and fluorescence anisotropy showed this binding to in fact be to micelles in the solution.

Chapter V: The synthesis of the switch axle

5.1 Introduction

In the initial plan the axle of the switch was to be constructed using SPPS. However during the preliminary studies and synthesis of the multivalent ligand some challenges to this method became apparent. For the multivalent ligand all of the reagents were synthesised in a single step from commercially available materials. This meant they could be produced in quantities of over a gram. While during the preliminary studies in chapter III it had become evident that the building block synthesis could not be scaled up to make SPPS practical. CuAAC was instead chosen as it had proved a reliable method to connect the pseudotetrasaccharide **4.18** together.

A retrosynthesis of the switch axle was performed with three main fragments connected via PEG-linkers **5.1** (Scheme 5-1). The PEG-linkers **5.1** were derivatised at one end with a Boc-protected oxyamine and an azide at the other. This allowed them to be attached via an oxime ligation to an unprotected sugar and via CuAAC to an alkyne. This method allowed the same linker to be used throughout the axle **5.6**.

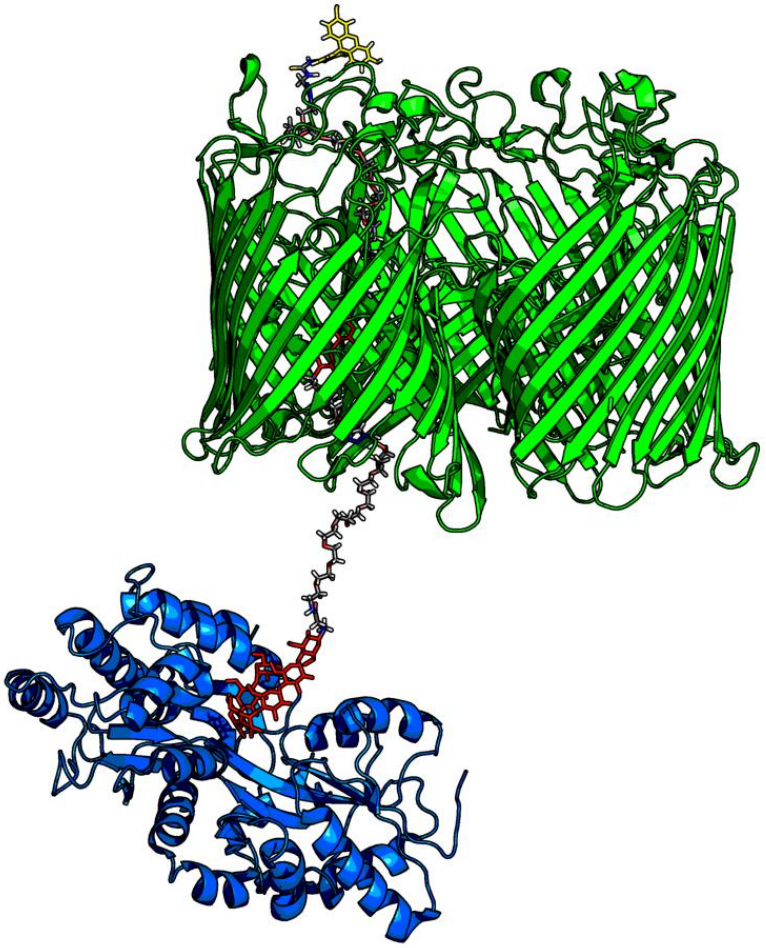


Scheme 5-1 Retrosynthesis of the switch axle

The fragment at the non-reducing end would be maltoheptaose **4.2** while a propargyl containing fluorescein group **5.2** would cap the axle at the reducing end. The central

fragment **5.5** consisted of unprotected maltose derivatised at the 4'-position with an alkyne. The construction of the axle **5.6** from the non-reducing end would allow for alternating methods of attachment.

The PEG-linkers **5.1** were chosen to have a chain length of 10 as this had been shown to be sufficient to allowing binding to both maltoporin and MBP (Scheme 5-2). Fluorescein was selected as it would be too large to fit through the pore of maltoporin and could be used in FRET experiments upon the switch's completion.

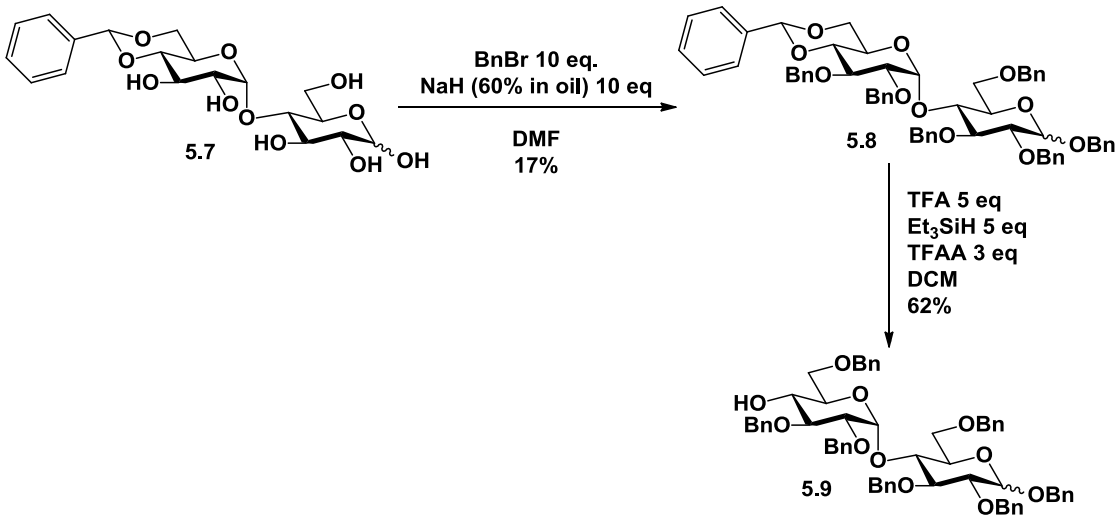


Scheme 5-2 A model of binding between the switch and maltoporin (green) and MBP (blue). The maltoheptaose fragment (red) can be seen binding to MBP while the smaller maltose fragment (red) can also be seen in the binding site of maltoporin. Fluorescein (yellow)

5.2 Synthesis of the maltose fragment

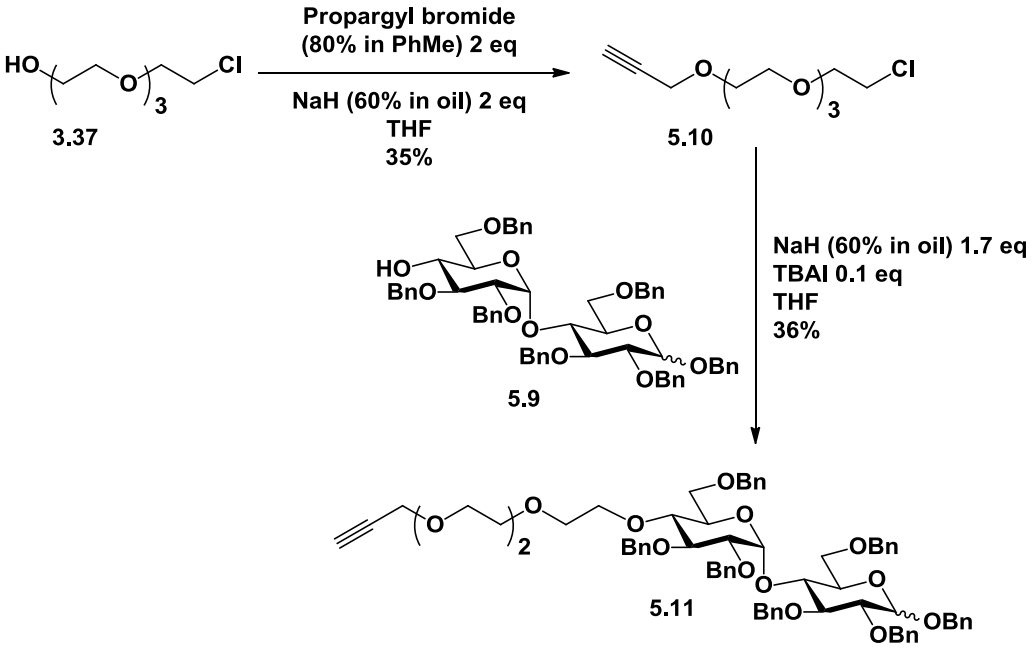
Here the complete synthesis of the maltose fragment is shown. The optimisation of the techniques used in this synthesis is shown in chapter III. It had been shown that the use of benzyl ethers was crucial for the alkylation step at the 4'-position and that an aminooxy ligation with the unprotected sugar was the most efficient method of attachment at the reducing terminus.

In the first step of the synthesis benzylidene acetal-protected maltoside **5.7** was benzylated before the benzylidene acetal was selectively opened using triethylsilane under acidic conditions. This left the 4'-position unprotected and available for derivatisation (Scheme 5-3).



Scheme 5-3 Benzylation of the benzylidene acetal protected maltose 10.

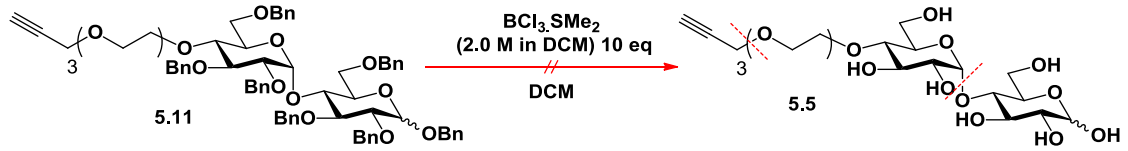
With the maltose fragment **5.9** selectively deprotected at the 4'-position, a propargyl group could be attached. It was decided to attach the propargyl group via a PEG-linker as this allowed the propargyl group to be placed distal from the sugar. This approach was considered to be advantageous as the triazole produced in the CuAAC reaction would not interrupt any binding between the sugar and maltoporin.



Scheme 5-4 The formation of the propargyl containing PEG and its attachment to the 4'-position of maltose.

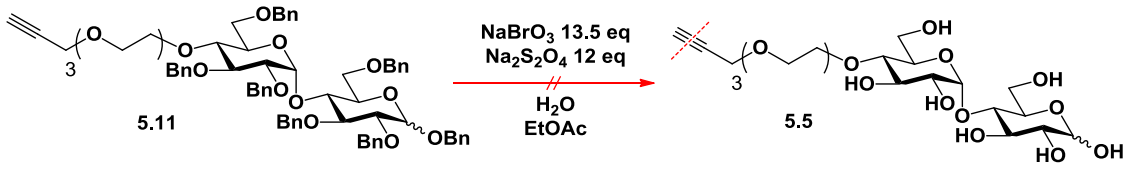
The product **5.11** was synthesised successfully in two successive base-promoted alkylations (Scheme 5-4). Both reactions were found to be low yielding due to the PEG-group **5.10** polymerising in the first reaction and the beta-effect in the subsequent reaction. Despite the low yield, the product **5.11** was formed on a large enough scale to avoid the need for optimisation. Using this method also avoided additional protection and deprotection steps that would be required if the propargyl group were added later.

Ideally the maltose fragment **5.11** could be deprotected at this stage as an oxime ligation requires a free hemiacetal at the reducing end. However, this was not possible under the standard conditions of H_2 with palladium on carbon as the alkyne would also be hydrogenated.



Scheme 5-5 The unsuccessful deprotection of benzylated maltoside **5.11** with boron trichloride-dimethyl sulfide.

Two milder methods were tested for the removal of benzyl ethers in the presence of a propargyl group. It has been shown that boron trichloride-dimethyl sulfide is an effective way to remove benzyl ethers in the presence of other functional groups.[129] A combination of sodium bromate and sodium dithionite has also been shown to remove benzyl groups from oligosaccharides in the presence of azides.[130] It was found that the use of boron trichloride-dimethyl sulfide removed the propargyl group but also resulted in cleavage of the glycosidic linkage (Scheme 5-5).

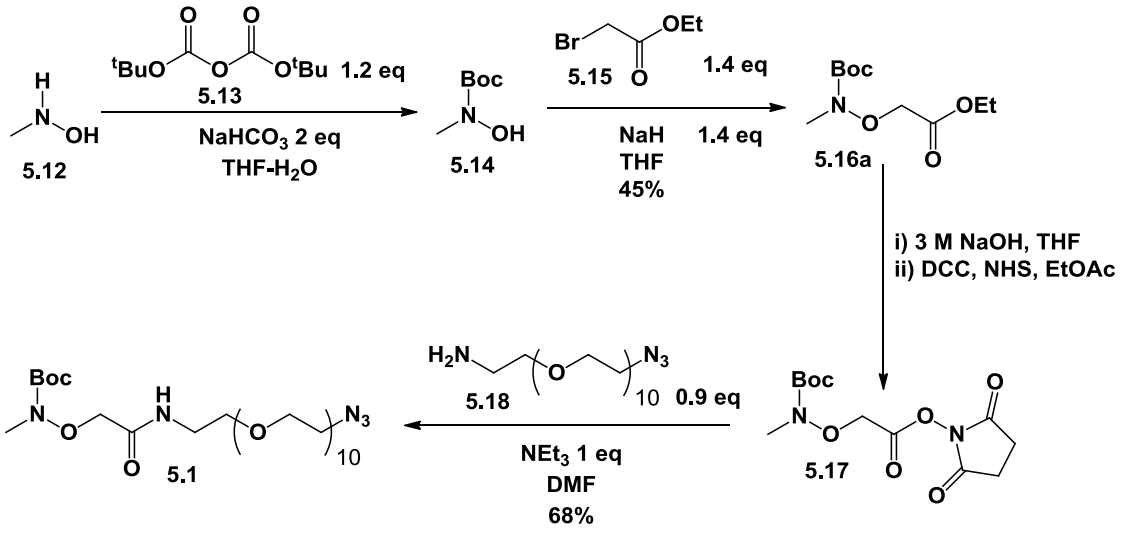


Scheme 5-6 The unsuccessful oxidative deprotection of benzylated maltoside **5.11** with sodium bromate and sodium dithionite.

The use of sodium bromate and sodium dithionite was found to have successfully deprotected the sugar as the product **5.5** was no longer visible under UV. Although an unknown addition to the molecule had occurred as the mass of the molecule was substantially higher than the desired product. It was clear that the benzyl groups could not be removed easily in the presence of the alkyne. There was a potential option to protect the alkyne before removal of the benzyl ethers but the additional steps and loss of yield was thought not to justify its use. As it was not possible to deprotect the sugar **5.11** without destruction of the propargyl group it was decided to remove the benzyl groups once the maltose fragment was integrated into the switch axle.

5.3 The synthesis of the linker

A linker was needed to connect the different fragments of the switch. A PEG-linker was selected due to its water solubility, suitable length and the commercial availability of heterobifunctional PEG-groups. An azide-amine PEG was used as it already contained the desired azide and the amine allowed ease of attachment of a Boc-protected aminooxy group.

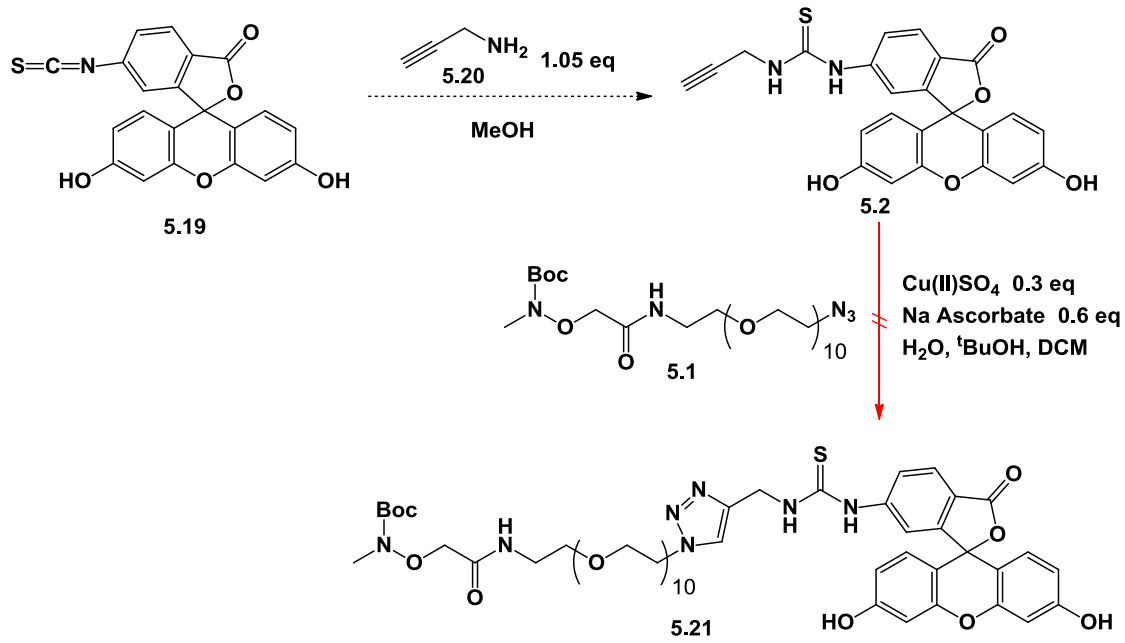


Scheme 5-7 Synthesis of the azide-aminooxy-functionalised PEG-linker.

An *N*-methylaminooxy compound was desired as these had been shown to cyclise on attachment to sugars producing the β-glycoside.[131] To form this product *N*-methylhydroxylamine **5.12** was protected with Boc-anhydride **5.13** before alkylation with ethyl bromoacetate **5.15**. The ester **5.16** was hydrolysed and an NHS ester **5.17** formed which was used to attach the protected aminoxyacetic acid to the amine of the PEG-linker **5.18**. The linker **5.1** had thus been successfully synthesised in 4 steps (Scheme 5-7).

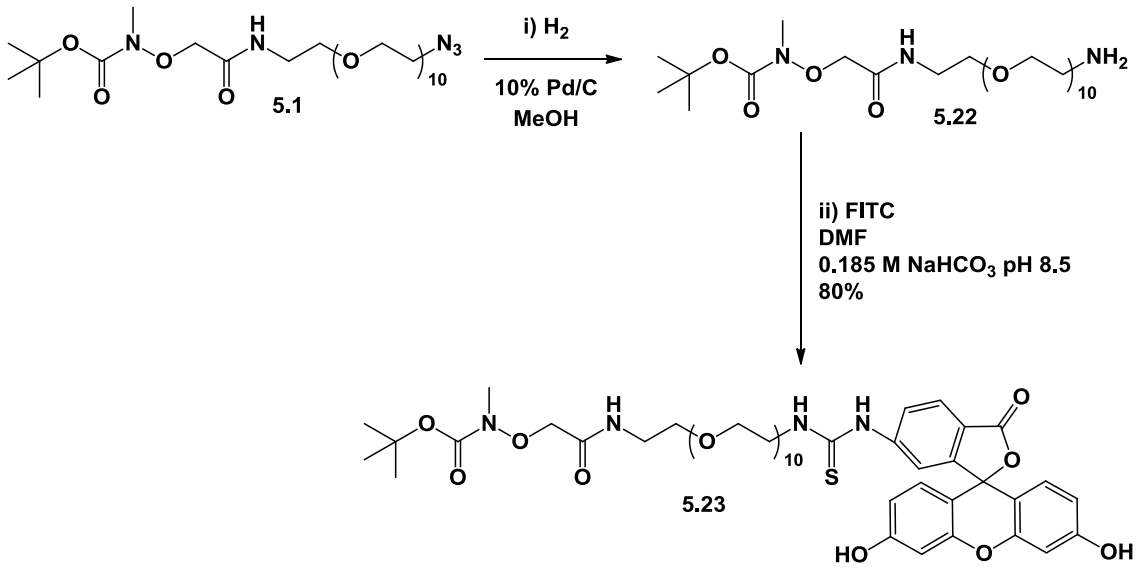
5.4 Synthesis of the fluorescein fragment

A large fluorescent molecule would be needed at one end of the switch to stop the switch threading all the way through the pore of maltoporin and also to be used for fluorescent binding studies. To attach fluorescein to the switch it would need to be functionalised with an alkyne as the end of the PEG-linker contained an azide. Fluorescein isothiocyanate **5.19** (FITC) was reacted with propargylamine in methanol (Scheme 5-8). Mass spectrometry showed the presence of the correct product **5.2** which was then attached to the PEG-linker **5.1** via a CuAAC reaction. The CuAAC reaction was not successful as the alkyne-fluorescein was shown to be insoluble in the mixture of solvents used. The reaction was also attempted in methanol but still no product was seen.



Scheme 5-8 The unsuccessful synthesis of the fluorescent fragment.

It had been shown that the propargyl-derivatised fluorescein **5.2** was insoluble in common solvents used for CuAAC reactions. Therefore the PEG-linker **5.1** would be modified first to allow its attachment directly to FITC **5.19**.

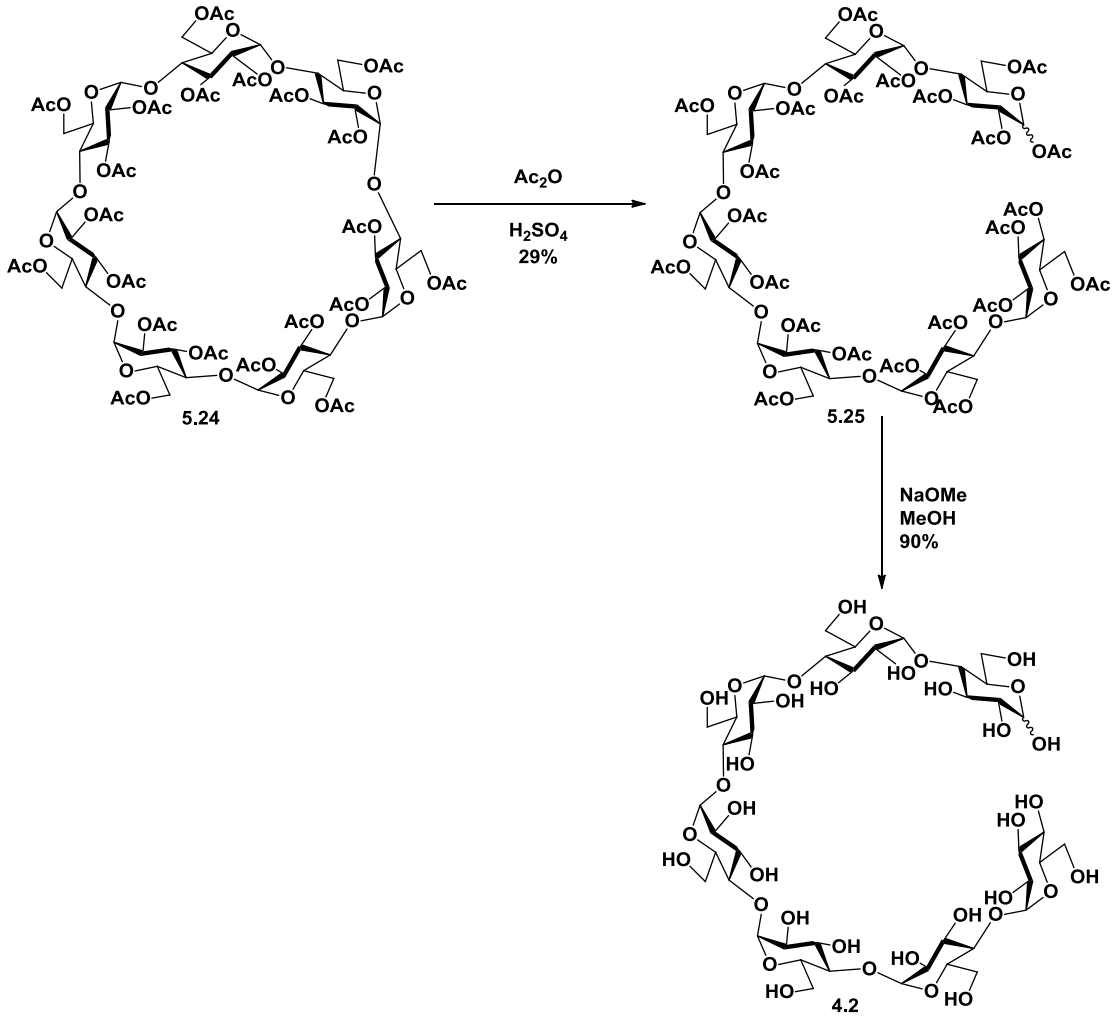


Scheme 5-9 The reduction and functionalisation of the PEG-linker with FITC.

The PEG-linker **5.1** was hydrogenated to produce an amine **5.22** to which FITC **5.19** was attached. The product was found to be soluble in MeOH and could be purified by size exclusion chromatography. This led to the successful synthesis of the fluorescent fragment **5.23** of the switch (Scheme 5-9).

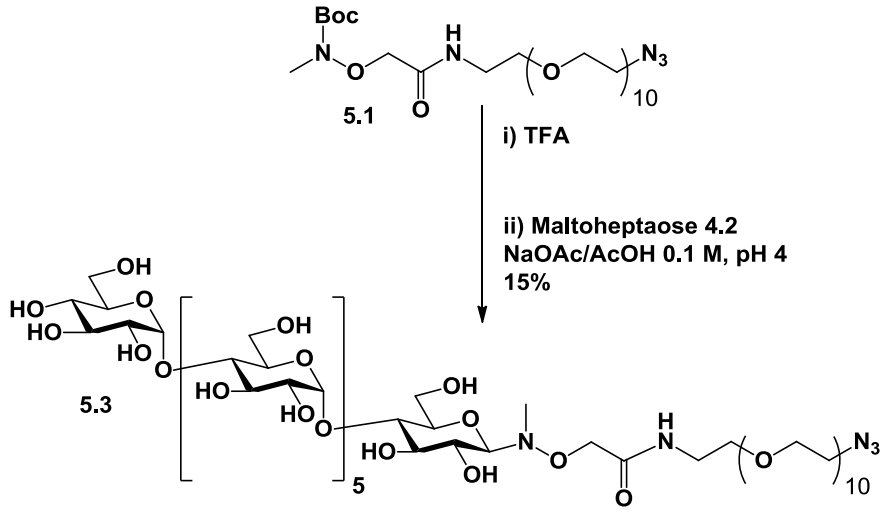
5.5 The maltoheptaose fragment

To allow the switch to bind to maltoporin a high affinity ligand was needed at the non-reducing end. As longer maltooligosaccharides were shown to bind more tightly,[68] the seven-mer maltoheptaose **4.2** was selected. This also had the advantage that it could be readily synthesised from β-cyclodextrin **5.24**.[81]



Scheme 5-10 Synthesis of maltoheptaose from acetylated β-cyclodextrin

Acetylated β-cyclodextrin **5.24** was ring opened in the presence of acetic anhydride and sulfuric acid. Per-acetylated maltoheptaose **5.25** is the most abundant product as ring strain causes the bonds in the β-cyclodextrin to be slightly more reactive than in the linear product (Scheme 5-10). Removal of the acetate groups with sodium methoxide and purification by size exclusion gave maltoheptaose **4.2** as the pure product.



Scheme 5-11 Attachment of maltoheptaose to the linker

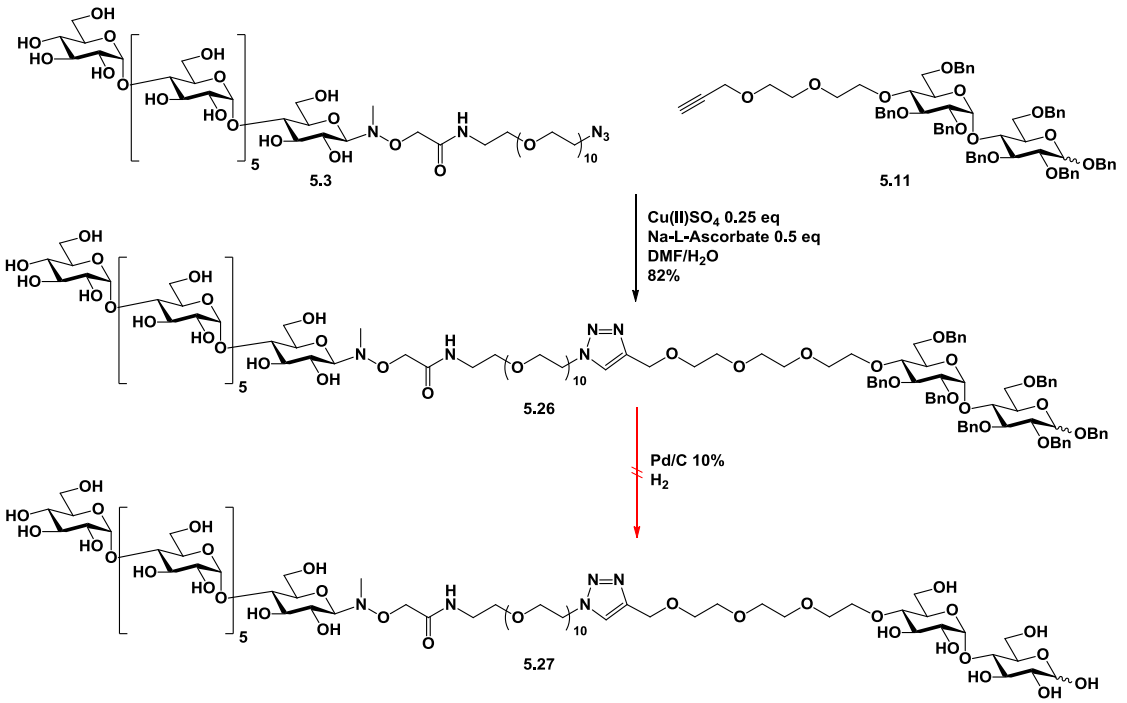
The PEG-linker **5.1** was attached to maltoheptaose **4.2** via an oxime ligation (Scheme 5-11). The Boc-group was removed from the linker by stirring in TFA which was subsequently removed under a stream of nitrogen. Maltoheptaose **4.2** was added in sodium acetate/acetic acid buffer at pH 4.0. This reaction took 8 days after which the reaction mixture was separated by size exclusion chromatography. It could be seen that a large amount of starting material **4.2** was still remaining and a low yield of 15% was achieved. Despite the low yield the final fragment **5.3** of the switch had now been made and the synthesis could be moved on to assemble the final product.

5.6 Assembly of the switch

The individual parts of the switch had been synthesised which now left the final construction of the polymer. This involved a copper catalysed click reaction to attach the maltoheptaose fragment to the maltose section followed by removal of the benzyl groups and a final oxime ligation to attach the fluorescein fragment.

A CuAAC reaction enabled connection of maltoheptaose and maltose through a PEG-linker. The benzyl groups were then removed with palladium on carbon under an atmosphere of hydrogen (Scheme 5-12). Unfortunately this reaction also led to

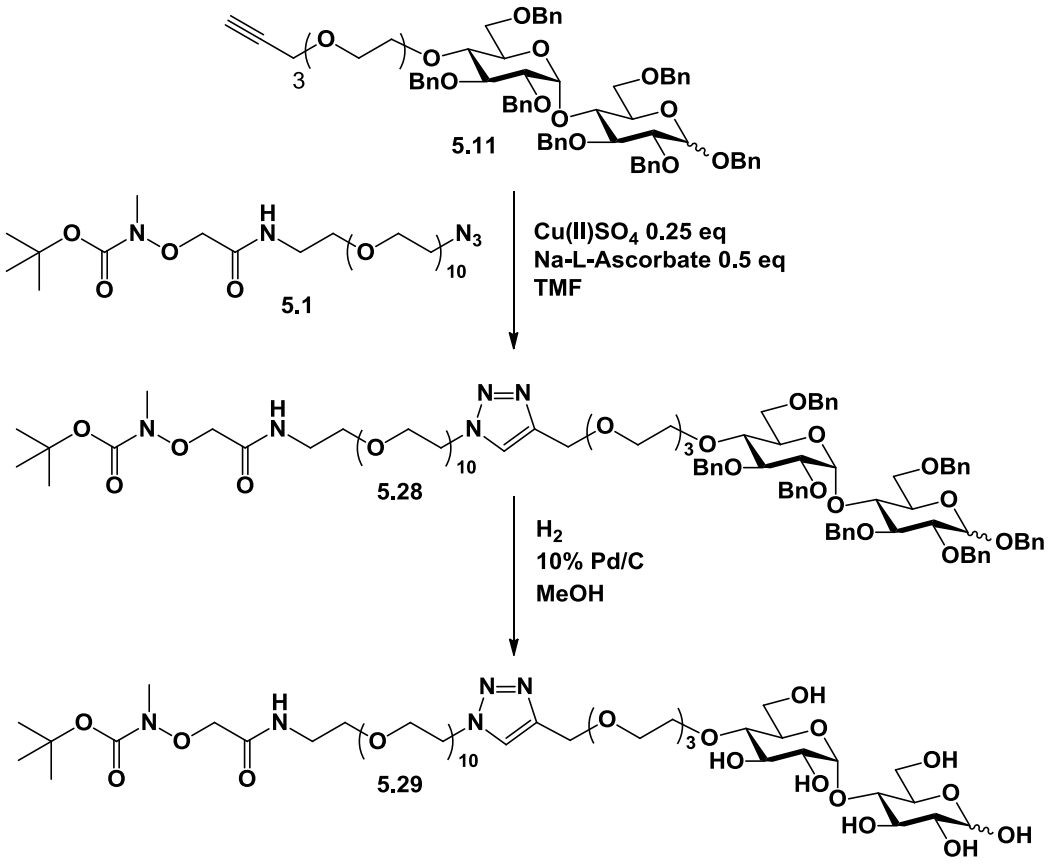
cleavage of the aminooxy bond. As the milder methods for removal of benzyl groups had already been shown to be ineffective with the kind of functional groups present, a different order was proposed to assemble the switch axle.



Scheme 5-12 The attempted synthesis of the unprotected sugar containing section of the switch.

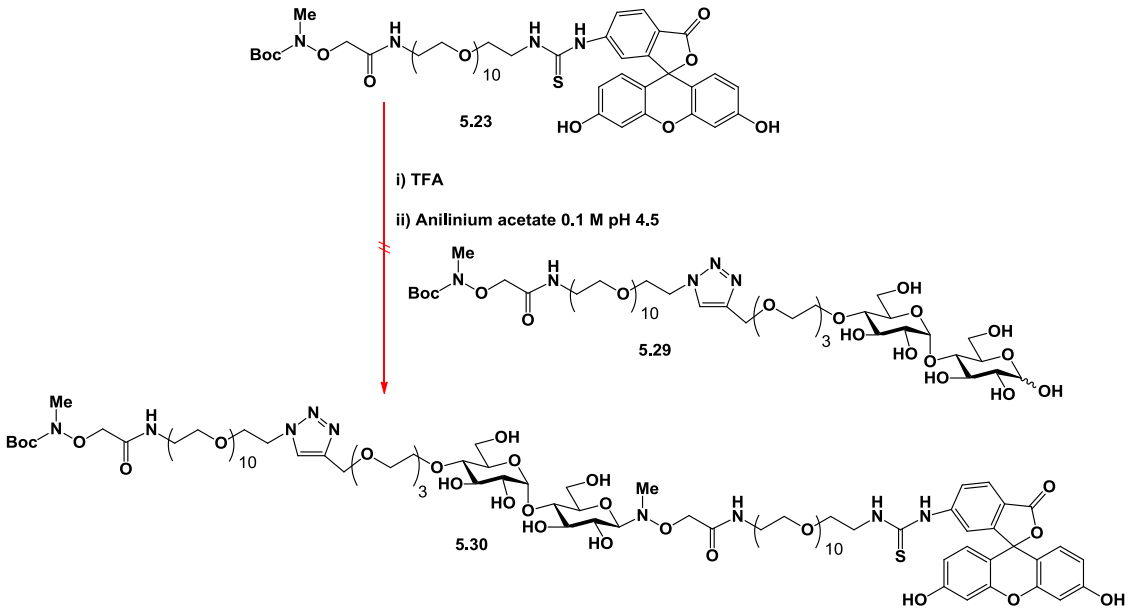
In the new method of construction the PEG-linker **5.1** is attached to the maltose fragment **5.11** first and the benzyl groups removed. In this order the hydroxylamine remains Boc-protected so the benzyl groups can be removed under hydrogen. In this manner the central part of the switch is constructed first and can be connected to the fluorescein **5.23** and maltoheptaose **5.23** fragments through oxime ligations.

This method was successful with the maltose fragment **5.11** connected with the linker **5.1** via a CuAAC reaction. The benzyl groups were then removed by hydrogenation without affecting the aminooxy functional group (Scheme 5-13). Now all that remained were the oxime ligations to attach the terminal sections of the switch.



Scheme 5-13 The attachment of the linker to maltose and its subsequent deprotection.

The fluorescent fragment was first deprotected with TFA which was shown to be successful by mass spectrometry. The product **5.29** was then immediately reacted with the fluorescein fragment **5.23** in anilinium acetate buffer at pH 4.5 (Scheme 5-14). Although after five days no reaction was seen.



Scheme 5-14 An oxime ligation between the fluorescein and central maltose fragments.

The reaction was repeated with *p*-phenylenediamine as this had been shown to be more effective than aniline due to its increased solubility allowing a higher concentration to be used.[132] Again LCMS showed no product formation after 48 hours and both starting materials remained. The reaction relies on sugars been in their acyclic form which they have been shown to only occupy for a tiny fraction of their time.[133,134] This is thought to be the reason behind the slow reaction times in the synthesis of the switch axle as oxime ligations are fast reactions in the presence of aniline.[135]

Although carbohydrates are slower to react with *N*-methyloxyamine compounds than aldehydes, in the presence of aniline, they have been found to react within 24 hours.[136] This has also been observed within this project where maltose has been derivatised in 24 h. The difference between these reactions and the connection of the central and fluorescent fragments is the complexity of the molecules.

As the compounds have grown larger the speed at which they react has decreased this can be prescribed to the large number of orientations the molecules can assume and the subsequent reduction in the probability of a collision occurring in the correct orientation for a reaction to occur.[137] This problem is compounded by the precious nature of the reagents involved which does not allow for a large excess of the carbohydrate to be used.

Based on these assertions a new method was needed for the connection of the fragments.

5.7 Conclusion

The synthesis of the building blocks was completed successfully. The synthesis of the most complex central fragment **5.11** was optimised from chapter IV by derivatising the PEG-chain at the 4'-position before attachment. As well as using a mixture of O-benzyl anomers thus simplifying the molecules protection. A variety of methods were attempted to remove the benzyl groups before its incorporation into the switch axel but this was not possible.

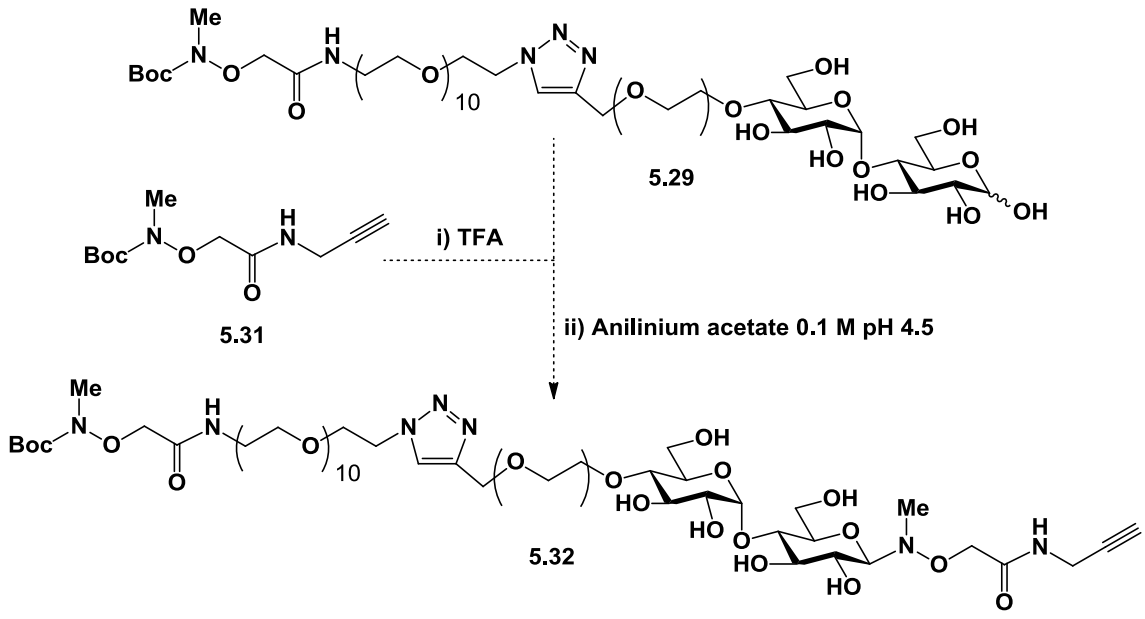
The use of a single PEG-linker **5.1** that could be used for both connections proved to be useful, reducing the total number of steps needed in the synthesis. The PEG-linker **5.1** was successfully connected to both maltoheptaose **4.2** and FITC **5.19** demonstrating its effectiveness.

CuAAC was found to be an effective method to connect fragments together although the synthesis was halted when it was found that an unprotected oxyamine could not survive hydrogenation. This resulted in a new order of assembly being followed. The PEG-linker was successfully attached to the 4'-position of the central fragment **5.11** via CuAAC and hydrogenated.

The limitation to this synthesis was the utility of the oxime ligation reaction when used with the compounds formed in this synthesis. It was thought that the short time carbohydrates reside in the open chain formation combined with the size and flexibility of the fragments resulted in the molecules not reacting. This obstacle ultimately requires a redesign of the current synthesis.

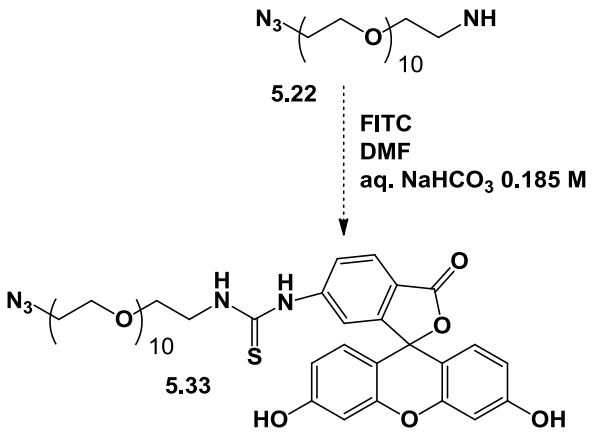
5.8 Future work

In the future to overcome the limitations of the oxime ligation a small alkyne containing oxyamine **5.31** could be reacted with the central fragment **5.29**. Used in a large excess it could push the reaction to completion and would not suffer the same problems as a large PEG-containing compound.



Scheme 5-15 Proposed reaction of the central fragment with a small alkyne containig oxyamine

Once formed the new central fragment **5.32** could be reacted with an excess of maltoheptaose **4.2**. Although the reaction of maltoheptaose **4.2** with the PEG-linker **5.1** was found to be slow it was also successful and an excess of maltoheptaose **5.1** could be used to push the reaction to completion.



Scheme 5-16 Proposed synthesis of an azide derivatised fluorescein

The final fluorescent fragment **5.33** could be attached through CuAAC chemistry which had proved to be reliable. This new synthesis requires the least amount of subsequent chemistry and makes use of the already synthesised central fragment.

Chapter VI: Conclusions and future work

6.1 Summary

The two principal proteins required for this project, maltoporin and MBP, were successfully expressed, purified and analysed. A variety of methods were tested and optimised for the expression of maltoporin and its purification from the membrane fraction of BL21(DE3)omp8 cells was found to be the most effective. A G382C mutant of maltoporin was also expressed and modified with tetramethylrhodamine-5-maleimide. The ligand binding of maltoporin was found to occur on a timescale too long to measure by ITC.

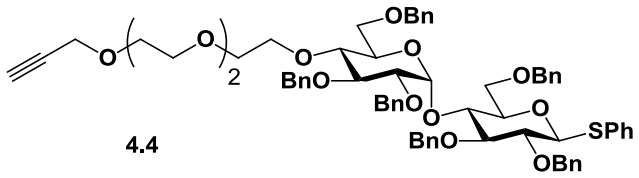


Figure 6-1 The maltose fragment

The synthesis of a maltose fragment with which to build the switch axle was optimised (Figure 6-1). During this procedure it was found that maltoporin was permeable to β-maltosides with the use of a liposome swelling assay.

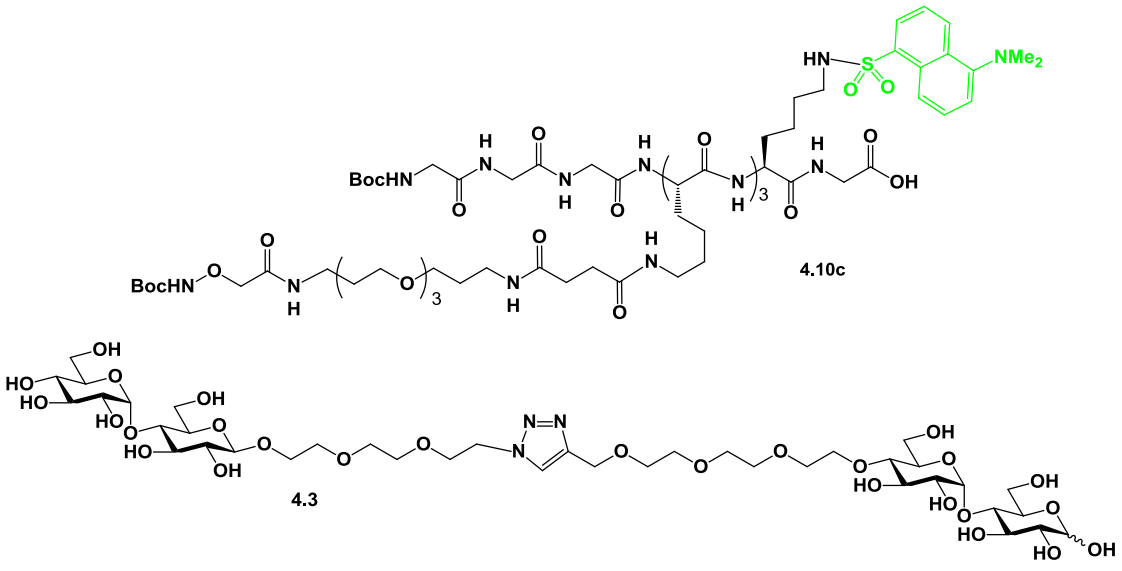


Figure 6-2 The pseudotetrasaccharide

A pseudotetrasaccharide **4.3** was synthesised with a long linker between the maltose stations to probe the functioning of the bind-and-slide mechanism (Figure 6-2). The synthesis of the trivalent ligand precursor **4.10c** was also optimised with a view to further binding studies comparing the affinities of the mono-,di- and trivalent ligands (Figure 6-3).

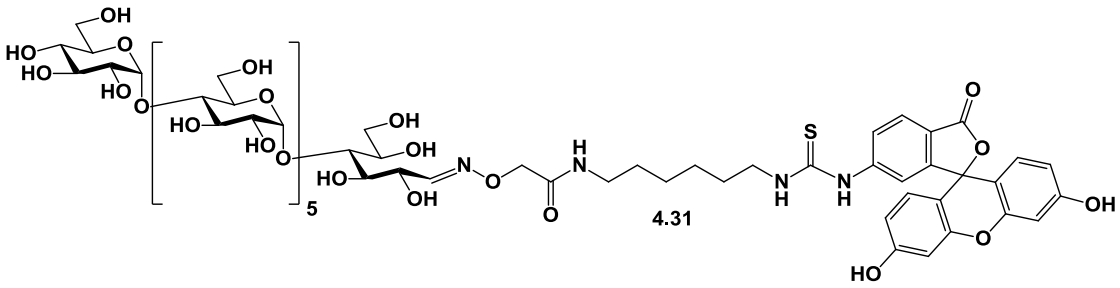


Figure 6-3 The fluorescent ligand

To test the binding of these molecules a fluorescent ligand **4.31** was synthesised and its binding investigated (Figure 6-3). Through fluorescence anisotropy and FRET assays its binding to micelles was discovered. Its use will be further investigated with the use of different strategies to solubilise maltoporin.

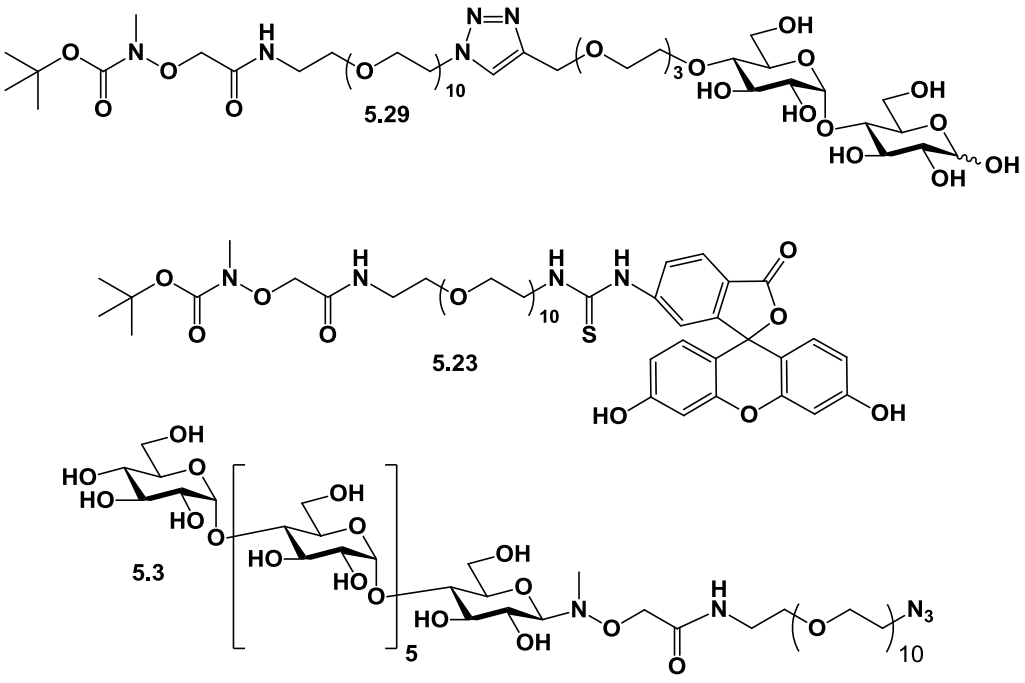


Figure 6-4 The synthesised molecular switch axle precursors

The building blocks required for the construction of the switch axle were successfully synthesised (Figure 6-4). The use of oxime ligations was found to need optimisation although the use of CuAAC proved efficient at connecting the molecules together. Significant progress has been made in the formation of the switch all that remains is the successful connection of the precursors before binding experiments can be performed.

6.2 Future Work

Several avenues of research are still available following this project. The use of biophysical techniques can now be used to investigate the binding of maltoporin as well as measuring the efficiency of the maltose responsive switch.

6.2.1 Binding studies on the bind-and-slide mechanism of maltoporin

Micelles have proven to interfere with fluorescence anisotropy and FRET so the experiments can be repeated with either Amphipol A8-35 or lipid vesicles. When these experiments have been optimised comparisons of the pseudotetrasaccharide **4.3** with maltotetraose and maltose can begin. This experiment could give insight into how the bind-and-slide mechanism functions. Will replacement of maltose units in the centre of a MOSs with PEG units result in a loss in binding or can maltoporin ‘slide’ past them to the next maltose unit it can bind? Other molecules with longer linkers between the maltose moieties could also be synthesised with more than two maltose stations and longer linkers between them.

6.2.2 Multivalent ligand

The oxime ligation reaction using the trivalent ligand will be re-attempted although with a much higher equivalence of maltoheptaose. Once synthesised the binding of the mono-, di- and trivalent ligands could be tested via fluorescence anisotropy. As the multivalent ligands already have a fluorescent dansyl group attached they should not require the use of the fluorescent ligand. This method should demonstrate the increase in binding affinity along with multivalency. If the binding affinity for the multivalent ligands was found to be in the nM range as could be expected their ability to bind live cells would be tested.

Flow cytometry is a technique that can sort cells and measure their fluorescence individually. Omp8 cells expressing WT maltoporin could be stained with 4′,6-diamidino-2-phenylindole (DAPI) which, on binding DNA, emits blue fluorescence. After incubating the stained cells with the multivalent ligand their co-fluorescence can be measured. Any instances of fluorescence from both the DAPI and dansyl group on the multivalent ligand would demonstrate binding to live cells.

6.2.3 Proto-cells

Once the switch has been synthesised following the protocols in the future works of Chapter V its binding can be investigated with a range of techniques.

To test the effectiveness of a maltose responsive switch FRET assays would be performed. These would be developed through the formation of unilamellar vesicles punctuated with G382C maltoporin and containing the switch axle. These would be formed by re-suspending a thin layer of dried 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in a solution containing G382C maltoporin and the switch axle. This should capture the switch axle in multilamellar vesicles punctuated by maltoporin. To transform these into unilamellar vesicles they would be subject to extrusion and could then be purified by gel filtration chromatography to remove any free axle.

The first FRET experiment to take place would be comparing the fluorescence of the free axle compared to the encapsulated axle. As the axle is so long and maltoporin should bind the highest affinity ligand on the axle (i.e. maltoheptaose) it is not thought that any FRET should occur in this experiment.

The second experiment would add MBP to the outside of the vesicles. If MBP can bind to the protruding axle it should pull the ligand through pushing fluorescein close to the pore of maltoporin and also the tetramethylrhodamine. This experiment would demonstrate the ability of the switch to form a rotaxane as if formed the proximity of the fluorescent groups should allow quenching and a FRET signal.

To demonstrate that the formation of the rotaxane was reversible and the system can act as a switch, maltose would be added to the system. At this point the MBP should preferentially bind the maltose releasing the axle and a loss of FRET should be seen.

6.2.4 Live cells

For the switch to act as a fully reversible system it needs to be on the surface of a living cell as the cells own maltose transport system will relieve MBP of the maltose it binds allowing it to rebind the axle.

For these experiments a small amount of G382C maltoporin would be expressed in BL21(DE3)omp8 cells using a low concentration of IPTG. The smaller amount of protein would ensure the cells viability. Maltoporin could then be labelled with tetramethylrhodamine-5-maleimide on the surface of the cells.

The same experiments as on a plate could then be repeated on live cells. Although in this case the experiment could be repeated more than once to test the ability of the system to return to equilibrium after addition of maltose.

Chapter VII: Experimental

7.1 Biology Experimental

7.1.1 Instrumentation

Analytical grade reagents were obtained from Sigma Aldrich, Fisher Scientific, Alfa Aesar and VWR International. Water was purified using the ELGA PURLAB Classic. Ampicillin sodium salt was obtained from Fisher Biotech. Protein molecμlar weight markers used during SDS-PAGE were obtained from Fermentas. Amylose resin was obtained from Sigma Aldrich. MBPTrap columns were obtained from GE Healthcare Life Sciences. Bacterial cμltures were grown using an ISF1-X shaker incubator (Kuhner). Centrifugation was performed using the Avanti J30-I (Beckman Coulter), Pico 17 and Fresco 21 centrifuges (Thermo Scientific). Gels were imaged and analysed using a Universal Hood II gel documentation system (BioRad).

Analytical grade reagents were supplied by Sigma-Aldrich, Fisher Scientific, Melford laboratories and VWR International. Pwo enzyme (DNA polymerase) (from *Pyrococcus woesei*) was made in the Turnbμll lab. Restriction enzymes, T4 DNA ligase, DpnI and CIP were purchased from NEB. Competent cells were purchased from Stratagene or made in the Turnbull Lab. Primers and DNA parts for gene synthesis were purchased from IDT.

7.1.2 Cell lines

BL21(DE3)omp8

Genotype**:** BL21(DE3), *ΔlamB ompF::Tn5ΔompAΔompC*[107]

This cell line was provided by the laboratory of Ralf Koebnik and does not produce any of the major outer membrane pore proteins which consist of OmpC, OmpA, OmpF, LamB.

7.1.3 The pMal-c5X plasmid

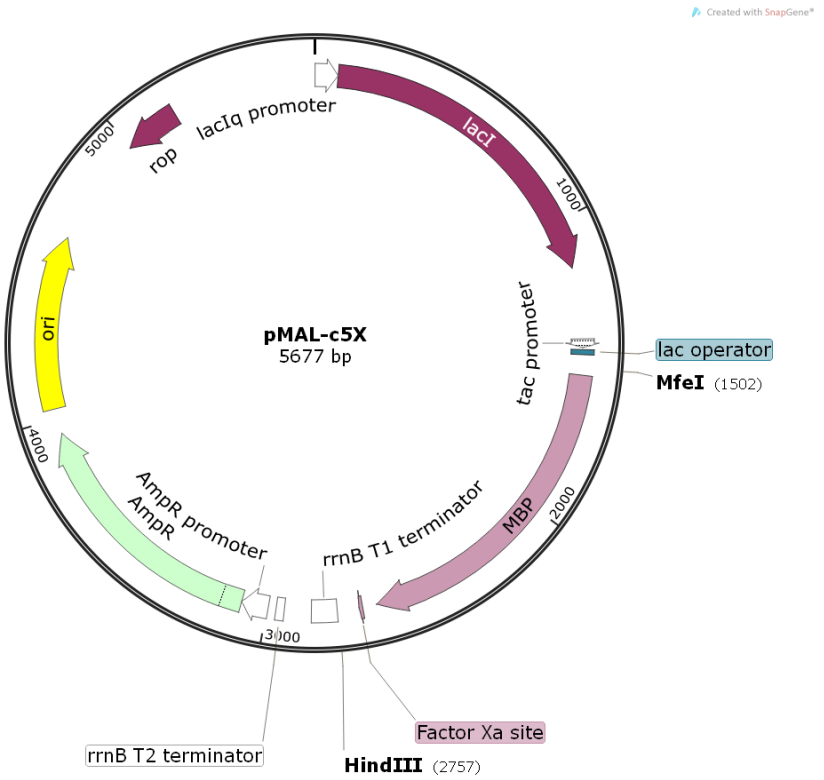


Figure 7-1 The pMal-c5X plasmid from New England Biolabs. This plasmid is used for the gene fusion of MBP to a protein of choice. Expression is controlled by the lac operon.

7.1.4 Primers

Forward Primers

Minus leader Mfe1 Forward (51 bases): CAC GAG **CAA TTG** AAC AAG GAC CAT AGA TT **ATG** GTT GAT TTC CAC GGC TAT G

Minus leader + 6His/TEV cleavage site Mfe1 Forward (90 bases): CAC GAG **CAA TTG** AAC AAG GAC CAT AGA TT **ATG** CAT CAC CAC CAT CAT CAC **GAA AAC CTG TAC TTT CAG GGC** GTT GAT TTC CAC GGC TAT G

G382C mutation forward (38 bases): GG GGT TAC GAC TAC ACC **TGC** AAC GCT GAT AAC AAC GCG

Mfe1 restriction site, ribosome binding site, **start codon**, overlap region, His Tag, **TEV cleavage, G382C mutation**

Reverse Primers

HindIII Reverse (37 bases): **GAT GGA AAT CTG GTG GTA A**TA AAT **AAG CTT** CAA ATA A

G382C mutation reverse (38 bases): CGC GTT GTT ATC AGC GTT **GCA** GGT GTA GTC GTA ACC CC

Overlap Region, **HindIII restriction site, G382C mutation**

7.1.5 Expressed sequences

Maltoporin

MMITLRKLPLAVAVAAGVMSAQAMAVDFHGYARSGIGWTGSGGEQQCFQTTGAQSK
YRLGNECETYAELKLGQEVWKEGDKSFYFDTNVAYSVAQQNDWEATDPAFREANVQ
GKNLIEWLPGSTIWAGKRFYQRHDVHMIDFYYWDISGPGAGLENIDVGFGKLSLAATR
SSEAGGSSSFASNNIYDYTNETANDVFDVRLAQMEINPGGTLELGVDYGRANLRDNY
RLVDGASKDGWLFTAEHTQSVLKGFNKFVVQYATDSMTSQGKGLSQGSGVAFDNEK
FAYNINNNGHMLRILDHGAISMGDNWDMMYVGMYQDINWDNDNGTKWWTVGIRPM
YKWTPIMSTVMEIGYDNVESQRTGDKNNQYKITLAQQWQAGDSIWSRPAIRVFATYA
KWDEKWGYDYTGNADNNANFGKAVPADFNGGSFGRGDSDEWTFGAQMEIWW

Leader sequence

Maltoporin without leader sequence

VDFHGYARSGIGWTGSGGEQQCFQTTGAQSKYRLGNECETYAELKLGQEVWKEGD
KSFYFDTNVAYSVAQQNDWEATDPAFREANVQGKNLIEWLPGSTIWAGKRFYQRHD
VHMIDFYYWDISGPGAGLENIDVGFGKLSLAATRSSEAGGSSSFASNNIYDYTNETAN
DVFDVRLAQMEINPGGTLELGVDYGRANLRDNYRLVDGASKDGWLFTAEHTQSVLK

GFNKFVVQYATDSMTSQGKGLSQGSGVAFDNEKFAYNINNNGHMLRILDHGAISMGD
NWDMMYVGMYQDINWDNDNGTKWWTVGIRPMYKWTPIMSTVMEIGYDNVESQRT
GDKNNQYKITLAQQWQAGDSIWSRPAIRVFATYAKWDEKWGYDYTGNADNNANFGK
AVPADFNGGSFGRGDSDEWTFGAQMEIWW

Maltoporin G382C mutant

VDFHGYARSGIGWTGSGGEQQCFQTTGAQSKYRLGNECETYAELKLGQEVWKEGD
KSFYFDTNVAYSVAQQNDWEATDPAFREANVQGKNLIEWLPGSTIWAGKRFYQRHD
VHMIDFYYWDISGPGAGLENIDVGFGKLSLAATRSSEAGGSSSFASNNIYDYTNETAN
DVFDVRLAQMEINPGGTLELGVDYGRANLRDNYRLVDGASKDGWLFTAEHTQSVLK
GFNKFVVQYATDSMTSQGKGLSQGSGVAFDNEKFAYNINNNGHMLRILDHGAISMGD
NWDMMYVGMYQDINWDNDNGTKWWTVGIRPMYKWTPIMSTVMEIGYDNVESQRT
GDKNNQYKITLAQQWQAGDSIWSRPAIRVFATYAKWDEKWGYDYTCNADNNANFGK
AVPADFNGGSFGRGDSDEWTFGAQMEIWW

Mutated residue

MBP

MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGP
DIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIY
NKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGK
YDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWS
NIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEA
VNKDKPLGAVALKSYEEELVKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINA
ASGRQTVDEALKDAQTNSSSNNNNNNNNNNLGIEGRISHMSMGGRDIVDGSEFPAG
N

7.1.6 Buffer solutions

Standard buffers

Elution buffer: HEPES (pH 7.0, 20 mM): 1% (v/v) Octyl-POE, 20% (w/v) maltose, 0.1 M NaCl.
Elution buffer (+DTT): HEPES (pH 7.0, 20 mM): 1% (v/v) Octyl-POE, 20% (w/v) maltose, 0.1 M NaCl, 10 mM DTT.
Labelling buffer (pH 7.4): Phosphate (pH 7.4, 100 mM): 0.5% (v/v) Octyl-POE, 0.1 M NaCl.
Labelling buffer (pH 8.4): Phosphate (pH 8.4, 100 mM): 0.5% (v/v) Octyl-POE, 0.1 M NaCl.
Lysis buffer (+DTT): Tris-HCl (pH 8.0, 50 mM): 10 mM $MgSO_4$, 0.1 M NaCl, 10 mM DTT.

MBP elution buffer: Tris-HCl (pH 7.4, 50 mM): 0.1 M NaCl, 20% (w/v) glucose.

Tris buffer: Tris-HCl (pH 7.4, 50 mM): 0.1 M NaCl.

Triton buffer: Tris-HCl (pH 8.0, 50 mM): 2% (v/v) Triton X-100, 10 mM EDTA, 0.1 M NaCl.

Triton buffer (+DTT): Tris-HCl (pH 8.0, 50 mM): 2% (v/v) Triton X-100, 10 mM EDTA, 0.1 M NaCl, 10 mM DTT.
Urea buffer: Tris-HCl (pH 8.0, 50 mM): 1 mM EDTA, 8 M Urea, 0.1 M NaCl.
Wash buffer: HEPES (pH 7.0, 20 mM): 1% (v/v) Octyl-POE, 0.1 M NaCl.
Wash buffer (+DTT): HEPES (pH 7.0, 20 mM): 1% (v/v) Octyl-POE, 0.1 M NaCl, 10 mM DTT.
Buffer N3 – 4.2 M Guanidine HCl, 0.9 M KAc

Buffer QG – Tris-HCl (pH 6.6, 20 mM), 5.5 M GuSCN

Buffer P1 – Tris-HCl (pH 8.0, 50 mM), 10 mM EDTA, 100 $\mu g.mL^{-1}$ RNase A

Buffer P2 – 200 mM NaOH, 1% (*w/v*) SDS

Buffer PB – 5.0 M Guanidine HCl, 30% (*v/v*) isopropanol

Buffer PE – Tris-HCl (pH 7.5, 10 mM), 80% (*v/v*) ethanol

Bacterial growth buffers

LB growth media (LB): LB Broth (25 g premixed; 10 g Tryptone, 5 g Yeast extract, 10 g NaCl), Ampicillin (100 mg).
SOC growth media (SOC): LB Broth (20 g Tryptone, 5 g Yeast extract, 0.58 g NaCl, 0.19 g KCl, $MgCl_2$ (0.95 g), $MgSO_4$ (1.20 g), glucose (3.60 g), Ampicillin (100 mg).
Anaerobic minimal media: LB Broth (5 g premixed; 2 g Tryptone, 1 g Yeast extract, 2 g NaCl), Cysteine HCl (500 mg), Maltose (4 g), Ampicillin (100 mg).
Agar gels: agar (1.5 g) added to 100 ml of LB.

Buffers for DNA analysis and manipulations

50 × TAE Buffer: 2 M Tris-HCl (314 g), concentrated acetic acid (57.1 mL) and EDTA (0.5 M at pH 8.0, 100 mL).

6 × Agarose electrophoresis *loading buffer (10 ml, pH 7.6):* 15% (*w/v*) Ficoll 400, 66 mM EDTA (193 mg), 19.8 mM Tris-HCl (31 mg), 0.12% (*w/v*) SDS and 0.9% (*w/v*) Orange G.

10 × EcoRI buffer (10 mL, pH 7.5): 1 M Tris-HCl (1.57 g), 500 mM NaCl (292 mg), 100 mM $MgCl_2$ (95 mg) and 0.25% (*v/v*) Triton X-100.

10 × Ligase Buffer (10 mL pH 7.5): 500 mM Tris-HCl (7.85 g), 100 mM $MgCl_2$ (95 mg) 10 mM adenosine triphosphate (ATP, 55 mg) and 100 mM dithiothreitol (DTT, 154 mg).

Protein analysis buffer

5 x SDS running buffer: 125 mM Tris (15.15 g), 960 mM glycine (72.1 g), 0.5% w/v SDS (5 g).

SDS loading buffer: Tris (0.5M, pH 6.8, 1.25 ml), SDS (0.3 g), DTT (0.47 g), glycerol (1 ml), bromophenol blue (~5 crystals), water (7.2 ml).

SDS loading buffer without DTT: Tris (0.5M, pH 6.8, 1.25 ml), SDS (0.3 g), glycerol (1 ml), bromophenol blue (~5 crystals), water (7.2 ml).

Coomassie stain: coomassie G250, methanol (400 ml), acetic acid (100 ml).

7.1.7 Molecular Biology

7.1.7.1 PCR

PCR was carried out using the pSAB2.2-M5 vector containing the Maltoporin gene and primers listed in the primers section of the standard protocols. The concentrations are shown in Table 7-1.

Table 7-1 Materials used in PCR experiments.

	Amount (µl)
H_2O	43.2
PCR Buffer	5
DNTPs	0.4
PWO	0.2
F primer	0.5
R primer	0.5
Template	0.2

The sample was initially heated for 5 min at 95 °C before 25 cycles of 30s at 95 °C, 30s at 50 °C and 2m at 70 °C. The sample was held for an additional 5 min at 70 °C before being stored at 4 °C.

7.1.7.2 Site directed mutagenesis

Site directed mutagenesis was carried out using the pSAB2.2-M5 vector and primers listed in the primers section of the standard protocols. The concentrations are shown in Table 7-2.

Table 7-2 Materials for site directed mutatgenesis

	Volume (μl)	Concentration
pMaltp Plasmid	0.48	0.105 mM
Forward Primer	0.7	10 mM
Reverse Primer	0.7	10 mM
PFU buffer (with $MgSO_4$)	5.0	10x stock
dNTPs	0.39	25 mM
PFU DNA polymerase	1.0	3 U / μl
H_2O	41.7	

The sample was initially heated for 1.5 min at 95 °C before 20 cycles of 20s at 95 °C, 20s at 55 °C and 12m at 70 °C. The sample was held for an additional 24 min at 72 °C before being stored at 4 °C.

7.1.7.3 Gel Extraction

This was conducted using a QIAquick gel extraction kit, following the manufacturer-recommended protocol. All centrifugation steps took place at 17,900 × *g* at room temperature. The gel generated from an agarose gel electrophoresis experiment was viewed under UV light, and the target band excised from the gel (with minimal gel) using a scalpel. Buffer QG (3 gel volumes) was added at 50 °C to dissolve the agarose. Isopropanol (1 gel volume) was added to increase the DNA yield, and the solution passed through a silica membrane by centrifugation for 1 min, to which DNA is adsorbed but RNA, protein and metabolites may pass through. The membrane was washed by centrifugation for 1 min with buffer PE (750 µL) to remove salts, before a further 1 min centrifugation to remove residual ethanol. The DNA was then eluted in water (30 µL) by centrifugation for 1 min.

7.1.7.4 PCR Clean-up

This was conducted using a QIAquick PCR purification kit, following the manufacturer-recommended protocol. All centrifugation steps took place at 17,900 × g at room temperature. Buffer PB (5 volumes) was added to the PCR reaction mix, and the solution passed through a silica membrane by centrifugation for 1 min, to which DNA is adsorbed. The membrane was washed by centrifugation for 1 min with buffer PE (750 µL) to remove salts, subjected to a further 1 min centrifugation to remove residual ethanol, and the DNA eluted by centrifugation for 1 min in water (30 µL).

7.1.7.5 Plasmid preparation

This was conducted using a QIAprep spin Miniprep kit, following the manufacturer-recommended protocol. All centrifugation steps took place at 17,900 × g at room temperature. Bacterial cells were pelleted from cµltures by centrifugation for 5 min, then resuspended in buffer P1 (250 µL). Buffer P2 (250 µL) was added in order to lyse the bacterial cells by alkaline lysis5, followed by buffer N3 (350 µL) to neutralise the suspension and adjust to high salt binding conditions, whilst precipitating SDS. The precipitate (SDS, genomic DNA, and cell debris) was pelleted by centrifugation for 10 min, and the supernatant passed through a silica membrane by centrifugation for 1 min, to which the DNA was adsorbed. The membrane was washed by centrifugation for 1 min with buffer PB (500 µL) to remove endonucleases, and PE (750 µL) to remove salts, before the DNA was eluted in water (50 µL) by centrifugation for 1 min.

7.1.7.6 Agarose Gel

DNA purity was analysed by agarose gel using Bio-Rad Subcell agarose gel electrophoresis systems. A 1% gel using agarose (443 mg) in TAE buffer (40 ml) was prepared by heating the solution in a microwave until all the agarose had dissolved. Ethidium bromide (1.2 µL, 0.3 µg / ml) was added and mixed. The solution was then immediately poured into a moµld and a comb added before the gel was left to set. Samples for analysis were prepared by mixing 5 µL of DNA sample with 1 µL DNA loading buffer and 5 µL aliquots were loaded into the sample wells. Electrophoresis

was performed at 100 V in TAE running buffer for 20 min. Gels were then visualised under UV light using the Gel Doc system.

7.1.7.7 DNA concentration

All DNA concentrations (ng μL^{-1}) were determined by spectrophotometry at 260 nm and calcμlated using the equation; [DNA] = 50 × (A_{260}- A_{320}).

7.1.7.8 Restriction digest

Insert preparation - A restriction digest was used to give the Maltoporin PCR product sticky ends. The DNA (30 μL) was added to a solution of MfeI (2 μL, 20 U), Hind III (2 μL, 20 U), 10 × buffer 4 (4 μL) and sterile water (2 μL). The reaction mixture was incubated at 37 °C for 1.5 h before the digested DNA was isolated by agarose gel electrophoresis and purified using a QIAquick Gel Extraction Kit.

Vector preparation - The pMal vector was prepared for DNA ligation using a slightly modified restriction digest procedure. The plasmid DNA (20 μL) was added to a solution of MfeI (3 μL, 20 U), Hind III (3 μL, 20 U), 10 × buffer 4 (4 μL) and sterile water (2.5 μL). The reaction mixture was incubated at 37 °C for 1 h before calf-intestinal phosphatase (3 μL, 10 U) was added. The incubation was continued at 37 °C for an additional hour before the linearised plasmid was isolated and purified as previously described.

7.1.7.9 Ligation

The Maltoporin gene insert and linear pMal vector were ligated together using the following procedure. A ligation mixture was prepared over ice using the linear plasmid vector (50 ng), gene insert (3:1 molar ratio of insert to vector), 10 × ligase buffer (1 μL), T4 ligase (1 μL, 20 U) and H_2O to make a total volume of 10 mL. The reaction mixture was incubated at 4 °C overnight before being transformed into chemically competent *E. coli* XL10 cells.

7.1.7.10 Transformation

The plasmid (1 μl) was added to chemically competent freshly thawed cells (10 μl) on ice and left for 10 min. The solution was then heat shocked at 42 °C for 45 s before being placed back on ice for 10 min. 900 μl LB was added and the solution incubated at 37 °C for 1 h before centrifugation at 18,000 x *g* for 4 min. 800 μl of the supernatant was discarded and the pellet re-suspended in the remaining 100 μl. The culture was spread onto an agar plate and incubated for 16 h. A colony was selected from the plate and used to inoculate a falcon tube of LB growth media (5 mL) with ampicillin (0.5 mg) to be incubated at 37 °C for 20 h. Aliquots (500 μl) of the culture were then mixed with 80% (v/v) glycerol (500 μl) and stored in the -80 °C freezer.

7.1.7.11 Heat competent procedure

Cells were grown up in 5 ml LB with 5 μl kanamycin. 100 ml of LB was then inoculated with 300 μl of the cells grow up. The 100 ml grow up was allowed to grow to OD_{600} = 0.4 at which point 50 ml of the solution was transferred to a 50 ml falcon tube which was cooled in ice for 10 min. The falcon tubes were then pelleted at 4,000 x g for 10 min at 4 °C and the supernatant poured off. The pellets were re-suspended in 10 ml ice-cold 0.1 M $CaCl_2$ and pelleted again at 4,000 x g for 10 min. The supernatant was poured off and the cells were re-suspended in 2 ml ice-cold 0.1 M $CaCl_2$. The solution was then split into 75 μl aliquots combined with 25μl 40% glycerol and stored in the -80 °C fridge.

7.1.7.12 DNA extraction and sequencing procedure

The DNA was extracted from the bacterial cell pellet using a QIAprep Spin Mini Prep Kit and sequenced by GATC Biotech.

7.1.7.13 Agar plate procedure

To make the plates agar mix (50 ml) and antibiotic (5 mg) were mixed in a falcon tube and poured onto a petri dish where the plate was allowed to cool.

7.1.8 Protein expression

LB Media

Maltoporin was expressed in the following way. A stab was taken from a stock of *E.coli* BL21(DE3)omp8 cells containing the plasmid encoding the protein to be expressed, and a falcon tube of LB growth media (5 mL) was inoculated. This starter culture was incubated at 37 °C for 20 h. This starter cμlture (3 mL) was added to 1 L LB growth media plus antibiotic (ampicillin or kanamycin as stated) (100 mg) and was then incubated at 37 °C for a further 3 h when the OD_{600} had reached ~0.8 and IPTG (120 mg) was added to each flask to induce protein over-expression. The incubation was continued for 4 h before isolating the bacterial cells by centrifugation at 10,000 x *g* for 15 min. The supernatant was discarded and the bacterial pellet was frozen at -80 °C until being purified.

SOC Media

Maltoporin was expressed in the following way. A stab was taken from a stock of *E.coli* BL21(DE3)omp8 cells containing the plasmid encoding the protein to be expressed, and a falcon tube of LB growth media (5 mL) was inoculated. This starter cμlture was incubated at 37 °C for 20 h. This starter culture (3 mL) was added to 1 L SOC growth media plus ampicillin (100 mg) and was then incubated at 16 °C for a further 14 h when the OD_{600} had reached ~0.6 and IPTG (24 mg) was added to each flask to induce protein over-expression. The incubation was continued at 16 °C for 12 h before isolating the bacterial cells by centrifugation at 10,000 x *g* for 15 min. The supernatant was discarded and the bacterial pellet was frozen at -80 °C until being purified.

Anaerobic maltoporin expression

Maltoporin was expressed in the following way. A stab was taken from a stock of *E.coli* BL21(DE3)omp8 cells containing the plasmid encoding the protein to be expressed, and a falcon tube of LB growth media (5 mL) was inoculated. This starter culture was incubated at 37 °C for 20 h. This starter cμlture (3 mL) was added to 1 L anaerobic minimal media plus ampicillin (100 mg); which had been sealed and purged with nitrogen. This was then incubated at 37 °C for a further 26 h when the OD_{600} had reached ~0.6 and IPTG (120 mg) was added to each flask to induce protein over-expression. The incubation was continued for 20 h before isolating the bacterial cells by centrifugation at 10,000 x *g* for 15 min. The supernatant was discarded and the bacterial pellet was frozen at -80 °C until being purified.

MBP Expression

MBP was expressed in the following way. A stab was taken from a stock of *E.coli* C43 cells containing an empty pMal-c5X plasmid encoding the protein to be expressed, and a falcon tube of LB growth media (5 mL) was inoculated. This starter culture was incubated at 37 °C for 20 h. This starter cμlture (3 mL) was added to 1 L LB growth media plus ampicillin (100 mg) and was then incubated at 37 °C for a further 3 h when the OD_{600} had reached ~0.6 and IPTG (120 mg) was added to each flask to induce protein over-expression. The incubation was continued for 20 h before isolating the bacterial cells by centrifugation at 10,000 x *g* for 15 min. The supernatant was discarded and the bacterial pellet was frozen at -80 °C until being purified.

7.1.9 Protein purification

Maltoporin purification

The frozen bacterial pellet was resuspended on ice in 10 mM $MgCl_2$ (30 mL). Cells were lysed using the sonicator for 2 x 4 min, the resulting lysate was cleared by centrifugation at 48,000 x *g* for 30 min and the supernatant discarded. The cell pellet was re-suspended in 2% (v/v) Triton X-100 in TEN buffer and the insoluble fraction separated by centrifugation at 48,000 x *g* for 30 min.

Maltoporin G382C mutant purification

The frozen bacterial pellet was resuspended on ice in Lysis buffer (+DTT) (30 mL). Cells were lysed using the sonicator for 2 x 4 min, the resulting lysate was cleared by centrifugation at 48,000 x *g* for 30 min and the supernatant discarded. The cell pellet was re-suspended in Triton buffer (+DTT) (30 ml) and the insoluble fraction separated by centrifugation at 48,000 x *g* for 30 min.

MBP Purification

The frozen bacterial pellet was resuspended on ice in Tris Buffer (30 mL). Cells were lysed using the sonicator for 3 x 2 min. The resulting lysate was cleared by centrifugation at 48,000 x *g* for 30 min and the pellet discarded.

7.1.10 Amylose column purification

Maltoporin amylose column

For the amylose column, the protein solution was loaded and non-binding proteins were removed by washing with wash buffer (40 mL). Maltoporin was eluted with elution buffer (40 mL) and fractions were collected (5 mL). The fractions containing protein were concentrated and purified by dialysis to remove maltose.

Maltoporin G382C mutant amylose column

For the amylose column, the protein solution was loaded and non-binding proteins were removed by washing with wash buffer (+DTT) (50 mL). Maltoporin was eluted with elution buffer (+DTT) (40 mL) and fractions were collected (5 mL). The fractions containing protein were concentrated and purified by dialysis to remove maltose.

MBP amylose column

For the amylose column, the protein solution was loaded and non-binding proteins were removed by washing with Tris buffer (40 mL). MBP was eluted with MBP elution buffer (40 mL) and fractions were collected (5 mL). The fractions containing protein were concentrated and purified by dialysis to remove glucose.

MBPTrap AKTA FPLC Purification

The Triton X-100 solubilised protein was passed through a 5 ml MBPTrap column with the use of an AKTA FPLC machine. The column was equilibrated in wash buffer, 100 ml of sample was passed through the column, washed with 15 ml wash buffer and the protein step eluted with 50 ml elution buffer. All flow through was kept and the process repeated.

7.1.11 Protein Modification

Solubilising maltoporin into amphipol A8-35

Maltoporin (27 uM) in wash buffer (670 μl) had 5:1 (w/w) amphipol A8-35 (4.3 mg) added. The solution was vortexed and allowed to stand for 15 min. 10:1 (w/w) SM2 biobeads (39 mg) were added and the solution was mixed for 2 h before the beads were removed by centrifugation at 3500 x *g* for 10 min.

Labelling of cysteine mutant

G382C maltoporin (72 uM) in wash buffer (400 μl) had its buffer exchanged for labeling buffer (500 μl) using a PD-10 column. To this solution was added TCEP (50 mM) in labeling buffer (4 μl) and tetramethylrhodamine maleimide (20 mM) in DMSO (1.7 μl). The mixture was left in the dark o.n.

7.1.12 Liposome swelling assay

To make the mμltilamellar liposomes 6.2 μl of dipalmitoyl phosphorycholine and 0.2 μl of dicetyl phosphate were dissolved in chloroform and dried under a stream of N_2. The lipids were then re-suspended by sonication with 0.2 μM Maltoporin (0 μM Maltoporin for the negative control) in 5 mM Tris, pH 8 and 0.1% Octyl-POE. The water was removed by heating to 45 °C under a stream of N_2 before re-suspension in 17% Dextran T9.9, 5 mM Tris, pH 7.5 and 5 mM $MgCl_2$ by vortexing for 2 h.

The sugar solutions were made up to 20 mM sugar, 5 mM $MgCl_2$ and 5 mM Tris, pH 7.5.

For the assay 600 μl of the sugar solution was placed in a curvette in a UV spectrometer and 20 μl of the liposome solution was added. The absorption of the liposomes was plotted every 5 seconds for 1 min and the gradient of the line recorded.

7.1.13 SDS-PAGE

Protein purity was assessed by sodium dodecyl sμlfate polyacrylamide gel electrophoresis (SDS-PAGE) using BioRad tetragel apparatus. A resolving gel (10%) was prepared using before pouring the gel into the plates. *Tert*-butanol was poured on top of the resolving gel to produce a flat surface and the gel was left to set.

The stacking gel (5%) was prepared using the materials in Table 7-3, with the TEMED left until last. The *tert*-butanol was decanted off of the set resolving gel, the stacking gel poured on top and the plastic comb inserted to create sample wells. The gel was again left to set at room temperature. Samples for analysis were prepared by mixing 10 μL of protein sample with 10 μL SDS loading buffer and 10 μL aliquots were loaded into the sample wells. Electrophoresis was performed at 180 V in SDS running buffer.

After 45 minutes the gels were removed and stained with instant blue for at least 1 h before being stored in water until the gel was imaged.

Table 7-3 Materials for preparing SDS-PAGEs.

	Resolving Gel / mL	Stacking Gel / mL
H_2O	4.93	3.24
3 M TRIS-HCl pH 8.8	1.25	-
0.5 M TRIS-HCl pH 6.8	-	1.25
10% w/v SDS	0.1	0.05
40% Acrylamide	2.5	0.5
TEMED	0.013	0.01
20% w/v APS	0.083	0.04

7.1.14 Protein concentration analysis

Protein concentrations were measured using UV absorption at 280 nm. The concentration was determined using the following equation:

$A = \varepsilon c l$

Where A is the absorbance, ε is the extinction coefficient, c is the concentration in mol L^{-1} and l is the pathlength in cm.

The theoretical extinction coefficient for maltoporin was obtained from ExPASy ProtParam. For the maltoporin monomer this was 137,280 mol^{-1} L cm^{-1}.

The theoretical extinction coefficient for MBP was obtained from ExPASy ProtParam. For the maltoporin monomer this was 66,350 mol^{-1} L cm^{-1}.

7.1.15 Biophysical experiments

7.1.15.1 Fluorescence anisotropy

Appropriate dilution series (110 µM to 0.2 µM, 25 µl) were set up in triplicate before addition of fluorescent ligand (1.2 µM stock, 5 µl) Total volume 30 µl, 200 nM probe.

Fluorescence Anisotropy measurements were taken in 96-well plate format using an Envision 2103 Mμltilabel Reader.

G-factor was calcμlated by the average of the readings on a blank probe sample where G=S/P

Anisotropy (r) of individual wells was then calcμlated using:

$$r = \frac{(S - G * P)}{(S + 2G * P)}$$

The total fluorescence (S+2G*P) was plotted vs protein concentration to confirm there was no observable quenching.

The anisotropy values for the triplicate wells were averaged and standard errors calcμlated. These values were used to produce a plot of anisotropy vs protein concentration. This plot was fitted with a logistic method (fixed power of 1) and this used to convert the anisotropy values to fraction bound.

7.1.15.2 FRET assay

Solutions of TAMRA labelled maltoporin (1.2 μM), fluorescent ligand (100 nM, 2 μM, 20 μM), in HEPES (20 mM, pH 7.0), NaCl (0.1 M) and Octyl-POE (0.5% v/v) had their emissions from 500 – 650 nm measured separately in a 600 μl quartz curvette with a HORIBA scientific Fluoromax-3 fluorimeter. The emission spectra of these combined provided a negative control.

A 500 μl solution of TAMRA labelled maltoporin (1.2 μM) and fluorescent ligand (100 nM, 2 μM, 20 μM) in HEPES (20 mM, pH 7.0), NaCl (0.1 M) and Octyl-POE (0.5% v/v) as well as a 500 μl solution of TAMRA labelled maltoporin (1.2 μM), fluorescent ligand (100 nM, 2 μM, 20 μM) and maltohetpaose (10 mM) in HEPES (20 mM, pH 7.0), NaCl (0.1 M) and Octyl-POE (0.5% v/v) where then also measured.

A decrease in fluorescence at 515 nm and an increase in fluorescence at 568 nm would be indicative of FRET occurring.

7.1.15.3 Isothermal titration calorimetry (ITC)

ITC titrations were performed using a Microcal ITC_{200} calorimeter (GE healthcare) with a cell volume of 0.2028 mL and data processed and fitted using Origin with the one site model.[138] Protein samples were dialysed into *phosphate buffer* (SnakeSkin® pleated dialysis tubing, Thermo Scientific, with 7000 MWCO) prior to analysis. The ligands were then dissolved in the dialysis buffer to ensure an exact match for the buffers during the titration. The reference cell was filled with water and the analysis cell was filled with the protein solution and both were allowed to reach thermal equilibrium at 25 °C before titrations were performed. Titrations typically comprised 20 injections of 2 µL at 2 minute intervals. Separate titrations of the ligand into buffer were used to subtract the heat of dilution from the integrated data prior to curve fitting.

7.2 Chemistry Experimental

7.2.1 General Methods

All solvents were dried prior to use, according to standard methods. Boron trifluoride diethyl etherate ($BF_3.OEt_2$) was distilled over calcium hydride, and all other commercially available reagents were used as received. Where appropriate anhydrous quality material was purchased. All solvents used for flash chromatography were GPR grade, except hexane and ethyl acetate, when HPLC grade was used. All concentrations were performed *in vacuo,* unless otherwise stated. All reactions were performed in oven dried glassware under a N_2(g) atmosphere, unless otherwise stated.

^{1}H NMR spectra were recorded at 500 MHz on a Bruker Avance 500 instrument or at 300 MHz on a Bruker Avance 300 instrument. ^{13}C NMR spectra were recorded at 75 MHz on a Bruker Avance 300 instrument. Chemical shifts are given in parts per million

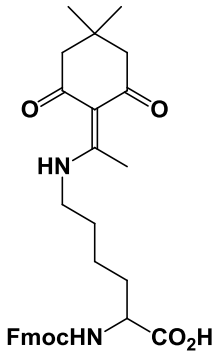
downfield from tetramethylsilane. The following abbreviations are used in ^{1}H NMR analysis: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, dt = double triplet, td = triple doublet, ddd = double double doublet.

Electrospray (ES+) ionisation mass spectra were obtained on a Bruker HCTUltra mass spectrometer, and high resolution ES+ were performed on a Bruker Daltonics MicroTOF mass spectrometer. Isotopic ratios were calculated by comparison of the ^{13}C peak area of the most abundant ion, using Bruker Compass Data Analysis 4.0 software. Infra-red spectra were recorded on a Bruker ALPHA FT-IR spectrometer with platinum ATR. Melting points were obtained on a Reichert hot-stage apparatus and are uncorrected. Optical rotations were measured at the sodium D-line with a Schmidt and Haensch Polartronic H 532. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}

Analytical TLC was performed on silica gel 60-F^{254} (Merck) with detection by fluorescence and/or charring following immersion in a 5% H_2SO_4/methanol solution, unless otherwise stated.

7.2.2 Experimental

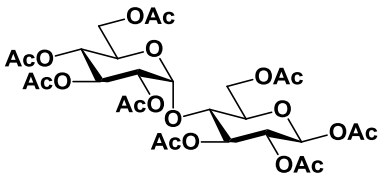
(1.17) Fmoc-lys(dde)-OH



2-(1-Hydroxyethylidene)-5,5-dimethyl-1,3-cyclohexanedione (1 g, 5.47 mmol), Fmoc-lys-OH (1.01 g, 2.73 mmol) and trifluoroacetic acid (21 µl, 0.27 mmol) in EtOH (22 ml) were stirred at 85 °C under reflux for 74 h. The solvent was removed under reduced pressure and the residue taken into EtOAc (25 ml), washed with 1 M $KHSO_4$ (20 ml), water (20 ml), sat. aq. NaCl (20 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 50:50:2 EtOAc-Hex-AcOH) to afford a colourless solid (1.37 g, 95%). [lit.[139] [α]d +3.1

(*c* 1.0, MeOH)]**;** [α]d +8.7 (*c* 1.0, $CHCl_3$); IR (cm^{-1}): 3303 (O-H), 1711 (C=O, ketone/carboxylic acid), 1625 (C=O, amide); HRMS: Found ($[M+H]^+$) 533.2646, $C_{31}H_{36}N_2O_6$ requires 533.2661; HRMS ^{1}H NMR (500 MHz, $CDCl_3$); δ_H **Dde:** 2.53 (s, 3H, C=CC<u>H</u>$_3$), 2.36 (s, 2H, C(O)C<u>H</u>$_2$), 2.35 (s, 2H, C(O)C<u>H</u>$_2$), 1.00 (s, 6H, C(C<u>H</u>$_3$)$_2$), **Fmoc**: 7.74 (d, *J* 7.6, 2H, Ar-H), , 7.58 (dd, *J* 7.4 Hz, *J* 10.7, 2H, Ar-H), 7.37 (t, *J* 7.4 Hz, 2H, Ar-H), 7.30-7.23 (m, 2H, Ar-H), 4.44 (d, *J* 6.9 Hz, 2H, C<u>H</u>2-CH), 4.21 (t, *J* 6.9 Hz, 1H, CH2-C<u>H</u>) **Lys**: 5.83 (d, *J* 8.1 Hz, 1H, C(O)N<u>H</u>), 4.44-4.40 (m, 1H, Hα), 3.39-3.35 (m, 2H, 2 x Hε), 2.00-1.93 (m, 1H, Hβ'), 1.75-1.70 (m, 1H, Hβ"), 1.74-1.68 (m, 2H, 2 x Hδ), 1.61-1.44 (m, 2H, 2 x Hγ). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = **Dde:** 198.4 (C=O), 141.3 (NH<u>C</u>=C), 108.3 ((O=C)$_2$<u>C</u>=C), 52.4 (O=C<u>C</u>H$_2$), 30.9 (<u>C</u>(CH$_x$)$_4$), 28.2 (C(<u>C</u>H$_3$)$_2$), 18.2 (C=C<u>C</u>H$_3$), **Fmoc**: 174.4 (NH<u>C</u>=O), 143.9 (Ar-C), 143.8 (Ar-C), 128.2, 127.7, 125.1, 120.0 (Ar-CH), 67.1 (<u>C</u>H$_2$-CH), 47.1 (CH$_2$-<u>C</u>H), **Lys:** 174.8 (<u>C</u>OOH), 53.7 (Cα), 43.3 (Cε), 32.0 (Cβ), 28.4 (Cδ), 22.6 (Cγ).

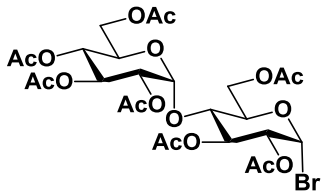
(3.2) β-D-Glucopyranose, 4-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-, 1,2,3,6-tetraacetate



Sodium acetate (50 g, 0.61 mol) in acetic anhydride (541 mL, 4.86 mol) was heated under reflux. The solution was removed from the heat and maltose (100 g, 0.29 mol) was added in portions to keep the solution boiling. The solution was allowed to cool to 75°C, poured into ice (2 L) and stirred for 19 hrs. The water was decanted off and extracted with DCM (200 mL), the remaining solid was dissolved in DCM (200 mL) and the extracts combined. The extracts were washed with water (2 x 100 mL), sat. aq. $NaHCO_3$ (3 x 100 mL), water (100 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was recrystallised from EtOH to give colourless crystals (139 g, 70%). Mp 125 – 134 °C; [α]$_D$ +65.3 (*c* 0.94, $CHCl_3$); [lit.[140] [α]$_D$ +63 (*c* 1, $CHCl_3$)]; MS: *m/z* ($[M+Na]^+$) = 701.1; IR (cm^{-1}): 1752 (C=O); ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.76 (d, 1H, $J_{1\text{-}2}$ 8.1 Hz, H-1), 5.42 (m, 1H, H-1'), 5.39 (m, 1H, H-3'), 5.33 (m, 1H, H-3), 5.06 (dd, 1H, $J_{4'\text{-}3'}$ 9.9 Hz, $J_{4'\text{-}5'}$ 9.9 Hz, H-4'), 4.99 (dd, 1H, $J_{2\text{-}1}$ 8.1 Hz, $J_{2\text{-}3}$ 9 Hz, H-2), 4.89 (dd, 1H, $J_{2'\text{-}1'}$ 4 Hz, $J_{2'\text{-}3'}$ 10.5 Hz, H-2'), 4.47 (dd, 1H, $J_{6a\text{-}5}$ 2.6 Hz, $J_{6a\text{-}6b}$ 12.3 Hz, H-6a), 4.25 (m, 2H, H-6b, H-6'a), 4.05 (m, 2H, H-4, H-6'b), 3.96 (ddd, 1H, $J_{5'\text{-}4'}$ 9.9 Hz, $J_{5'\text{-}6'a}$ 6 Hz, $J_{5'\text{-}6'b}$ 3 Hz, H-5'), 3.85 (ddd, 1H, $J_{5\text{-}4}$ 9.6 Hz, $J_{5\text{-}6a}$ 4.4 Hz, $J_{5\text{-}6b}$ 2.6 Hz, H-5),

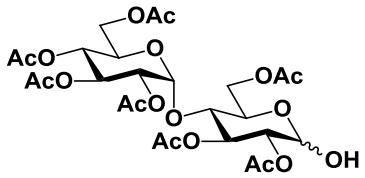
1.9-2.1 (m, 24H, C(O)CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.6, 170.5, 170.4, 170.1, 169.9, 169.5, 168.9 (8 x $\underline{C}OCH_3$), 95.7 (CH-1'), 91.2 (CH-1), 75.2, 72.9, 72.3, 70.9, 69.9, 69.3, 68.5, 67.9, 68.6, 67.8 (8 x CH), 61.8, 61.3 (2 x CH_2), 20.9, 20.8, 20.7, 20.6, 20.5, 18.4 (6 x CO$\underline{C}H_3$).

(3.5) 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranose



HBr-HOAc (33% w/v) (21.6 mL, 124 mmol), was added to a solution of maltose (20.0 g, 29.6 mmol) in DCM (200 ml) at 0°C, the reaction mixture was then stirred for 3 hrs. The mixture was poured into DCM (1.2 L) and washed with water (2 x 200 ml), sat. aq. $NaHCO_3$ (300 ml), sat. aq. NaCl (300 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 3:2 (v/v) ethyl acetate-hexane) to afford a white solid (7.9g, 38%). R_f (EtOAc–Hex, 2:1) 0.43; [α]D +146.3 (*C* 1.1, $CHCl_3$) [lit.[141] [α]D +172 (*C* 1, $CHCl_3$)]; MS: *m/z* ([M+Na]$^+$) = 722.4; IR (cm^{-1}): 1749 (C=O); ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 6.50 (d, 1H, $J_{1\text{-}2}$ 4 Hz, H-1), 5.61 (dd, 1H, $J_{3\text{-}2}$ 9.5 Hz, $J_{3\text{-}4}$ 9.5 Hz, H-3), 5.43 (d, 1H, $J_{1'\text{-}2'}$ 4 Hz, H-1'), 5.38 (dd, 1H, $J_{3'\text{-}2'}$ 10 Hz, $J_{3'\text{-}4'}$ 10Hz, H-3'), 5.08 (dd, 1H, $J_{4'\text{-}3'}$ 10 Hz, $J_{4'\text{-}5'}$ 10 Hz, H-4'), 4.88 (dd, 1H, $J_{2'\text{-}1'}$ 4 Hz, $J_{2'\text{-}3'}$ 11 Hz, H-2'), 4.71 (dd, 1H, $J_{2\text{-}1}$ 4 Hz, $J_{2\text{-}3}$ 10 Hz, H-2), 4.53 (dd, 1H, $J_{6a\text{-}5}$ 4 Hz, $J_{6a\text{-}6b}$ 14 Hz, H-6a), 4.23-4.28 (m, 3H, H-5, H-6b, H-6'a), 4.05-4.13 (m, 2H, H-4, H-6'b), 3.94-4.05 (m, 1H, H-5'), 2.01-2.15 (m, 21H, C(O)CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.6, 170.5, 170.4, 170.1, 169.9, 169.5, 168.9 (8 x $\underline{C}OCH_3$), 95.7 (C-1'), 86.6 (C-1), 72.5 (C-5), 72.3 (C-3), 71.5 (C-4), 71.0 (C-2), 70.0 (C-2'), 69.2 (C-3'), 68.6 (C-5'), 67.8 (C-4'), 61.8 (C-6), 61.3 (C-6'), 21.0, 20.8, 20.7, 20.6, 20.6, 20.5, 20.5 (7 x CO$\underline{C}H_3$).

(3.8) 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α,β-D-glucopyranose

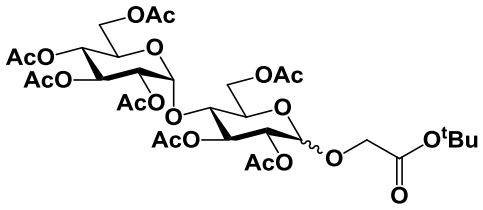


2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-α-D-glucopyranose (5 g, 7.36 mmol) and hydrazine acetate (0.75 g, 8.1 mmol) in DMF (70 ml) were heated to 50°C for 1 hr 10 min and then allowed to stir at r.t. for 23.5 hrs. The solvent was removed under reduced pressure and the solid recrystallised from EtOH to give an inseperable mixture of anomers (dr: 59:41) as a white solid (3.56 g, 76%). Mp 174 – 176 °C; $[\alpha]_D$ +78.8 (*C* 1.00, $CHCl_3$); [Lit.[142] $[\alpha]_D$ +86 (*C* 1.04, $CHCl_3$)]**;** MS: *m/z* ($[M+Na]^+$) = 659.1; IR (cm^{-1}): 1748 (C=O), 3473 (O-H);

α - Major diastereoisomer: ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.59 (t, 1H, $J_{3,2}$ 9 Hz, $J_{3,4}$ 9 Hz, H-3), 5.45 (d, 1H, $J_{1',2'}$ 4 Hz, H-1'), 5.38 (t, 1H, $J_{3',2'}$ 10 Hz, $J_{3',4'}$ 10 Hz, H-3'), 5.34 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1), 5.08 (t, 1H, $J_{4',3'}$ 9 Hz, $J_{4',5'}$ 9 Hz, H-4'), 4.88 – 4.85 (m, 1H, H-2'), 4.80 – 4.76 (m, 1H, H-2), 4.52 – 4.48 (m, 1H, H-6a), 4.28 – 4.21 (m, 3H, H-5, H-6b, H-6a'), 4.07 – 3.96 (m, 5.1H, H-4, H-5', H-6b'), 2.15 – 1.84 (m, 21H, 7 x $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.8, 170.8, 170.7, 170.4, 170.2, 170.1, 169.6 (7 x $\underline{C}OCH_3$), 95.5 (C-1'), 89.9 (C-1), 72.6 (C-2, C-4), 72.4 (C-3), 70.0 (C-2'), 69.4 (C-3'), 68.5 (C-5'), 68.4 (C-4'), 67.5 (C-5), 62.9 (C-6), 61.4 (C-6'), 21.0, 20.9, 20.9, 20.7, 20.6, 20.6 (7 x $CO\underline{C}H_3$).

β - Minor diastereoisomer: ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.41 (d, 1H, $J_{1',2'}$ 4 Hz, H-1'), 5.40 – 5.35 (m, 1H, H-3'), 5.29 (t, 1H, $J_{3,2}$ 9 Hz, $J_{3,4}$ 9 Hz, H-3), 5.06 (t, 1H, $J_{4',3'}$ 9.5 Hz, $J_{4',5'}$ 9.5 Hz, H-4'), 4.88 – 4.85 (m, 1H, H-2'), 4.80 – 4.76 (m, 2H, H-1, H-2), 4.52 – 4.48 (m, 1H, H-6a), 4.28 – 4.21 (m, 2H, H-6b, H-6a'), 4.07 – 3.96 (m, 3H, H-4, H-5', H-6b'), 3.76 – 3.70 (m, 1H, H-5), 2.15 – 1.84 (m, 21H, 7 x $COCH_3$,). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.8, 170.8, 170.7, 170.4, 170.2, 170.1, 169.6 (7 x $\underline{C}OCH_3$), 95.5 (C-1'), 94.8 (C-1), 75.0 (C-3), 72.6 (C-4), 72.3 (C-5), 70.2 (C-2), 70.0 (C-2'), 69.3 (C-3'), 68.5 (C-5'), 67.9 (C-4'), 62.9 (C-6), 61.4 (C-6'), 21.0, 20.9, 20.9, 20.7, 20.6, 20.6 (7 x $CO\underline{C}H_3$).

(3.9) 2-(*tert*-Butyl acetyl) 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*O*-α,β-D-glucopyranoside[143]



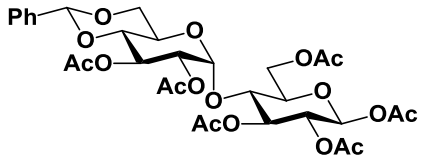
2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α,β-D-glucopyranose (2.4 g, 3.77 mmol), K_2CO_3 (1.56 g, 3.77 mmol) and tert-butyl bromoacetate (1.11 ml, 7.54 ml) in DMF (40 ml) were stirred for 19 hr 15 min. The mixture was then filtered, the solid washed with DCM and the filtrate concentrated under reduced pressure. The solid was taken into DCM (50 ml), washed with H_2O (2 x 50 ml), dried (Na_2SO_4) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 1:1 (v/v) EtOAc–Hex) to give an inseparable mixture of diastereoisomers (dr: 80:20) as a white solid (1.56 g, 55%). MS: *m/z* ($[M+Na]^+$) = 773.3; IR (cm^{-1}): 1747 (C=O).

α - Major diastereoisomer: ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.59 (t, 1H, $J_{3,2}$ 10 Hz, $J_{3,4}$ 10 Hz, H-3), 5.44 (d, 1H, $J_{1',2'}$ 4 Hz, H-1'), 5.37 (t, 1H, $J_{3',2'}$ 10 Hz, $J_{3',4'}$ 10 Hz, H-3'), 5.10 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1), 5.07 (t, 1H, $J_{4',3'}$ 10 Hz, $J_{4',5'}$ 10 Hz, H-4'), 4.86 (dd, 1H, $J_{2',1'}$ 4 Hz, $J_{2',3'}$ 9.5 Hz, H-2'), 4.78 (dd, 1H, $J_{2,1}$ 3.5 Hz, $J_{2,3}$ 10, H-2), 4.46 (dd, 1H, $J_{6a,5}$ 2.5 Hz, $J_{6a,6b}$ 12.5, H-6a), 4.28 – 4.23 (m, 2H, H-6b, H-6a'), 4.18 – 4.13 (m, 1H, H-5), 4.08 (s, 2H, $OCH_2C(O)$), 4.06 – 4.02 (m, 1H, H-6b'), 4.00 (t, 1H, $J_{4,3}$ 9.5 Hz, $J_{4,5}$ 9.5 Hz, H-4), 3.99 – 3.95 (m, 1H, H-5'), 2.08 – 1.94 (m, 21H, 7 x $C(O)CH_3$), 1.40 (s, 9H, $OC(CH_3)_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 171.0, 170.6, 170.4, 170.4, 170.4, 170.3, 170.0, 169.8, 169.8, 169.6, 169.4, 169.3, (7 x $\underline{C}OCH_3$) 168.1 ($\underline{C}$(O)OtBu), 95.4, 95.4 (C-1', C-1), 82.0 ($O\underline{C}(CH_3)_3$), 72.5 (C-4), 72.2 (C-3), 70.8 (C-2), 69.9 (C-2'), 69.3 (C-3'), 68.4 (C-5'), 68.2 (C-5), 68.0 (C-4'), 64.5 ($O\underline{C}H_2C(O)$), 62.6 (C-6), 61.5 (C-6'), 28.0 ($OC(\underline{C}H_3)_3$), 20.9, 20.9, 20.8, 20.7, 20.6, 20.6, 20.5, 20.5 (7 x $C(O)\underline{C}H_3$).

β - Minor diastereoisomer: ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.41 (d, 1H, $J_{1',2'}$ 4 Hz, H-1'), 5.36 (t, 1H, $J_{3',2'}$ 9.5 Hz, $J_{3',4'}$ 9.5 Hz, H-3'), 5.28 (t, 1H, $J_{3,2}$ 9 Hz, $J_{3,4}$ 9 Hz, H-3), 5.05 (t, 1H, $J_{4',3'}$ 9.5 Hz, $J_{4',5'}$ 9.5 Hz, H-4'), 4.88 (t, 1H, $J_{2,1}$ 9 Hz, $J_{2,3}$ 9 Hz, H-2), 4.90 – 4.84 (m, 1H, H-2'), 4.69 (d, 1H, $J_{1,2}$ 9 Hz, H-1), 4.49 (dd, 1H, $J_{6a.5}$ 2.5 Hz, $J_{6a,6b}$ 12.5, H-6a), 4.28 – 4.23 (m, 2H, H-6b, H-6a'), 4.08 (s, 2H, $OCH_2C(O)$), 4.06 – 4.02 (m, 2H, H-4, H-6b'), 3.99 – 3.95 (m, 1H, H-5'), 3.70 (ddd, 1H, $J_{5,4}$ 9.5 Hz, $J_{5,6a}$ 3.5, $J_{5,6b}$ 9.5 Hz, H-5). 2.08 – 1.94 (m, 26.25H, 7 x $C(O)CH_3$,), 1.41 (s, 9H, $OC(CH_3)_3$). ^{13}C NMR (75 MHz,

$CDCl_3$): δ_C = 171.0, 170.6, 170.4, 170.4, 170.4, 170.3, 170.0, 169.8, 169.8, 169.6, 169.4, 169.3, (7 x $\underline{C}(O)CH_3$) 168.0 ($\underline{C}(O)OtBu$), 99.5 (C-1), 95.5 (C-1'), 82.0 ($O\underline{C}(CH_3)_3$), 75.1 (C-3), 72.5 (C-4), 72.2 (C-5), 70.8 (C-2), 69.9 (C-2'), 69.3 (C-3'), 68.4 (C-5'), 68.0 (C-4'), 64.5 ($O\underline{C}H_2CO$), 62.7 (C-6), 61.4 (C-6'), 27.9 ($OC(\underline{C}H_3)_3$), 20.9, 20.9, 20.8, 20.7, 20.6, 20.6, 20.5, 20.5 (7 x $CO\underline{C}H_3$).

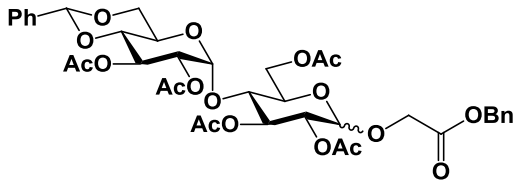
(3.10) 2,3-Di-*O*-acetyl-(*R*)-4,6-*O*-benzylidene-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose[86]



Maltose (10.0 g, 29.2 mmol), α,α-dimethoxy toluene (10.9 ml, 73 mmol) and *p*-toluenesulfonic acid (0.64g, 5.84 mmol) were dissolved in DMF (72 ml) and rotated on a rotary evaporator at 48°C and at 30 Torr for 6.5 h. The solution was neutralised with triethylamine and the solvent removed under reduced pressure. The concentrate was taken up into DMF (70 ml) and TFA (1 ml) was stirred for 6 h 10 min. The solution was neutralised with triethylamine and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 4:1 (v/v) $CHCl_3$ –MeOH) to afford a white solid. The solid, (*R*)-4,6-*O*-benzylidene–α-D-glucopyranosyl-(1→4)-D-glucose (9.30 g, 21.6 mmol), was added in portions to a solution of sodium acetate (8.87 g, 108 mmol) in acetic anhydride (88 ml) which had been heated at reflux. The solution was heated to boiling for an additional 15 min. The solution was allowed to cool and then poured into crushed ice (300 ml). The mixture was stirred for 3 hr 20 min, extracted into DCM (100 ml), washed with sat. aq. $NaHCO_3$ (30 ml), H_2O (30 ml), sat. aq. $NaHCO_3$ (30 ml), sat. aq. NaCl (30 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The solid was recrystallised from ethanol to give a white solid (1.31 g, 26%). Mp 206 – 207 °C [lit.[144] Mp 209 – 210]; $[\alpha]_D$ +36.5 (*C* 1.0, $CHCl_3$) [lit.[145] $[\alpha]_D$ +35 (*C* 1.1, $CHCl_3$)]**;** MS: *m/z* ($[M+Na]^+$) = 705.2; IR (cm^{-1}): 1751 (C=O); ^1H NMR (500 MHz, $CDCl_3$); δ_H = 7.43-7.42 (m, 2H, Ar-*o*), 7.37-7.34 (m, 3H, Ar-*m*, Ar-*p*), 5.74 (d, 1H, $J_{1,2}$ = 8 Hz, H-1), 5.48 (s, 1H, Bz-H), 5.46 (t, 1H, $J_{3',2'}$ 10 Hz, $J_{3',4'}$ 10 Hz, H-3'), 5.36 (d, 1H, $J_{1',2'}$ 4 Hz, H-1'), 5.31 (t, 1H, $J_{3,2}$ 9 Hz, $J_{3,4}$ 9 Hz, H-3), 4.98 (t, 1H, $J_{2,1}$ 8 Hz, $J_{2,3}$ 8 Hz, H-2), 4.88 (dd, 1H, $J_{2',3'}$ 10, $J_{2',1'}$ 4 Hz, H-2'), 4.50 (dd, 1H, $J_{6a,6b}$ 12.5, $J_{6a,5}$ 2 Hz, H-6a), 4.28 – 4.23 (m, 2H, H-6b, H-6a'), 4.05 (t, 1H, $J_{4,3}$ 9 Hz, $J_{4,2}$ 9 Hz, H-4), 3.86 – 3.81 (m,

2H, H-5, H-5'), 3.72 (t, 1H, $J_{6b',6a'}$ 10.5 Hz, $J_{6b',5'}$ 10.5 Hz, H-6b'), 3.63 (t, 1H, $J_{4',3'}$ 10 Hz, $J_{4',5'}$ 10 Hz, H-4'), 2.15 – 2.00 (m, 18H, 6 x $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.8, 170.3, 170.1, 169.7, 169.7, 168.8 (6 x $\underline{C}OCH_3$), 136.7 (Ar-*i*C), 129.2 (Ar-*p*C), 128.3 (Ar-*o*C), 126.2 (Ar-*m*C), 101.6 (Bz-C), 96.6 (C-1'), 91.2 (C-1), 78.7 (C-4'), 75.3 (C-3), 73.0 (C-4), 72.5 (C-5), 71.0 (C-2), 70.8 (C-2'), 68.4 (C-3', C-6'), 63.8 (C-5'), 62.3 (C-6), 20.9, 20.8, 20.8, 20.7, 20.6 (6 x $CO\underline{C}H_3$).

(3.12) 2-(*tert*-Butyl acetyl) 2,3-di-*O*-acetyl-(*R*)-4,6-*O*-benzylidene-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*O*-α,β-D-glucopyranoside



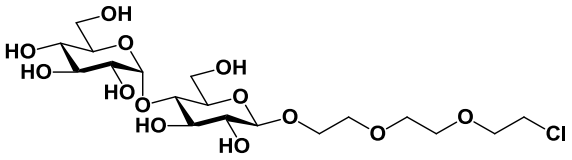
2,3-Di-*O*-acetyl-(*R*)-4,6-*O*-benzylidene-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (500 mg, 0.74 mmol) and hydrazine acetate (75 mg, 0.81 mmol) in DMF (7 ml) were stirred at 58 °C for 1 h. The solvent was removed under reduced pressure and the solid recrystallised from ethanol to give a white solid. The solid, 2,3-Di-*O*-acetyl-(*R*)-4,6-*O*-benzylidene-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α,β-D-glucopyranose (150 mg, 0.23 mmol), caesium carbonate (228 mg, 0.70 mmol), 2-bromo benzylacetate (60 μl, 0.47 mmol) and tetrabutylammonium iodide (26 mg, 0.07 mmol) in DMF (2 ml) were stirred at r.t. for 24 h. The reaction mixture was filtered under reduced pressure, taken into DCM (20 ml), washed with H_2O (3 x 10 ml), the aqueous washes were then extracted with DCM (10 ml) and the organic extracts combined and dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 2:3 (v/v) EtOAc–Hex) to give a white solid (135 mg, 74%).

α - Major diastereoisomer: ^1H NMR (500 MHz, $CDCl_3$); δ_H = 7.45-7.28 (m, 5H, Ar-H), 5.58 (t, 1H, J_{3-2} 10 Hz, J_{3-4} 10 Hz, H-3), 5.48 (s, 2H, 2 x Bz-H'), 5.47 (t, 1H, $J_{3'-2'}$ 10 Hz, $J_{3'-4'}$ 10 Hz, H-3'), 5.38 (d, 1H, $J_{1'-2'}$ 4 Hz, H-1'), 5.16 (s, 2H, 2 x Bz-H), 5.12 (d, 1H, J_{1-2} 4 Hz, H-1), 4.90-4.87 (m, 1H, H-2'), 4.78 (dd, 1H, $J_{2,1}$ 4 Hz, $J_{2,3}$ 10 Hz, H-2), 4.47 (dd, 1H, J_{6a-5} 2 Hz, $J_{6a,6b}$ 12 Hz, H-6a), 4.30 (s, 2H, $OCH_2C(O)$), 4.22-4.19 (m, 2H, H-5, H-6a'), 4.11 (m, 1H, H-6b), 3.99 (t, 1H, $J_{4,3}$ 10 Hz, $J_{4,5}$ 10 Hz, H-4), 3.84 (m, 1H, H-5'), 3.73 (t, 1H, $J_{6b'-5'}$ 10 Hz, $J_{6a'-6b'}$ 10 Hz, H-6b'), 3.63 (t, 1H, $J_{4'-3'}$ 10 Hz, $J_{4'-5'}$ 10 Hz, H-4'), 2.20-1.85 (m, 15H, 5 x $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.9, 170.5, 170.3,

169.7, 169.1 (5 x $\underline{C}OCH_3$, 1 x $\underline{C}OCH_2$), 136.8, 135.1, (2 x Ar-C), 129.1, 135.1, 129.1, 128.7, 128.6, 128.5, 128.4, 128.2, 126.2 (10 x Ar-CH), 101.6 (Bz-C'), 96.4 (C-1'), 95.6 (C-1), 78.2 (C-4'), 72.4 (C-4), 71.9 (C-3), 70.9 (C-2), 70.8 (C-2'), 66.9 (Bz-H, C-3', C-5, C-6'), 64.2 ($O\underline{C}H_2C(O)$), 63.7 (C-5'), 62.4 (C-6), 20.9, 20.8, 20.7, 20.6 (5 x $CO\underline{C}H_3$).

β - Minor diastereoisomer: ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.48 (s, 2H, 2 x Bz-H'), 5.45 (t, 1H, $J_{3'-2'}$ 10 Hz, $J_{3'-4'}$ 10 Hz, H-3'), 5.36 (d, 1H, $J_{1'-2'}$ 4 Hz, H-1'), 5.28 (t, 1H, J_{3-2} 9 Hz, $J_{3,4}$ 9 Hz, H-3), 5.17 (s, 2H, 2 x Bz-H), 4.90-4.87 (m, 2H, H-2, H-2'), 4.68 (d, 1H, $J_{1,2}$ 8 Hz, H-1), 4.53 (dd, 1H, J_{6a-5} 2 Hz, J_{6a-6b} 12 Hz, H-6a), 4.32 (s, 2H, $OCH_2C(O)$), 4.22-4.19 (m, 2H, H-6b, H-6a'), 4.02 (t, 1H, $J_{4,3}$ 9 Hz, $J_{4,5}$ 9 Hz, H-4), 3.84 (m, 1H, H-5'), 3.73 (t, 1H, $J_{6b'-5'}$ 10 Hz, $J_{6b'-6a'}$ 10 Hz, H-6b'), 3.66 (m, 1H, H-5), 3.63 (t, 1H, $J_{4'-3'}$ 10 Hz, $J_{4'-5'}$ 10 Hz, H-4'), 2.20-1.85 (m, 15H, 5 x $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.9, 170.5, 170.3, 169.7, 169.1 (5 x $\underline{C}OCH_3$, 1 x $\underline{C}OCH_2$), 136.8, 135.1, (2 x Ar-C), 129.1, 135.1, 129.1, 128.7, 128.6, 128.5, 128.4, 128.2, 126.2 (10 x Ar-CH), 101.6 (Bz-C'), 99.5 (C-1), 96.4 (C-1'), 78.8 (C-4'), 75.1 (C-3), 72.2 (C-4), 71.9 (C-5), 70.8 (C-2, C-2'), 66.9 (C-3', C-6'), 65.2 (Bz-H), 64.2 ($O\underline{C}H_2C(O)$), 63.7 (C-5', C-6), , 20.9, 20.8, 20.7, 20.6 (5 x5 x $CO\underline{C}H_3$).

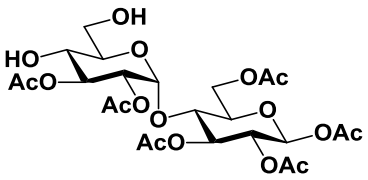
(3.13) 2-(2-(2-chloroethoxy)ethoxy)ethoxy α-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (7)



Iodine (crystal) and silver carbonate (3.9 g, 14.4 mmol) in DCM (25 ml) were added to acetobromomaltose **5** (5 g, 7.2 mmol) and 2-(2-(2-chloroethoxy)ethoxy)ethanol **17** (5.15 ml, 36 mmol) in DCM (25 ml). The solution was stirred at r.t. for 19.5 h before being filtered and concentrated under reduced pressure. The resulting syrup was taken up into acetic acid (10 ml) and pyridine (10 ml) and stirred for 25 h before being concentrated under reduced pressure. The solid was purified by flash column chromatography (silica, 1:1 → 2:1 EtOAc-Hex). The resulting solid was taken up into methanol (10 ml) with sodium methoxide (150 mg) and stirred at r.t for 18.5 h. The solution was neutralised with IRC-120 H^+ resin, filtered and concentrated under reduced pressure. The resulting syrup was purified by size exclusion chromatography (MeOH, Sephadex LH-20) to give a clear oil (1.11 g, 32%). $[\alpha]_D$ +113.3 (*c* 1.0,

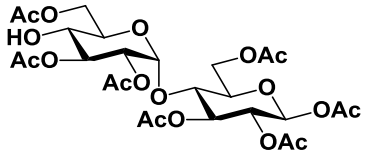
MeOH); HRMS: Found ([M+Na]$^+$) 515.1503, $C_{18}H_{33}ClO_{13}$ requires 515.1502; IR (cm^{-1}): 3350 (OH); ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.15 (d, $J_{1',2'}$ = 3.8 Hz, 1H, H-1'), 4.27 (d, $J_{1,2}$ = 8.0 Hz, 1H, H-1), 3.83 (dt, *J* 11.4 Hz, *J* 4.1 Hz, 1H, PEG-H), 3.70 (dd, $J_{6a,5}$ = 2.0 Hz, $J_{6a,6b}$ = 12.2 Hz, 1H, H-6a), 3.66-3.58 (m, 4H, H-6a', 3 x PEG-H), 3.58-3.46 (m, 12H, H-3, H-5', H-6b, H-6b', 8 x PEG-H), 3.45 (t, $J_{3',2'}$ = 9.5 Hz, $J_{3',2'}$ = 9.5 Hz, 1H, H-3'), 3.39 (t, $J_{4,3}$ = 9.5 Hz, $J_{4,5}$ = 9.5 Hz, 1H, H-4), 3.37-3.33 (m, 1H, H-5), 3.35 (dd, $J_{2',1'}$ = 3.8 Hz, $J_{2',3'}$ = 9.5 Hz, 1H, H-2'), 3.18 (t, $J_{4',3'}$ = 9.5 Hz, $J_{4',5'}$ = 9.5 Hz, 1H, H-4'), 3.10 (dd, $J_{2,1}$ = 8.0 Hz, $J_{2,3}$ = 9.5 Hz, 1H, H-2); ^{13}C NMR (75 MHz, $CDCl_3$): δ_C =75.2 (C-1), 72.8 (C-1'), 50.2 (C-4), 49.3 (C-3), 47.7 (C-5), 46.1 (C-3'), 46.0 (C-2), 45.9 (C-5'), 44.9 (C-2'), 44.0 (PEG-C), 42.9, 42.7, 42.6 (PEG-C), 42.5 (C-4'), 41.8 (PEG-C), 33.9 (PEG-C), 33.6 (C-6), 16.3 (C-6').

(3.14) 2,3-Di-*O*-acetyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose[86]



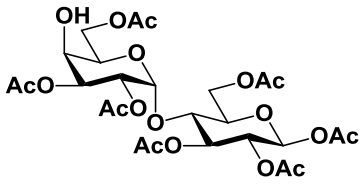
To a solution of 2,3-Di-*O*-acetal-(*R*)-4,6-*O*-Benzylidene–α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (1.2 g, 1.76 mmol) in MeCN (34 ml), HBF_4–H_2O (1:1 v/v) (0.34 ml, 2.72 mmol) was added. The mixture was stirred for 10 min before being neutralised with triethylamine. The solvent was removed under reduced pressure and the solid was purified by flash column chromatography (silica, EtOAc) to afford an amorphous solid (0.75 g, 72%). [α]D +50.4 (*C* 1.00, $CHCl_3$), [lit.[145] [α]D +53.1 (*C* 2.1, $CHCl_3$)]**;** MS: *m/z* ([M+Na]$^+$) = 617.1; IR (cm^{-1}): 1747 (C=O), 3445 (O-H); ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.75 (d, 1H, $J_{1,2}$ 8 Hz, H-1), 5.35 (d, 1H, $J_{1',2'}$ 4 Hz, H-1'), 5.31 (t, 1H, $J_{3,2}$ 9 Hz, $J_{3,4}$ 9 Hz, H-3), 5.23 - 5.19 (m, 1H, H-3'), 4.98 (t, 1H, $J_{2,1}$ 9 Hz, $J_{2,3}$ 9 Hz, H-2), 4.77 (dd, 1H, $J_{2',3'}$ 10.5, $J_{2',1'}$ 4 Hz, H-2'), 4.49 (dd, 1H, $J_{6a,6b}$ 10.5, $J_{6a,5}$ 2 Hz, H-6a), 4.21 (dd, 1H, $J_{6a',6b'}$ 12.5, $J_{6a',6b'}$ 4 Hz, H-6a'), 4.03 (t, 1H, $J_{4,3}$ 9.5 Hz, $J_{4,5}$ 9.5 Hz, H-4), 3.85 – 3.81 (m, 3H, H-5, H-6b, H-6b'), 3.69 – 3.67 (m, 2H, H-4', H-5'), 3.21 (bs, 1H, OH-4'), 3.21 (bs, 1H, OH-6'), 2.23 – 1.97 (m, 18H, 6 x $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 171.3, 171.3, 170.8, 170.2, 169.7, 168.9 (6 x $\underline{C}OCH_3$), 95.8 (C-1'), 91.3 (C-1), 75.3 (C-3), 73.1 (C-5'), 72.8 (C-5), 72.0 (C-3'), 71.9 (C-4), 70.9 (C-2), 70.3 (C-2'), 69.5 (C-4'), 62.7 (C-6), 62.0 (C-6'), 20.9, 20.9, 20.8, 20.7, 20.6 (6 x CO$\underline{C}H_3$).

(3.16) 2,3,6-Tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose[86]



EDC (1.08 g, 5.65 mmol) and HOBt (0.7 g, 5.18 mmol) were added successively to a stirred solution of acetic acid (0.27 ml, 4.71 mmol) in DCM (10 ml). After 23 hrs the solution was washed with aq. HCl (10%, 5 ml), sat. aq. $NaHCO_3$ (5 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give **7** as an amorphous solid. 2,3-Di-*O*-acetal-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (0.67 g, 1.13 mmol) and the solid (0.24 g, 1.35 mmol) were dissolved in DCM (6 ml). Triethylamine (0.25 ml, 1.66 mmol) was added and the solution was stirred for 19 hr 40 min. The solvent was removed under reduced pressure and the solid purified by flash column chromatography (silica, 3:2 (v/v) EtOAc–Hex) to afford a white solid (269 mg, 37%). $[\alpha]_D$ +35.5 (*C* 1.0, $CHCl_3$), [lit.[146] $[\alpha]_D$ +46.5 (*C* 1.0, $CHCl_3$)]**;** IR (cm^{-1}): 1747 (C=O), 3479 (O-H); ^1H NMR (500 MHz, $CDCl_3$); δ_H = 5.72 (d, 1H, $J_{1,2}$ 8 Hz, H-1), 5.35 (d, 1H, $J_{1',2'}$ 4 Hz, H-1'), 5.30 (t, 1H, $J_{3,2}$ 9 Hz, $J_{3,4}$ 9 Hz, H-3), 5.22 (t, 1H, $J_{3',2'}$ 9.5 Hz, $J_{3',4'}$ 9.5 Hz, H-3'), 4.97 (t, 1H, $J_{2,1}$ 9 Hz, $J_{2,3}$ 9 Hz, H-2), 4.78 (dd, 1H, $J_{2',1'}$ 10.5, $J_{2',1'}$ 4 Hz, H-2'), 4.49 – 4.53 (m, 2H, H-6a, H-6a'), 4.23 – 4.19 (m, 2H, H-6b, H-6b'), 4.04 (t, 1H, $J_{4,3}$ 9.5 Hz, $J_{4,5}$ 9.5 Hz, H-4), 3.84 (ddd, 1H, $J_{5,6a}$ 2, $J_{5,6b}$ 4.5, $J_{5,4}$ 9.5 Hz, H-5), 3.81 – 3.79 (m, 1H, H-5'), 3.53 (dt, 1H, $J_{4',3'}$ 9.5, $J_{4',5'}$ 9.5, $J_{4',OH-4'}$ 6 Hz, H-4'), 3.28 (bd, 1H, $J_{OH-4',4'}$ 6 Hz, OH-4'), 2.23 – 1.96 (m, 21H, 7 x $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 171.5, 171.1, 170.8, 170.6, 170.1, 169.7, 168.8 (7 x $\underline{C}OCH_3$), 95.9 (C-1'), 91.2 (C-1), 75.3 (C-3), 73.1 (C-5), 72.3 (C-4), 71.6 (C-3'), 71.2 (C-2), 70.9 (C-5'), 70.0 (C-2'), 68.7 (C-4'), 62.6 (C-6'), 62.5 (C-6), 20.9, 20.8, 20.7, 20.6 (7 x $CO\underline{C}H_3$).

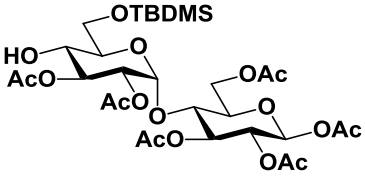
(3.17) 2,3,6-Tri-*O*-acetyl-α-D-galactopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose[86]



2,3,6-Tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (109 mg, 0.17 mmol) in DCM (4 ml) was stirred and cooled to 0 °C. Pyridine (30 μl, 0.24 mmol) and trifluoromethanesulfonic anhydride (40 μl, 0.29 mmol) were added and the mixture stirred for 1 h 20 min. The mixture was diluted with DCM (8 ml), washed with H_2O (4 ml), sat. aq. $NaHCO_3$ (4 ml), 5% aq. $KHSO_4$ (4 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was taken up into DMF (6 ml) and tetrabutylammonium nitrite (1.00 g, 3.4 mmol) added. The mixture was stirred for 18 h 45 min. The mixture was diluted with EtOAc (10 ml), washed with sat. aq. NaCl (2 x 8 ml), H_2O (8 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 1:1 (v/v) EtOAc–Hex) to give a white solid (47 mg, 43%). 10:1 ax-eq. $[\alpha]_D$ +62.0 (*c* 0.7, $CHCl_3$); MS: *m/z* ($[M+Na]^+$) = 659.2; IR (cm^{-1}): 1747 (C=O), 3483 (O-H).

ax - Major diastereoisomer - ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 5.73 (d, 1H, $J_{1,2}$ 8 Hz, H-1), 5.42 (d, 1H, $J_{1',2'}$ 4 Hz, H-1'), 5.28 (t, 1H, $J_{3,2}$ 8 Hz, $J_{3,4}$ 8 Hz, H-3), 5.25 (dd, 1H, $J_{2',1'}$ 4 Hz, $J_{2',3'}$ 10.5 Hz, H-2'), 5.17 (dd, 1H, $J_{3',2'}$ 10.5 Hz, $J_{3',4'}$ 3 Hz, H-3'), 4.98 (t, 1H, $J_{2,1}$ 8 Hz, $J_{2,3}$ 8 Hz, H-2), 4.48 (dd, 1H, $J_{6a,5}$ 2 Hz, $J_{6a,6b}$ 12 Hz, H-6a), 4.33 (dd, 1H, $J_{6a',5'}$ 6.5 Hz, $J_{6a',6b'}$ 11.5 Hz, H-6a'), 4.19 (dd, 1H, $J_{6b,5}$ 5 Hz, $J_{6b,6a}$ 12 Hz, H-6b), 4.16-4.10 (m, 2H, H-4', H-6b'), 4.04-3.99 (m, 2H, H-4, H-5'), 3.83-3.81 (m, 1H, H-5), 2.75 (d, 1H, $J_{OH-4',4'}$ 3.5 Hz, OH-4'), 2.17-1.99 (m, 21H, 7 x $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.9, 170.8, 170.5, 170.1, 169.9, 169.6, 168.8 (7 x $CO\underline{C}H_3$), 96.3 (C-1), 91.3 (C-1'), 75.3 (C-3), 73.2 (C-5), 72.1 (C-4), 71.0 (C-2), 69.1 (C-3'), 68.7 (C-5'), 67.4 (C-4'), 67.2 (C-2'), 62.7 (C-6), 62.3 (62.3), 21.0, 20.9, 20.8, 20.7, 20.7, 20.5 (7 x $CO\underline{C}H_3$).

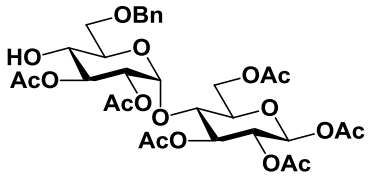
(3.30) 2,3-Di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose



2,3-Di-*O*-acetyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (435 mg, 0.84 mmol), *tert*-butyldimethylsilyl chloride (515 mg, 4.2 mmol) and 4-dimethylaminopyridine (35 mg, 0.29 mmol) were stirred in pyridine (8 ml) at r.t. for 17 h

45 min. The solvent was removed under reduced pressure and the residue taken up into DCM (20 ml), washed with H_2O (3 x 10 ml) and the aqueous washes were extracted with DCM (10 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 3:2 (v/v) EtOAc–Hex) to give a white solid (270 mg, 45%). $[\alpha]_D$ +32.1 (*C* 0.7, $CHCl_3$); MS: *m/z* ($[M+Na]^+$) = 659.2; IR (cm^{-1}): 1751 (C=O), 3475 (O-H). ^1H NMR (500 MHz, $CDCl_3$); δ_H = 5.64 (d, 1H, $J_{1,2}$ 8.5 Hz, H-1), 5.22 (d, 1H, $J_{1',2'}$ 3.5 Hz, H-1'), 5.20 (t, 1H, $J_{3,2}$ 9 Hz, $J_{3,4}$ 9 Hz, H-3), 5.14 (t, 1H, $J_{3',2'}$ 10 Hz, $J_{3',4'}$ 10 Hz, H-3'), 4.87 (t, 1H, $J_{2,1}$ 8.5 Hz, $J_{2,3}$ 8.5 Hz, H-2), 4.67 (dd, 1H, $J_{2',1'}$ 3.5 Hz, $J_{2',3'}$ 10.5 Hz, H-2'), 4.37 (dd, 1H, $J_{6a,5}$ 2 Hz, $J_{6a,6b}$ 12.5 Hz, H-6a), 4.11 (dd, 1H, $J_{6b,5}$ 4.5 Hz, $J_{6b,6a}$ 12.5 Hz), 3.92 (t, 1H, $J_{4,3}$ 9 Hz, $J_{4,5}$ 9 Hz, H-4), 3.83 (dd, 1H, $J_{6a',5}$ 4.5 Hz, $J_{6a',6b'}$ 10.5 Hz, H-6a'), 3.73 (ddd, 1H, $J_{5,4}$ 9Hz, $J_{5,6a}$ 2 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 3.65 (dd, 1H, $J_{6b',5'}$ 4 Hz, $J_{6b',6a'}$ 10.5 Hz, H-6b'), 3.62 (t, 1H, $J_{4',3'}$ 10 Hz, $J_{4',5'}$ 10 Hz, H-4'), 3.56-3.54 (m, 1H, H-5'), 3.12 (s, 1H, OH-4'), 2.08-2.19 (m, 18H, 6 x $COCH_3$), 0.79 (s, 9H, $C(CH_3)_3$), 0.00 (s, 6H, 2 x $Si(CH_3)$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 171.0, 170.8, 170.4, 170.1, 169.7, 168.8 (6 x $\underline{C}OCH_3$), 95.9 (C-1'), 91.3 (C-1), 75.3 (C-3), 73.2 (C-5), 72.2 (C-4), 72.1 (C-5'), 71.9 (C-3'), 71.0 (C-2), 70.2 (C-2'), 70.0 (C-5'), 63.0 (C-6), 62.6 (C-6'), 20.9, 20.7, 20.7, 20.6, 20.5 (6 x $CO\underline{C}H_3$).

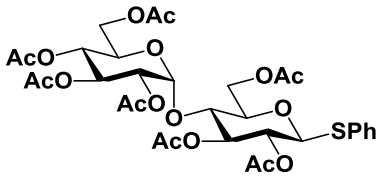
(3.31) 2,3-Di-*O*-acetyl-6-*O*-benzyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose



2,3-Di-*O*-acetyl-(*R*)-4,6-*O*-benzylidene-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (270 mg, 0.40 mmol) and sodium cyanoborohydride (216 mg, 3.48 mmol) were stirred in THF (2.7 ml) at r.t. in the presence of methyl orange. HCl in ether was added in portions until TLC showed the reaction had gone to completion. The solvent was removed under reduced pressure, taken into DCM (5 ml) and washed with sat. aq. $NaHCO_3$ (10 ml). The aqueous layer was extracted with DCM (3 x 5 ml) before the organic extracts were combined and washed with sat. aq. NaCl (10 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 1:1 (v/v) EtOAc–Hex) to give a white solid (137 mg, 50%). $[\alpha]_D$ +38.5 (*C* 1.1, $CHCl_3$); MS: *m/z* ($[M+Na]^+$) = 707.2; IR (cm^{-1}):

1747 (C=O). ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 7.37-7.28 (m, 5H, Ar), 5.73 (d, 1H, $J_{1,2}$ 8 Hz, H-1), 5.36 (d, 1H, $J_{1',2'}$, H-1'), 5.28 (t, 1H, $J_{3,2}$ 8 Hz, $J_{3,4}$ 8 Hz, H-3), 5.22 (t, 1H, $J_{3',2'}$ 8 Hz, $J_{3',4'}$ 3 Hz, H-3'), 4.96 (t, 1H, $J_{2,1}$ 8 Hz, $J_{2,3}$ 8 Hz, H-2), 4.80 (dd, 1H, $J_{2',1'}$ 3 Hz, $J_{2',3'}$ 8 Hz, H-2'), 4.58 (s, 2H, Bn-H), 4.46 (d, 1H, $J_{6a,6b}$ 12 Hz, H-6a), 4.18 (dd, 1H, $J_{6b,6a}$ 12.5 Hz, $J_{6b,5}$ 3.5 Hz, H-6b), 4.03 (t, 1H, $J_{4,3}$ 8 Hz, $J_{4,5}$ 8 Hz, H-4), 3.80-3.70 (m, 1H, H-5, H-4', H-6a', H-5'), 3.62 (d, 1H, $J_{6b',6a'}$ 10 Hz, H-6b'), 3.19 (s, 1H, OH-4'), 2.21-1.93 (m, 18H, 6 x $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 171.1, 170.7, 170.6, 170.1, 169.6, 168.8 (6 x $\underline{C}OCH_3$), 137.6 (Ar-*i*C), 128.5 (2 x Ar-*m*C), 127.8 (Ar-*p*C), 127.7 (2 x Ar-*o*C), 95.8 (C-1'), 91.3 (C-1), 75.4 (C-3), 73.8 (Bz-C), 73.2 (C-5), 72.0 (C-3'), 71.8 (C-4), 71.0 (C-2, C-4'), 70.2 (C-2'), 68.7 (C-6'), 62.6 (C-6), 58.3 (C-5'), 20.8, 20.8, 20.7, 20.6, 20.5 (6 x $CO\underline{C}H_3$).

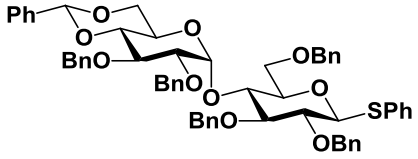
(3.32) Phenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-1-thio-1-deoxy-β-D-glucopyranoside



2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-α-D-glucopyranose **10** (19 g, 27.17 mmol) in DCM (100 ml) had 33% hydrogen bromide in acetic acid (35 ml, 118 mmol) added over 30 min. The solution was stirred at r.t. for 5 h and poured into a brine/ice slurry (100 ml). The DCM layer was extracted, washed with sat. aq. $NaHCO_3$ (100 ml), sat. aq. NaCl (100 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The solid was taken into ethyl acetate (237.5 ml) with thiophenol (8.37 ml, 81.5 mmol), tetrabutylammonium sulphate (10.15 g, 29.9 mmol) and 1 M sodium carbonate solution (380 ml). The solution was stirred at r.t for 30 min, the organic layer was extracted and washed with 1 M NaOH (100 ml), H_2O (100 ml x 3), sat. aq. NaCl (100 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The solid was purified by flash column chromatography (silica, 95:5→9:1 DCM-MeOH) to give a white solid (10.9 g, 55%). [lit.[147] $[\alpha]_D$ -19.6 (*C* 1.0, $CHCl_3$)]**;** $[\alpha]_D$ -20.5 (*C* 1.0, $CHCl_3$); HRMS: Found ($[M+Na]^+$) 751.1900, $C_{32}H_{40}O_{17}SNa$ requires 751.1878; IR (cm^{-1}): 1740 (C=O); ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 7.47-7.45 (m, 2H, ArH), 7.33-7.29 (m, 3H, ArH), 5.37 (d, $J_{1',2'}$ 4.0 Hz, 1H, H-1'), 5.32 (t, $J_{3',2'}$ 9.0 Hz, $J_{3',4'}$ 9.0 Hz, 1H, H-3'), 5.28 (t,

$J_{3,2}$ 9.0 Hz, $J_{3,4}$ 9.0 Hz, 1H, H-3), 5.02 (t, $J_{4',3'}$ 9.0 Hz, $J_{4',5'}$ 9.0 Hz, 1H, H-4'), 4.83 (dd, $J_{2',1'}$ 4.0 Hz, $J_{2',3'}$ 9.0 Hz, 1H, H-2'), 4.78 (t, $J_{2,1}$ 9.0 Hz, $J_{2,3}$ 9.0 Hz, 1H, H-2), 4.74 (d, $J_{1,2}$ 9.0 Hz, 1H, H-1), 4.53 (dd, $J_{6a,5}$ 2.6 Hz, $J_{6a,6b}$ 12.0 Hz, 1H, H-6a), 4.24-4.19 (m, 2H, H-6a', H-6b), 4.04 (dd, $J_{6b',5'}$ 2.3 Hz, $J_{6b',6a'}$ 12.4 Hz, 1H, H-6b'), 3.96-3.92 (m, 1H, H-5'), 3.92 (t, $J_{4,3}$ 9.0 Hz, $J_{4,5}$ 9.0 Hz, 1H, H-4), 3.71 (ddd, J 9.0, 4.9, 2.6 Hz, 1H, H-5), 2.20-1.97 (m, 21H, 7 x COC$\underline{H}_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 170.5, 170.4, 170.3, 170.1, 169.8, 169.5, 169.4 (7 x $\underline{C}OCH_3$), 133.4 (Ar-*m*C), 131.2 (Ar-*i*C), 128.9, 128.4 (Ar-*oC,* Ar-*p*C), 95.6 (C-1'), 84.9 (C-1), 76.4 (C-3), 76.1 (C-5), 72.6 (C-4), 70.7 (C-2), 70.0 (C-2'), 69.3 (C-3'), 68.5 (C-5'), 68.1 (C-4'), 62.8 (C-6), 61.5 (C-6'). 21.0, 20.9, 20.8, 20.7, 20.6, 20.6, 20.6, 20.5 (7 x CO$\underline{C}H_3$).

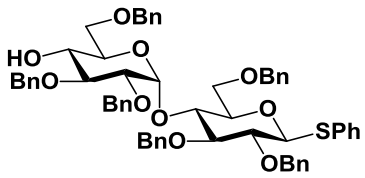
(3.35) Phenyl 2,3-di-*O*-benzyl-(*R*)-4,6-*O*-benzylidene-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-1-thio-1-deoxy-β-D-glucopyranoside



Phenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-1-thio-1-deoxy-β-D-glucopyranoside **12** (11 g, 15.09 mmol) and sodium methoxide (1.1 g) were stirred at r.t. for 20 h. The solution was neutralised with Amberlite IRC-120 H^+ resin, filtered and the solvent removed under reduced pressure. The resulting syrup, α,α-dimethoxy toluene (5.66 ml, 37.7 mmol) and *p*-toluenesulfonic acid (0.33 g, 3.00 mmol) were dissolved in DMF (80 ml) and rotated on a rotary evaporator at 48°C and at 30 Torr for 6.5 h. The solution was neutralised with triethylamine and the solvent removed under reduced pressure. The concentrate was taken up into DMF (70 ml) and TFA (1 ml) was stirred for 6 h 10 min. The solution was neutralised with triethylamine and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 95:5→9:1 DCM-MeOH) to afford a white solid. The solid in DMF (20 ml) was added to sodium hydride (60% in mineral oil) (1.73 g, 43.2 mmol) in DMF (23 ml). Benzyl bromide (5.14 ml, 43.2 mmol) was added dropwise to the solution. Excess sodium hydride was quenched with methanol and diluted with Et_2O (100 ml), washed with sat. aq. NaCl (100 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The solidf was purified by flash column chromatography (silica, 4:1→3:1 Hex-EtOAc) to afford a colourless oil (2.15 g, 13%). [lit.[148] [α]d +6.4 (*c* 1.0,

$CHCl_3$)]**;** [α]d +3.7 (*c* 1.0, $CHCl_3$); HRMS: Found ([M+Na]$^+$) 993.4195, $C_{61}H_{62}NaO_{11}$ requires 993.4184; IR (cm^{-1}): 1739 (C=O); ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 7.66-7.64 (m, 2H, ArH), 7.56-7.54 (m, 2H, ArH), 7.45-7.29 (m, 26H, ArH), 7.22-7.19 (m, 7H, ArH), 5.73 (d, $J_{1',2'}$ 4.0 Hz, 1H, H-1'), 5.58 (s, 1H, Benzylidene acetal-H), 4.96 (d, *J* 10.5 Hz, 1H, Bn-H), 4.94 (d, *J* 10.5 Hz, 1H, Bn-H), 4.87 (d, *J* 10.5 Hz, 1H, Bn-H), 4.89 (d, *J* 10.5 Hz, 1H, Bn-H), 4.78 (d, *J* 10.5 Hz, 1H, Bn-H), 4.75-4.72 (m, 3H, H-1, 2 x Bn-H), 4.65 (d, *J* 10.5 Hz, 1H, Bn-H), 4.61 (d, *J* 10.5 Hz, 1H, Bn-H), 4.58 (d, *J* 10.5 Hz, 1H, Bn-H), 4.23-4.19 (m, 2H, H-4, H-6a), 4.05 (t, $J_{3',2'}$ 9.0 Hz, $J_{3',4'}$ 9.0 Hz, 1H, H-3'), 3.96-3.85 (m, 4H, H-3, H-6a', H-6b, H-6b'), 3.70-3.60 (m, 4H, H-2, H-4', H-5, H-5'), 3.55 (dd, $J_{3',2'}$ 9.0 Hz, $J_{3',4'}$ 9.0 Hz, 1H, H-2'). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 138.7, 138.6, 138.4, 138.3, 137.9,137.8, 137.6, 134.5, 133.5, 132.2, 132.1, 129.8, 129.0, 128.4, 128.4, 128.4, 128.3, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.7, 127.6, 127.6, 127.5, 126.2, 126.1 (42 x Ar-C), 101.2 (C-1'), 97.6 (Benzylidene acetal-H), 87.2 (C-1), 86.9 (C-3), 82.4 (C-5'), 81.0 (C-2), 78.8 (C-5'), 78.8 (C-2), 78.5 (C-5), 75.3 (Bn-C), 74.2 (Bn-C), 74.0 (Bn-C), 73.5 (Bn-C), 72.2 (Bn-C), 71.9 (C-4), 69.0 (C-4', C-6), 63.4 (C-6').

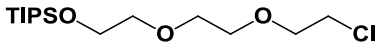
(3.36) Phenyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-1-thio-1-deoxy-β-D-glucopyranoside



Phenyl 2,3-di-*O*-benzyl-(*R*)-4,6-*O*-benzylidene-α-D-glucopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-1-thio-1-deoxy-β-D-glucopyranoside (2.05 g, 2.10 mmol), trifluoroacetic anhydride (0.89 ml, 6.31 mmol), triethylsilane (1.67 ml, 10.51 mmol) in DCM (20 ml) were stirred at 0 °C. Trifluoroacetic acid (0.80 ml, 10.51 mmol) was added dropwise to the solution over 15 min. The solution was diluted with EtOAc (100 ml), washed with sat. aq. $NaHCO_3$ (100 ml), sat. aq. NaCl (100 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The syrup was purified by flash column chromatography (silica, 4:1→2:1 Hex-EtOAc) to afford a colourless oil (1.11 g, 54%). [lit.[149] [α]d -11.0 (*c* 1.0, $CHCl_3$)]**;** [α]d -9.80 (*c* 1.0, $CHCl_3$); IR (cm^{-1}): 1740 (C=O); HRMS: Found ([M+Na]$^+$) 997.3956, $C_{32}H_{40}O_{17}SNa$ requires 997.3966; ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 7.65-7.63 (m, 2H, Ar-H), 7.38-7.27 (m, 26H, Ar-H), 7.24-7.18 (m, 7H, Ar-

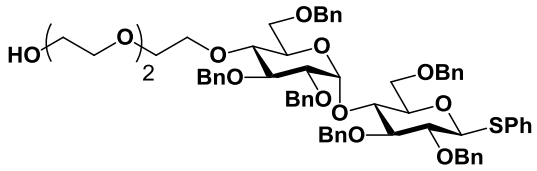
H), 5.68 (d, *J* 3.6 Hz, 1H, H-1'), 4.97 (d, *J* 12.0 Hz, 1H, Bn-H), 4.93-4.88 (m, 3H, Bn-H), 4.76 (d, *J* 12.0 Hz, 1H, Bn-H), 4.74 (d, $J_{1,2}$ = 9.0 Hz, 1H, H-1), 4.64-4.60 (m, 4H, Bn-H), 4.55 (d, *J* 11.8 Hz, 1H, Bn-H), 4.52 (d, *J* 11.8 Hz, 1H, Bn-H), 4.44 (d, *J* 12.1 Hz, 1H, Bn-H), 4.15 (t, $J_{4,3}$ = 9.2 Hz, $J_{4,5}$ = 9.2 Hz, 1H, H-4), 3.94 (dd, $J_{6a,5}$ = 4.2 Hz, $J_{6a,6b}$ = 11.1 Hz, 1H, H-6a), 3.90-3.78 (m, 4H, H-3, H-3', H-5', H-6b), 3.73-3.59 (m, 4H, H-2, H-4', H-5, H-6a'), 3.54 (dd, $J_{6b,5}$ = 3.8 Hz, $J_{6b,6a}$ = 10.2 Hz, 1H, H-6b'), 3.50 (dd, $J_{2',1'}$ = 3.6 Hz, $J_{2',3'}$ = 9.7 Hz, 1H, H-2'). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 138.7, 138.6, 138.4, 137.9, 137.8, 137.8, 133.6, 132.0, 128.9, 128.4, 128.3, 128.3, 128.2, 127.8, 127.7, 127.6, 127.6, 127.4, 127.4, 127.2, 126.4 (Ar-C), 96.9 (C-1'), 87.2 (C-1), 86.7 (C-3), 81.2 (C-3'), 80.9 (C-2), 78.9 (C-5), 78.7 (C-2'), 75.2 (Bn-C), 75.2 (Bn-C), 74.2 (Bn-C), 73.5 (Bn-C), 73.2 (Bn-C), 73.2 (Bn-C), 72.6 (C-4), 71.4 (C-4'), 70.7 (C-5'), 69.7 (C-6'), 69.1 (C-6).

(3.38) 2-(2-(2-chloroethoxy)ethoxy)ethoxytriisopropylsilyl ether



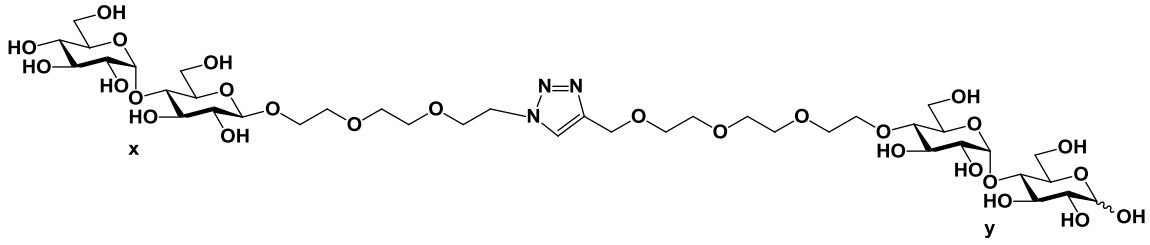
2-(2-(2-chloroethoxy)ethoxy)ethanol **17** (0.86 ml, 5.9 mmol), imidazole (0.80 g, 11.8 mmol) and triisopropylsilyl chloride (2.53 ml, 11.8 mmol) in DCM (6 ml) were stirred at r.t. for 21 h. The solvent was removed under reduced pressure and the remaining oil taken into Et_2O (30 ml), washed with sat. aq. $NaHCO_3$ (30 ml) and extracted with Et_2O (30 ml). The combined organic fractions were washed with sat. aq. NaCl (30 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The oil was purified by flash column chromatography (silica, 4:1→3:1 Hex-EtOAc) to afford a colourless oil (2.01 g, 98%). IR (cm^{-1}): 2941, 2891, 2865 (C-H); HRMS: Found ($[M+Na]^+$) 347.1791, $C_{32}H_{40}O_{17}SNa$ requires 347.1780; ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 3.83 (t, *J* 5.5 Hz, 2H, $ClC\underline{H}_2$), 3.74 (t, *J* 5.5 Hz, 2H, $SiOC\underline{H}_2$), 3.69-3.64 (m, 4H, $CH_2OC\underline{H}_2C\underline{H}_2OCH_2$), 3.61 (t, *J* 5.5 Hz, 1H, $SiOCH_2C\underline{H}_2$), 3.58 (t, *J* 5.5 Hz, 1H, $ClCH_2C\underline{H}_2$), 1.09-1.03 (m, 21H, $(C\underline{H}_3)_2C\underline{H}Si$). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 72.9 ($ClCH_2\underline{C}H_2$), 71.5 ($SiO\underline{C}H_2$), 70.9, 70.8 ($CH_2O\underline{C}H_2\underline{C}H_2OCH_2$), 63.1 ($ClC\underline{H}_2$), 42.7 ($SiOCH_2\underline{C}H_2$), 18.2, 18.1, 18.0, 17.9, 17.8, 12.4, 12.3, 12.1, 12.0 ($(\underline{C}H_3)_2\underline{C}HSi$).

(3.39) Phenyl 2,3,6-tri-*O*-benzyl-4-2-(2-(2-(chloroethoxy)ethoxy)ethoxytriisopropylsilyl ether)-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-1-thio-1-deoxy-β-D-glucopyranoside



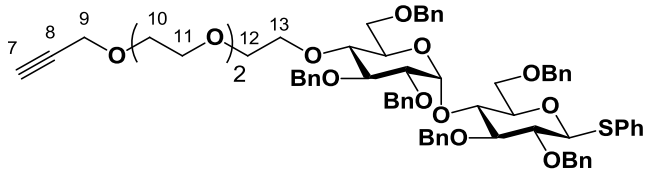
Phenyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-1-thio-1-deoxy-β-D-glucopyranoside **16** (50 mg, 0.05 mmol) in DMF (2.5 ml) had sodium hydride (60% in mineral oil) in DMF (2.5 ml) added at 0 °C. Tetrabutylammonium iodide (3.7 mg, 0.01 mmol) and 2-(2-(2-chloroethoxy)ethoxy)ethoxytriisopropylsilyl ether (65 mg, 0.20 mmol) were added dropwise to the stirred solution. An additional aliquot of sodium hydride (60% in mineral oil) (25 mg) and tetrabutylammonium iodide (4 mg) were added to the solution. The reaction was then quenched with MeOH (4 ml) and the solvent removed under reduced pressure. The resulting oil was purified by flash column chromatography (silica, 5:1→3:1 Hex-EtOAc) to afford a colourless oil. The oil and tetrabutylammonium fluoride (22 µl, 0.05 mmol) were taken into THF (1.5 ml) and stirred at r.t. for 18 h. The oil was purified by flash column chromatography (silica, 5:1→3:1 Hex-EtOAc) to afford a colourless oil (20 mg, 20%). IR (cm^{-1}): 3468 (O-H); HRMS: Found ($[M+Na]^+$) 1129.4751, $C_{32}H_{40}O_{17}SNa$ requires 1129.4742; ^1H NMR (500 MHz, $CDCl_3$); δ_H = 7.60-7.57 (m, 2H, Ar-H), 7.35-7.22 (m, 26H, Ar-H), 7.18-7.10 (m, 7H, Ar-H), 5.58 (d, *J* 3.7 Hz, 1H, H-1'), 4.93-4.78 (m, 5H, Bn-H), 4.69 (d, *J* 9.8 Hz, 1H, H-1'), 4.63-5.53 (m, 5H, Bn-H), 4.46 (d, *J* 12.0 Hz, 1H, Bn-H), 4.37 (d, *J* 12.0 Hz, 1H, Bn-H), 4.08 (t, *J* 9.0 Hz, 1H, H-4), 3.95-3.71 (m, 6H, H-3, H-3', H-5', H-6a, 2 x PEG-H), 3.70-3.40 (m, 17 H, H-2, H-2', H-4', H-5, H-6a', H-6b, H-6b', 10 x PEG-H).

(4.3) Psuedotetrasaccharide



Copper sulfate (0.92 mg, 6 μmol) in H_2O (2 ml) followed by sodium D-ascorbate in H_2O (2 ml) were added to SUGAR X (35 mg, 30 μmol) and SUGAR X (20 mg, 17 μmol) in DCM (4 ml) and ^{t}BuOH (4 ml) and stirred for 24 h. DMF (2 ml) was added and the solution stirred for another 24 h before Copper sulfate (0.92 mg, 6 μmol) in H_2O (2 ml) followed by sodium D-ascorbate in H_2O (2 ml) were added again. The solution was stirred for a final 4 days before being diluted with DCM (15 ml) and washed with H_2O (15 ml), brine (15 ml) and dried ($MgSO_4$) before being concentrated under reduced pressure. The resulting oil was taken into acetone (0.7 ml) and H_2O (30 μl) before NBS (4.7 mg, 18 μmol) was added. The solution was quenched with EtOAc (10 ml) after 20 min and the solvent removed under reduced pressure. The resulting oil was taken into methanol (10 ml) with palladium on carbon (10 wt. %, 30 mg) was added and the solution put under an atmosphere of hydrogen before stirring for 48 h. The solution was then filtered and concentrated under reduced pressure before purification by size exclusion chromatography (LH-20) to give the product as a clear oil (5 mg, 27%). HRMS: Found ($[M+H]^+$) 1012.4203, $C_{39}H_{70}N_3O_{27}^+$ requires 1012.4192; ^{1}H NMR (500 MHz, D_2O) δ 8.12 (s, 1H, triazole-H), 5.41 (d, $J_{1,2}$ = 4.9 Hz, 1H, H-1y'), 5.40 (d, $J_{1,2}$ = 4.9 Hz, 1H, H-1x'), 5.24 (d, *J* 3.8 Hz, 1H, H-1yα), 4.66 (m, 1H, H-1yβ), 4.49 (d, $J_{1,2}$ = 7.9 Hz, 1H, H-1x), 3.98 – 3.46 (m, 17H), 3.36 (t, *J* 9.5 Hz, 1H), 3.26 (dd, *J* 9.6, 8.2 Hz, 2H), 3.21 (dd, *J* 9.5, 8.0 Hz, 1H).

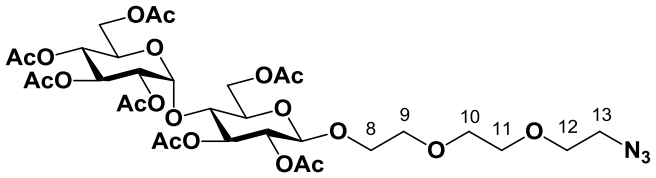
(4.4) Phenyl 2,3,6-tri-*O*-benzyl-4-2-(2-(2-(ethoxy)ethoxy)ethoxypropargyl ether)-α-D-glucopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-1-thio-1-deoxy-β-D-glucopyranoside



Sodium hydride in oil (30 mg, 76.5 mmol) was added to Phenyl 2,3,6-tri-*O*-benzyl-4-2-(2-(2-(chloroethoxy)ethoxy)ethoxytriisopropylsilyl ether)-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-1-thio-1-deoxy-β-D-glucopyranoside (500 mg, 0.45 mmol) in DMF (5 ml) at 0 °C. Propargyl bromide (334 mg, 2.25 mmol) was then added dropwise and the solution allowed to stir for 1 h. The reaction was then quenched with methanol and the solution concentrated under reduced pressure. The oil was taken into EtOAc (200 ml), washed with H_2O (200 ml), sat. aq. NaCl (200 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The oil was purified by flash column chromatography (silica,

3:1→2:1 Hex-EtOAc) to afford a colourless oil (141 mg, 27%); HRMS: Found ($[M+NH_4]^+$) 1162.5363, $C_{69}H_{80}NO_{13}S^+$ requires 1162.5345; ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 7.60-7.57 (m, 2H, Ar-H), 7.35-7.22 (m, 26H, Ar-H), 7.18-7.10 (m, 7H, Ar-H), 5.54 (d, *J* 3.7 Hz, 1H, H-1'), 4.93-4.78 (m, 5H, Bn-H), 4.66 (d, *J* 9.8 Hz, 1H, H-1'), 4.63-5.53 (m, 5H, Bn-H), 4.46 (d, *J* 12.0 Hz, 1H, Bn-H), 4.37 (d, *J* 12.0 Hz, 1H, Bn-H), 4.11 (d, *J* 2.4 Hz, 2H, H-9), 4.08 (t, *J* 9.0 Hz, 1H, H-4), 3.95-3.71 (m, 6H, H-3, H-3', H-5', H-6a, 2 x PEG-H), 3.70-3.40 (m, 17 H, H-2, H-2', H-4', H-5, H-6a', H-6b, H-6b', 10 x PEG-H), 2.37 (t, *J* 2.4 Hz, 1H, H-7); ^{13}C NMR (126 MHz, $CDCl_3$) δ_H = 138.7, 138.6, 138.4, 138.0, 137.9, 137.7 (6 x Ar-C), 133.6 (Ph-C), 131.9 (Ph-CH), 128.8, 128.2, 128.2, 128.1, 128.1, 128.1, 127.8, 127.7, 127.7, 127.5, 127.4, 127.4, 127.3, 127.0, 126.4 (30 x Ar-CH, 4 x Ph-CH), 97.2 (C-1'), 87.1 (C-1), 86.5 (C-3), 81.6 (C-3'), 80.8 (C-2), 79.5 (C-7), 79.1 (C-2'), 78.7, 78.2 (C-4'), 75.3, 75.1, 74.4, 74.3, 73.3, 73.3, 73.2 (6 x Bn-CH_2, C-4), 72.0 (C-5), 71.0, 70.6, 70.5, 70.3, 70.3, 69.1 (6 x PEG-CH_2), 69.0 (C-6), 58.2 (C-9).

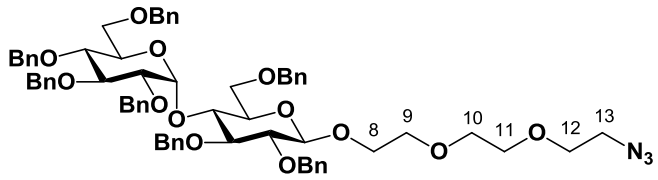
(4.6) 2-(2-(2-azidoethoxy)ethoxy)ethoxy 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside



2-(2-(2-chloroethoxy)ethoxy)ethoxy 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (1 g, 2.03 mmol), sodium azide (1.32 g, 20.3 mmol) and tetrabutylammonium iodide (74 mg, 0.20 mmol) were stirred in DMF (30 ml) at 80 °C for 26 h. The solution was then diluted with Et_2O (200 ml), washed with H_2O (100 ml x 3), dried ($MgSO_4$) and concentrated under reduced pressure. The resulting solid was purified by flash column chromatography (silica, 2:1 EtOAc–Hex) to afford a colourless oil (0.57 g, 55%). HRMS: Found ($[M+NH_4]^+$) 811.3092, $C_{32}H_{47}N_3NaO_{20}$+ requires 811.3112; ^{1}H NMR (500 MHz, Chloroform-d) δ_H 5.42 (d, $J_{1',2'}$ = 4.0 Hz, 1H, H-1'), 5.37 (dd, $J_{3',2'}$ = 10.6, $J_{3',4'}$ = 9.5 Hz, 1H, H-3'), 5.26 (t, $J_{3,2}$ = 9.1 Hz, $J_{3,4}$ = 9.1 Hz, 1H, H-3), 5.06 (t, $J_{4',3'}$ = 9.9 Hz, $J_{4',5'}$ = 9.9 Hz, 1H, H-4'), 4.87 (dd, $J_{2',1'}$ = 4.0 Hz, $J_{2',3'}$ = 10.6 Hz, 1H, H-2'), 4.82 (dd, $J_{2,1}$ = 7.8 Hz, $J_{2,3}$ = 9.1 Hz, 1H, H-2), 4.64 (d, $J_{1,2}$ = 7.8 Hz, 1H, H-1), 4.49 (dd, $J_{6a,5}$ = 2.8 Hz, $J_{6a,6b}$ = 12.1 Hz, 1H, H-6a), 4.26 (dd, $J_{6a',5'}$ = 4.2 Hz, $J_{6a',6b'}$ = 12.3 Hz, 1H, H-6a'), 4.23 (dd, $J_{6b,5}$ = 4.2 Hz, $J_{6b,6a}$ = 12.1 Hz, 1H, H-6b), 4.05 (dd, $J_{6b',5'}$ = 2.3 Hz, $J_{6b',6a'}$ = 12.3 Hz, 1H, H-6b'), 4.01 (dd, $J_{4,3}$ = 9.1 Hz, $J_{4,5}$ = 8.8 Hz, 1H, H-

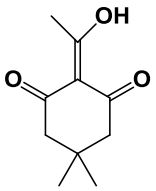
4), 3.98 – 3.90 (m, 2H, H-5', H-8a), 3.79 – 3.62 (m, 10H, H-5, H-8b, H-9, H-10, H-11, H-12), 3.42 (t, $J_{13,12}$ = 5 Hz, 2H, H-13), 2.20 – 1.97 (m, 21H, 7 x COC$\underline{H}_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 170.5, 170.5, 170.2, 169.9, 169.6, 169.4, (7 x $\underline{C}OCH_3$), 100.3 (C-1), 95.54 (C-1'), 75.4 (C-3), 72.8 (C-4), 72.16 (C-2), 72.1, 70.7, 70.4, 70.0, (C-8, C-9, C-10, C-11, C-12), 70.00 (C-2'), 69.3 (C-5), 69.1 (C-3'), 68.5 (C-5'), 68.0 (C-4'), 62.9 (C-6), 61.5 (C-6'), 50.7 (C-12), 20.90, 20.83, 20.67, 20.64, 20.56 (7 x CO$\underline{C}H_3$).

(4.7) 2-(2-(2-azidoethoxy)ethoxy)ethoxy 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside



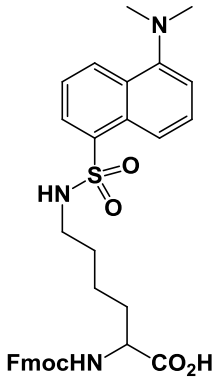
2-(2-(2-azidoethoxy)ethoxy)ethoxy α-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (0.56 g, 1.13 mmol) in DMF (10 ml) had sodium hydride (60% in oil) (0.63 g, 15.82 mmol) added at 0 °C. The solution was stirred for 40 min before the dropwise addition of benzyl bromide (1.88 ml, 15.82 mmol). The solution was stirred for a subsequent 4 h before being quenched with MeOH (10 ml). The solvent was removed under reduced pressure and the resulting oil taken into EtOAc (50 ml) and washed with H_2O (50 ml), brine (50 ml) and dried ($MgSO_4$) before concentration under reduced pressure. The resulting oil was purified by flash column chromatography (silica, 4:1 Hex–EtOAc) to afford a colourless oil (0.33 g, 27%); HRMS: Found ($[M+NH_4]^+$) 1147.5673, $C_{67}H_{79}N_4O_{13}^+$ requires 1147.5639; ^{1}H NMR (500 MHz, Chloroform-d) δ_H 7.37 – 7.05 (m, 35H, Ar-H), 5.68 (d, $J_{1',2'}$ = 3.7 Hz, 1H, H-1'), 4.95 (d, *J* 11.2 Hz, 1H, Bn-H), 4.93 (d, *J* 11.2 Hz, 1H, Bn-H), 4.88 (d, *J* 10.8 Hz, 1H, Bn-H), 4.81 – 4.73 (m, 3H, Bn-H), 4.64 – 4.43 (m, 9H, 8 x Bn-H, H-1), 4.32 (d, *J* 12.2 Hz, 1H, Bn-H), 4.09 – 4.02 (m, 2H, H-3, H-8a), 3.90 (t, $J_{3',2'}$ = 9.4 Hz, $J_{3',4'}$ = 9.4 Hz, 1H, H-3'), 3.84 – 3.69 (m, 7H, H-2, H-6a', H-8b, H-9, H-12), 3.67 – 3.61 (m, 3H, H-4', H-5, H-6a), 3.60 – 3.51 (m, 7H, H-4, H-5', H-6b, H-10, H-11), 3.49 (dd, $J_{2',1'}$ = 3.7 Hz, $J_{2',3'}$ = 9.9 Hz, 1H, H-2'), 3.44 (dd, $J_{6b,5}$ = 2.0 Hz,$J_{6b,6a}$ = 10.7 Hz1H, H-6b'), 3.26 (dd, *J* 5.8, 4.4 Hz, 2H, H-13).

(4.13) 2-(1-Hydroxyethylidene)-5,5-dimethyl-1,3-cyclohexanedione



Dimedone (5 g, 35.7 mmol), acetic acid (2.04 ml, 35.7 mmol), dimethylamino pyridine (4.36 g, 35.7 mmol) and N,N'-Dicyclohexylcarbodiimide (7.35 g, 35.7 mmol) in DMF were stirred at r.t. for 21.5 h. The solution was filtered and the solvent removed under reduced pressure, the residue taken into EtOAc (100 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was again taken into EtOAc (100 ml), washed with sat. aq. NaCl, dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, EtOAc) to afford a colourless solid (1.01 g, 20%). IR (cm^{-1}): 3458 (O-H), 1710 (C=O); HRMS: Found ($[M+Na]^+$) 183.1015, $C_{10}H_{15}O_3$ requires 183.1016; ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 2.59 (s, 3H, C=CC<u>H</u>$_3$), 2.55 (s, 2H, C<u>H</u>$_2$), 2.37 (s, 2H, C<u>H</u>$_2$), 1.09 (s, 6H, C(C<u>H</u>$_3$)); ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 202.2 (C=O), 197.7 (C=O), 194.8 (C=<u>C</u>OH), 112.2 (<u>C</u>=COH), 52.3 (<u>C</u>H$_2$), 46.7 (<u>C</u>H$_2$), 30.5 (<u>C</u>(CH$_3$)$_2$), 28.4 (C=C<u>C</u>H$_3$), 28.0 (C(<u>C</u>H$_3$)$_2$).

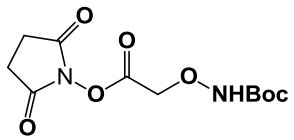
(4.15) Fmoc-lys(dansyl)-OH



Lys(dansyl)-OH (0.5 g, 2.45 mmol) in 10% aq. Na_2CO_3 (6.5 ml) had Fluorenylmethyloxycarbonyl chloride (0.64 g, 2.45 mmol) in dioxane (4.5 ml) added at 0 °C, the solution was allowed to warm to r.t. and stirred for 21 h. The solution was diluted with EtOAc (25 ml), washed with 5% aq. $KHSO_4$ (50 ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 1:0→9:1 DCM-MeOH then 50:50:2 EtOAc-Hex-AcOH) to give

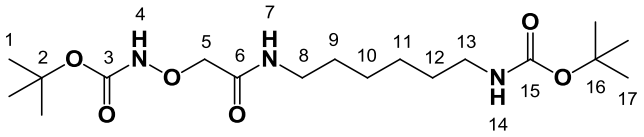
a fluorescent green solid (630 mg, 43%). [lit.[150] [α]d +1.7 (*c* 1.0, CH_2Cl_2)]; [α]d +5.0 (*c* 1.0, $CHCl_3$); IR (cm^{-1}): 3276 (O-H), 1698 (C=O); HRMS: Found ($[M+H]^+$) 602.2333, $C_{33}H_{36}N_3O_6S$ requires 602.2319; ^{1}H NMR (500 MHz, $CDCl_3$); δ_H Dansyl: 8.52 (d, *J* 8.5 Hz, 1H, Ar-H), 8.37 (d, *J* 8.5 Hz, 1H Ar-H), 8.21 (d, *J* 6.8 Hz, 1H Ar-H), 7.58-7.54 (m, 1H, Ar-H), 7.33-7.21 (m, 1H, Ar-H), 7.13 (d, *J* 7.6 Hz, 1H, Ar-H), 2.86 (6H, s, $N(CH3)_2$) Fmoc: 7.76 (d, *J* 7.7 Hz, 2H, Ar-H), 7.60 (d, *J* 7.7 Hz, 2H, Ar-H), 7.38-7.36 (m, 2H, Ar-H), 7.33-7.21 (m, 2H, Ar-H), 4.44 (d, *J* 6.9 Hz, 2H, CH2-CH), 4.21 (t, *J* 6.9 Hz, 1H, CH2-CH) Lys: 5.30 (d, *J* 8.1 Hz, 1H, C(O)NH), 5.05 (t, *J* 6.1 Hz, 1H, SO_2NH), 4.30-4.22 (m, 1H, Hα), 2.92-3.83 (m, 2H, 2 x Hε), 1.74-1.66 (m, 1H, Hβ'), 1.55-1.44 (m, 1H, Hβ''), 1.44-1.33 (m, 2H, 2 x Hδ), 1.33-1.13 (m, 2H, 2 x Hγ). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = Dansyl: 151.3, 129.9, 129.6 (Ar-C), 130.1, 129.6, 126.9, 123.3, 119.0, 115.3 (Ar-CH), 30.9 (NCH_3), Fmoc: 176.5 (NHC=O), 143.7 (Ar-C), 143.6 (Ar-C), 128.2, 127.7, 125.1, 119.8 (Ar-CH), 67.0 (CH_2-CH), 47.0 (CH_2-CH), Lys: 176.1 (COOH), 53.3 (Cα), 42.6 (Cε), 31.5 (Cβ), 28.8 (Cδ), 22.0 (Cγ).

(4.17) *N'*-Boc-aminooxyacetyl *N*-hydroxysuccinimide ester[151]



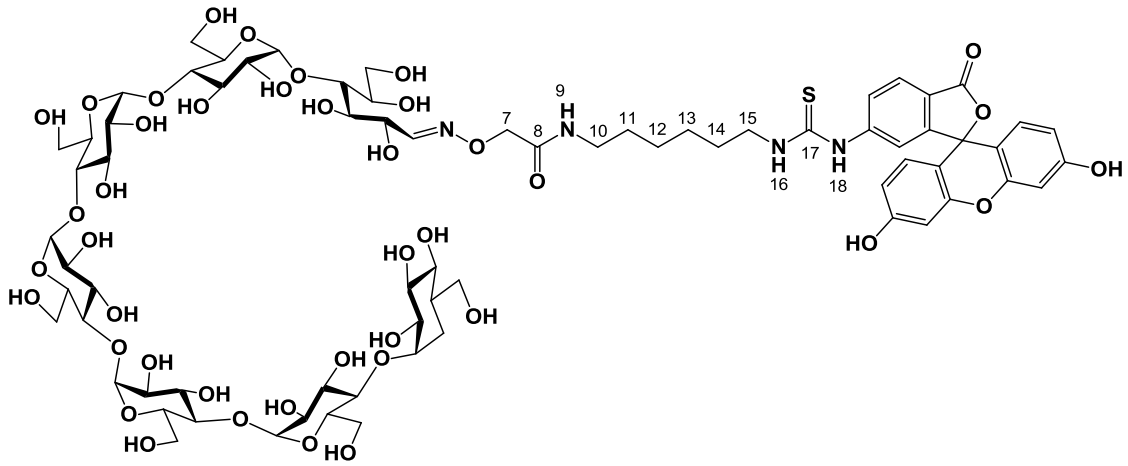
A solution of Boc-aminooxyacetic acid (953 mg, 4.98 mmol), *N*-hydroxysuccinimide (631 mg, 5.48 mmol) and dicyclohexylcarbodiimide (1.13 g, 5.48 mmol) in 1:1 (v/v) ethyl acetate-hexane (20 mL) was stirred for 3 h at r.t. and then filtered through a celite pad. The filtrate was re-dissolved in ethyl acetate (25 mL), washed with 1% aq. $NaHCO_3$ (2 x 25 mL), water (2 x 25 mL) and aq. NaCl (2 x 25 mL), and azeotroped three times with toluene to afford the product (1.178 g, 78%) as a crude colourless foam, which was used in SPPS without any further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.96 (s, 1H, NH), 4.78 (s, 2H, CH_2), 2.88 (s, 4H, CH_2), 1.49 (s, 9H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.88, 165.06, 156.35, 82.63, 70.79, 28.11, 25.57.

(4.29) 1-(*N*-Boc-(carboxymethoxy)amino)-heptane-7-((*N*-Boc-(amine))



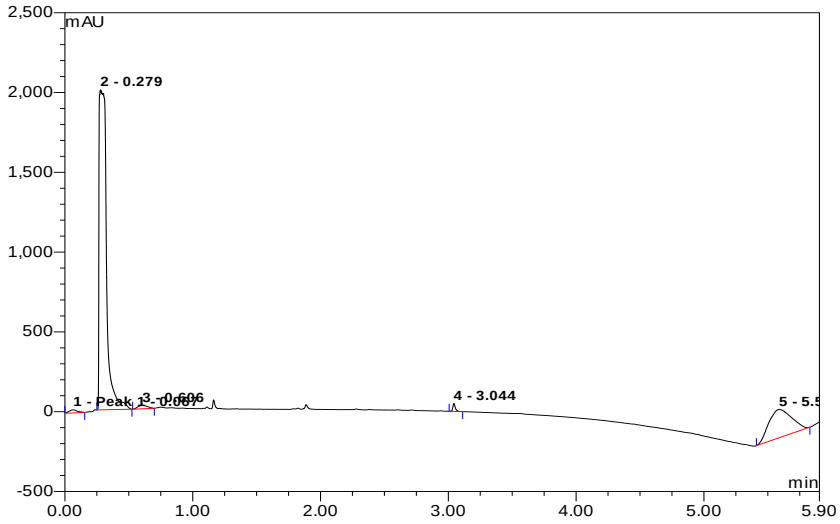
N-Boc-1,6-diaminohexane (183 μl, 0.82 mmol), Boc-aminooxy-acetic acid NHS ester (250 mg, 0.82 mmol) and triethylamine (114 μl, 0.82 mmol) in DMF (1 ml) were stirred at r.t. for 20 h. MS: Found ($[M+Na]^+$) 412.2, $C_{18}H_{35}N_3NaO_6^+$ requires 412.2. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.34 (s, 1H, H-4), 8.09 (s, 1H, H-14), 4.66 (s, 1H, H-7), 4.25 (s, 2H, H-5), 3.24 (dt, *J* 6.7 Hz, *J* 6.7 Hz, 2H, H-13), 3.05 (dt, *J* 6.8 Hz, *J* 6.8 Hz, 2H, H-8), 1.43 – 1.53 (m, 2H, H-12), 1.45 – 1.37 (m, 2H, H-9), 1.43 (s, 9H, H-1), 1.39 (s, 9H, H-17), 1.35 – 1.23 (m, 4H, H-10, H-11). ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 168.8 (C-3), 157.8 (C-15), 156.0 (C-6), 82.6 (C-2), 78.9 (C-16), 75.9 (C-5), 40.3 (C-8), 38.7 (C-13), 29.8 (C-9), 29.0 (C-12), 28.3 (C-1), 28.0 (C-17), 26.3 (C-10), 26.2 (C-11).

(4.31) 6-(1-((carboxymethoxy)amino)-heptane-7-(((amino))) fluorescein maltoheptaose

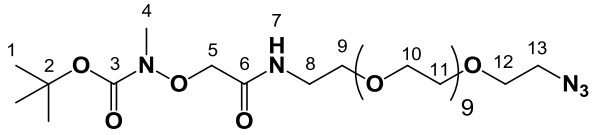


1-(*N*-Boc-(carboxymethoxy)amino)-heptane-7-((*N*-Boc-(amine)) (50 mg, 0.13 mmol) was stirred in TFA (200 μl) for 15 min. The TFA was removed under a stream of N_2 and maltoheptaose (75 mg, 0.065 mmol) in sodium acetate buffer (500 μl, 0.1 M, pH 4.0) was added and allowed to stir for 6 d. The solution was purified by size exclusion chromatography (LH-20) before FITC (11.8 mg, 0.03 mmol) in DMF (1.2 ml) was added in sodium bicarbonate (1.2 ml, pH 8.5, 0.185 M) and the solution stirred for 24 h. To purify the product it was applied directly to a size exclusion column (LH-20) yielding the

product as an orange oil (45 mg, 38%). HRMS: Found ([M+H] $^{+}$) 1713.5601, $C_{71}H_{101}N_4O_{42}S^{+}$ requires 1713.5606.

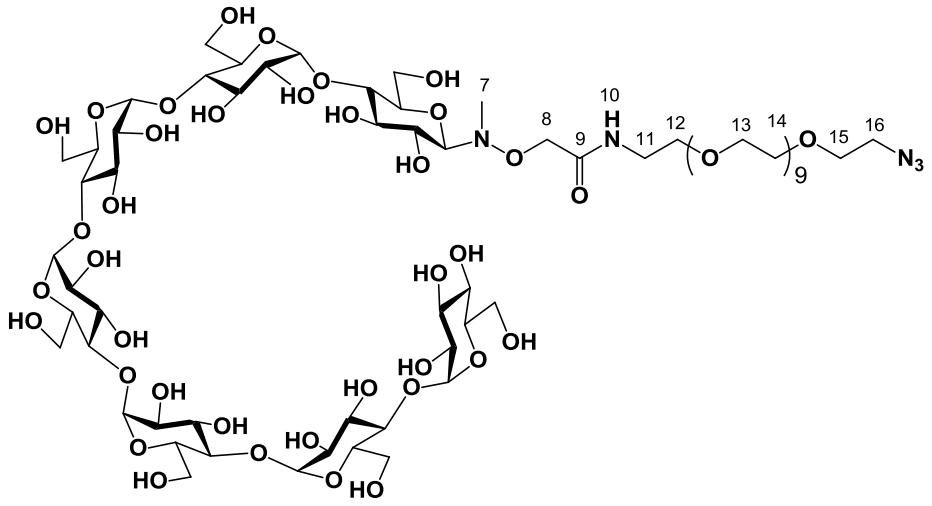


(5.1) *O*-(2-(*t*-Butoxycarbonyl-*N*′-methylaminooxyacetyl)-aminoethyl)-*O*′-(2-azidoethyl)nonaethylene glycol



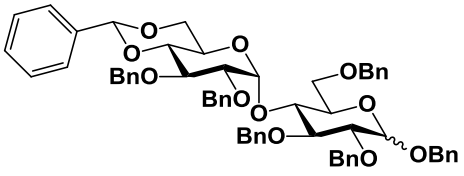
(*t*-Butoxycarbonyl-*N*′-methylaminooxyacetyl)-*N*-hydroxysuccinimide ester (180 mg, 0.61 mmol), *O*-(2-aminoethyl)-O'-(2-azidoethyl)nonaethylene glycol (200 mg, 0.38 mmol) and triethylamine (29 μl, 0.21 mmol) were stirred in DMF (1 ml) for 48 h. The product was concentrated under reduced pressure and taken into EtOAc (20 ml), washed with sat. aq. $NaHCO_3$ (15 ml), sat. aq. NaCl (15 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by flash column chromatography (silica, 4:1 EtOAc–MeOH) to afford a colourless oil (201 mg, 74%). HRMS: Found ([M+NH_4]$^{+}$) 731.4433, $C_{30}H_{63}N_6O_{14}^{+}$ requires 731.4397; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.04 (s, 1H, H-7), 4.32 (s, 2H, H-5), 3.71 – 3.61 (m, 38H, H-9, H-10, H-11), 3.58 (t, *J* 5.6 Hz, 2H,), 3.50 (t, *J* 5.6 Hz, 3H), 3.39 (t, *J* 5.1 Hz, 3H), 3.11 (s, 3H, H-4), 1.50 (s, 9H, H-1). ^{13}C NMR (126 MHz, MeOD) δ_C 170.4 (C-3), 158.4 (C-6), 82.9 (C-2), 73.8 (C-5), 70.7, 70.5, 70.4 (C-10, C-11, C-12, C-13), 51.0 (C-8), 39.2 (C-7), 37.0 (C-4), 27.7 (C-1).

(5.3) *O*-(2-(*N'*-methylaminooxyacetyl)-aminoethyl)-*O'*-(2-azidoethyl)nonaethylene glycol maltoheptaose



O-(2-(*t*-Butoxycarbonyl-*N'*-methylaminooxyacetyl)-aminoethyl)-*O'*-(2-azidoethyl)nonaethylene glycol (10 mg, 0.014 mmol) was stirred in TFA (200 μl) for 30 min before the TFA was removed under a stream of nitrogen. The residue was taken into sodium acetate buffer (100 μl, 0.1 M, pH 4.0) containing maltoheptaose (16.1 mg, 0.014 mmol) and stirred for 7 days. The solution was applied directly to a size exclusion column (LH-20) to yield the product as a colourless oil (16 mg, 15%). HRMS: Found ($[M+NH_4+NH_4]^{2+}$) 891.8955, $C_{67}H_{129}N_7O_{47}^{2+}$ requires 891.8954; ^{1}H NMR (500 MHz, Deuterium Oxide) δ 5.34 (d, *J* 3.8 Hz, 6H, 6 x H-1'), 4.27 (s, 2H, H-8), 3.90 (t, *J* 9.5 Hz, 7H, 7 x H-3'), 3.84 – 3.70 (m, 21H, 6 x H-5', 6 x H-6a', 6 x H-6b', H-5, H-6a, H-6b), 3.70 – 3.47 (m, 56H, 5 x H-4', H-4, 6 x H-2', PEG-C$\underline{H}_2$), 3.37 (t, $J_{4',3'}$ = 9.6 Hz, $J_{4',5'}$ = 9.6 Hz, 1H, H-4'), 3.29 (t, $J_{2,1}$ = 8.0 Hz, $J_{2,3}$ = 8.0 Hz, 1H, H-2β), 2.75 (s, 3H, H-7).

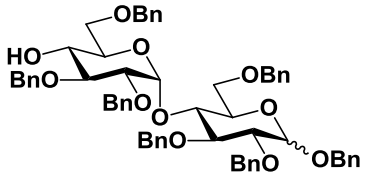
(5.8) Benzyl 2,3-di-*O*-benzyl-(R)-4,6-O-benzylidene-α-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-*O*-α-D-glucopyranose



Maltose (1.05 g, 2.92 mmol), α,α-dimethoxy toluene (1.09 ml, 7.3 mmol) and p-toluenesulfonic acid (0.06 g, 0.58 mmol) were dissolved in DMF (7.2 ml) and rotated on a rotary evaporator at 48°C and at 30 Torr for 6.5 h. The solution was neutralised with

triethylamine and the solvent removed under reduced pressure. The concentrate was taken up into DMF (7 ml) and TFA (0.1 ml) was stirred for 6 h 10 min. The solution was neutralised with triethylamine and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 4:1 (v/v) CHCl3 –MeOH) to afford a white solid. The solid and sodium hydride (60% in mineral oil) (1.17 g) were stirred in DMF (25 ml) at 0 °C. Benzyl bromide (3.47 ml) was added dropwise and the solution allowed to stir at r.t. for 20 h. Excess sodium hydride was quenched with methanol and diluted with Et2O (20 ml), washed with sat. aq. NaCl (20 ml), dried (MgSO4) and the solvent removed under reduced pressure. The solid was purified by flash column chromatography (silica, 4:1→3:1 Hex-EtOAc) to afford a colourless oil (626 mg, 21%). HRMS: Found ($[M+NH_4]^+$) 988.4654, $C_{61}H_{66}NO_{11}^+$ requires 988.4631; IR (cm-1): 1740 (C=O); 1H NMR (500 MHz, $CDCl_3$); δH = 7.81 (m, 7H Ar-H), 7.69-7.38 (m, 130H, Ar-H), 6.09 (d, J 3.8 Hz, 1H, Hβ-1'), 6.03(d, J 3.9 Hz, 1H, Hα-1'), 5.78 (s, 1H, benzylidene acetal), 5.38 (d, J 11.8 Hz, 1H, Bn-H), 5.32-4.70 (m, 15H, Bn-H), 4.49-4.44 (m, 2H), 4.36-4.01 (m, 5H), 3.92-3.87 (m, 4H), 3.79 (dd, J 9.4 Hz, J 4.2 Hz, 1H, H-2); 13C NMR (75 MHz, $CDCl_3$): δC = 139.3, 139.0, 139.0, 139.0, 138.5, 138.5, 138.5, 138.2, 138.2, 138.0, 137.9, 137.7, 137.4, 129.3, 129.2, 129.1, 128.7, 128.7, 128.6, 128.6, 128.5, 128.5, 128.5, 128.4, 128.4, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.8, 127.8, 127.7, 127.5, 127.4, 127.4, 126.9, 126.8, 126.3, 125.6 (42 x Ar-C), 102.6 (C-1α), 101.3 (benzylidene acetal-C), 97.5 (C-1'), 95.3 (C-1β), 85.2, 82.4, 82.5, 82.5, 82.6, 80.6, 79.1, 79.0, 79.0, 78.9, 77.7, 77.6, 77.4, 77.2, 75.4, 75.4, 74.9, 74.6, 74.4, 74.1, 73.9, 73.9, 73.7, 73.6, 73.4, 73.1, 73.1, 72.3, 72.0, 71.2, 70.0, 69.4, 69.2, 69.1, 68.9, 63.5, 29.9, 21.7.

(5.9) Benzyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-*O*-α-D-glucopyranose



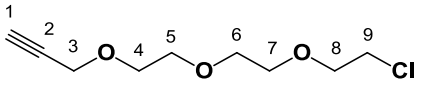
TFA (2 ml, 23.38 mmol) was added dropwise to Benzyl 2,3-di-*O*-benzyl-(R)-4,6-O-benzylidene-α-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-*O*-α-D-glucopyranose (4.70 g, 6.67 mmol), triethylsilane (4.20 ml, 23.38 mmol) and TFAA (2.23 ml, 14.03 mmol) in DCM (50 ml). The solution was stirred for 20 h before dilution into EtOAc (200 ml) and

washing with sat. aq. $NaHCO_3$ (200 ml), sat. aq. NaCl (200 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by flash column chromatography (silica, 3:1 Hex-EtOAc) to afford a colourless oil (2.8 g, 59%). HRMS: Found ($[M+NH_4]^+$) 990.4820, $C_{61}H_{68}NO_{11}^+$ requires 990.4787;

Major isomer – ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 – 7.13 (m, 35H, Ar-H), 5.77 (d, $J_{1,2}$ = 3.6 Hz, 1H, H-1), 5.73 (d, *J* 3.6 Hz, 1H, H-1'), 5.13 (d, *J* 11.7 Hz, 1H, Bn-H), 5.05 – 4.85 (m, 5H, Bn-H), 4.84 – 4.36 (m, 9H, Bn-H), 4.19 – 4.09 (m, 1H, H-4), 3.92 (dd, $J_{6a,6b}$ = 11.0 Hz, $J_{6a,5}$ = 4.4 Hz, 1H, H-6a), 3.89 – 3.78 (m, 4H, H-3, H-3', H-5', H-6b), 3.73 – 3.61 (m, 2H, H-4', H-5), 3.56 (m, 2H, H-6a', H-6b'), 3.53 – 3.47 (m, 1H, H-2), 3.49 (dd, $J_{2,1}$ = 3.6 Hz, $J_{2,3}$ = 9.7 Hz, 1H, H-2'), 2.69 – 2.61 (m, 1H, 4'-OH). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.8, 138.6, 138.6, 138.2, 138.1, 138.0, 137.8, 137.7, 137.7, 137.2, 136.9 (Ar-C), 133.9, 128.2, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 127.5, 127.4, 127.4, 127.3, 127.2, 127.2, 127.1, 126.9, 126.9, 126.5, 126.4 (Ar-CH), 99.6 (C-1'), 96.4 (C-1), 84.6 (C-3), 81.0 (C-3'), 80.0 (C-5), 78.8 (C-2), 78.7 (C-2'), 75.0, 74.4, 74.0, 73.6, 73.3, 73.3, 73.1, (Bn-C), 72.9 (C-4), 72.8 (Bn-C), 72.4, 72.2 (Bn-C), 71.1 (C-4'), 70.7 (C-5'), 69.5 (C-6'), 68.9 (C-6).

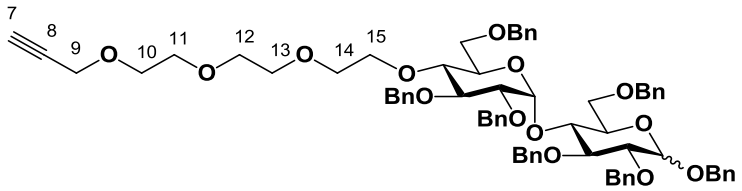
Minor isomer – ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 – 7.13 (m, 35H, Ar-H), 5.73 (d, *J* 3.6 Hz, 1H, H-1'), 5.13 (d, *J* 11.7 Hz, 1H, Bn-H), 5.05 – 4.85 (m, 6H, Bn-H, H-1), 4.84 – 4.36 (m, 9H, Bn-H), 4.23 (t, *J* 9.1 Hz, 1H, H-3), 4.19 – 4.09 (m, 1H, H-4), 4.03 – 3.98 (m, 1H, H-5), 3.92 (dd, $J_{6a,6b}$ = 11.0 Hz, $J_{6a,5}$ = 4.4 Hz, 1H, H-6a), 3.89 – 3.78 (m, 2H, H-3', H-5'), 3.73 – 3.61 (m, 3H, H-2, H-4', H-6b), 3.56 (m, 2H, H-6a', H-6b'), 3.49 (dd, $J_{2,1}$ = 3.6 Hz, $J_{2,3}$ = 9.7 Hz, 1H, H-2'), 2.69 – 2.61 (m, 1H, 4'-OH). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.8, 138.6, 138.6, 138.2, 138.1, 138.0, 137.8, 137.7, 137.7, 137.2, 136.9 (Ar-C), 133.9, 128.2, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 127.5, 127.4, 127.4, 127.3, 127.2, 127.2, 127.1, 126.9, 126.9, 126.5, 126.4 (Ar-CH), 99.6 (C-1'), 94.8 (C-1), 82.0 (C-2), 81.8 (C-3), 81.0 (C-3'), 78.7 (C-2'), 75.0, 74.4, 74.0, 73.6, 73.3, 73.3, 73.1, (Bn-C), 72.8 (Bn-C), 72.6 (C-4), 72.4, 72.2 (Bn-C), 71.1 (C-4'), 70.7 (C-5'), 70.5 (C-5), 69.5 (C-6'), 68.9 (C-6).

(5.10) 2-(2-(2-chloroethoxy)ethoxy)ethoxypropargyl ether



A stirred solution of sodium hydride (0.94 g, 23.6 mmol) in THF (20 ml) had 2-[2-(2-chloroethoxy)ethoxy]ethanol (2 g, 11.8 mmol) added dropwise at 0 °C. After 30 min propargyl bromine (2.3 ml, 23.6 mmol) was added dropwise, the solution was stirred for 1.5 h before more propargyl bromide (0.8 ml, 8.2 mmol) was added. The resulting solution was stirred for 1.5 h before being diluted into EtOAc (200 ml), washed with H_2O (200 ml), sat. aq. NaCl (200 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow oil. The oil was purified by flash column chromatography (silica, 3:1 Hex-EtOAc) to afford a yellow oil (400 mg, 35%); HRMS: Found ($[M+NH_4]^+$) 224.1061, $C_9H_{19}ClNO_3^+$ requires 224.1048; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 4.21 (d, *J* 2.5 Hz, 2H, H-3), 3.77 (t, 2H *J* 6.0 Hz, 1H, H-8), 3.75 – 3.61 (m, 10H, H-4, H-5, H-6, H-7, H-9), 2.60 (t, *J* 2.5 Hz, 1H, H-1). ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 79.8 (C-2), 74.9 (C-1), 71.3 (C-8), 70.5, 70.3, 69.0 (C-4, C-5, C-6, C-7), 58.2 (C-3), 42.9 (C-9).

(5.11) Benzyl 2,3,6-tri-*O*-benzyl-4-2-(2-(2-(ethoxy)ethoxy)ethoxypropargyl ether)-α-D-glucopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-β-D-glucopyranoside



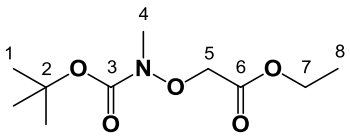
To a stirred solution of Benzyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-*O*-α-D-glucopyranose (1.0 g, 1.02 mmol), 2-(2-(2-chloroethoxy)ethoxy)ethoxypropargyl ether (426 mg, 2.04 mmol) and tetrabutylammonium iodide (37 mg, 0.10 mmol) in DMF (50 ml) sodium hydride (60% in oil) (70 mg, 1.73 mmol) was added at 0 °C. The solution was diluted into EtOAc (200 ml), washed with H_2O (200 ml), sat. aq. NaCl (200 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The oil was purified by flash column chromatography (silica, 3:1→2:1 Hex-EtOAc) to afford a colourless oil (422 mg, 36%); HRMS: Found ($[M+NH_4]^+$) 1160.5770, $C_{70}H_{82}NO_{14}^+$ requires 1160.5730;

β - **Major isomer:** ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.52 – 7.06 (m, 35H, Ar-H), 5.61 (d, $J_{1',2'}$ 3.7 Hz, 1H, H-1'), 5.03 (d, *J* 11.6 Hz, 1H, Bn-H), 4.99 – 4.89 (m, 3H, Bn-H), 4.83 (m, 2H, H-1, Bn-H), 4.77 (d, *J* 11.7 Hz, 1H, Bn-H), 4.74 – 4.44 (m, 6H, Bn-H), 4.39 (d, *J* 12.1 Hz, 1H, Bn-H), 4.35 (d, *J* 12.1 Hz, 1H, Bn-H), 4.14 (d, *J* 2.4 Hz, 2H, H-9), 4.12 (t, $J_{3,2}$ 10.0 Hz, $J_{3,4}$ 10.0 Hz, 1H, H-3), 4.04 (t, $J_{4,3}$ 10.0 Hz, $J_{4,5}$ 10.0 Hz, 1H, H-4), 3.90

(ddd, $J_{5,4}$ 10.0 Hz, $J_{5,6a}$ 5.9 Hz, $J_{5,6b}$ 3.7 Hz, 1H, H-5), 3.87 – 3.66 (m, 2H, H-3', H-5'), 3.66 – 3.51 (m, 17H, H-2, H-4', H-6a', H-6b, H-6b', H-10, H-11, H-12, H-13 , H-14, H-15), 3.51 – 3.39 (m, 2H, H-2', H-6b'), 2.40 (t, *J* 2.4 Hz, 1H, H-7); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.8, 138.4, 138.2, 138.0, 137.9, 137.4 (Ar-C), 128.3, 128.1, 128.1, 127.9, 127.8, 127.8, 127.5, 127.5, 127.4, 126.9, 126.6, 126.5 (Ar-CH), 96.8 (C-1'), 95.2 (C-1), 82.2 (C-3'), 81.6 (C-3), 79.1 (C-2'), 75.3, 74.6, 74.6, 74.4, 73.9, 73.3, 73.3, 73.2, 73.1, 73.1 (7 x Bn-C, C-2, C-4, C-4'), 72.0 , 71.0, 70.9, 70.6, 70.5, 70.3, 70.3 (6 x PEG-C, C-5, C5'), 69.2 (C-6'), 69.0 (C-6), 58.3 (C-9).

α - **Minor isomer:** ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.52 – 7.06 (m, 35H, Ar-H), 5.64 (d, $J_{1,2}$ = 3.7 Hz, 1H, H-1), 5.61 (d, $J_{1',2'}$ = 3.7 Hz, 1H, H-1'), 5.03 (d, *J* 11.6 Hz, 1H, Bn-H), 4.99 – 4.89 (m, 3H, Bn-H), 4.83 (m, 1H, Bn-H), 4.77 (d, *J* 11.7 Hz, 1H, Bn-H), 4.74 – 4.44 (m, 6H, Bn-H), 4.39 (d, *J* 12.1 Hz, 1H, Bn-H), 4.35 (d, *J* 12.1 Hz, 1H, Bn-H), 4.14 (d, *J* 2.4 Hz, 2H, H-9), 4.06 (t, $J_{4,3}$ = 10.0 Hz, $J_{4,5}$ = 10.0 Hz, 1H, H-4), 3.87 – 3.66 (m, 4H, H-3', H-3, H-5', H-6a), 3.66 – 3.51 (m, 16H, H-4', H-5, H-6a', H-6b, H-10, H-11, H-12, H-13 , H-14, H-15), 3.51 – 3.39 (m, 3H, H-2, H-2', H-6b'), 2.40 (t, *J* 2.4 Hz, 1H, H-7); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 138.8, 138.4, 138.2, 138.0, 137.9, 137.4 (Ar-C), 128.3, 128.1, 128.1, 127.9, 127.8, 127.8, 127.5, 127.5, 127.4, 126.9, 126.6, 126.5 (Ar-CH), 96.8 (C-1, C-1'), 84.6 (C-3), 82.2 (C-3'), 79.6 (C-2), 79.1 (C-2'), 75.3, 74.6, 74.6, 74.4, 73.9, 73.3, 73.3, 73.2, 73.1, 73.1 (7 x Bn-C, C-4, C-4', C-5), 72.0 , 71.0, 70.9, 70.6, 70.5, 70.3, 70.3 (6 x PEG-C, C5'), 69.2 (C-6'), 68.2 (C-6), 58.3 (C-9).

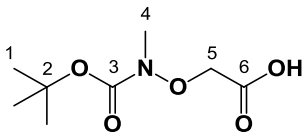
(5.16a) Ethyl (*t*-butoxycarbonyl-*N*-methylaminooxy)acetate[152]



Sodium hydrogen carbonate (2.50 g, 30.0 mmol) and Di-*tert*-butyl dicarbonate (3.92 g, 17.9 mmol) were added to *N*-methylhydroxylamine (1.25 g, 15.0 mmol) in THF–H_2O (27.5 ml, 10:1). The solution was stirred for 20 h at r.t., diluted with H_2O (25 ml), extracted with DCM (3 x 15 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was added dropwise to sodium hydride (60% in oil, 1.44 g, 36.1 mmol) in THF (15 ml) at 0 °C. After stirring for 10 min 2-bromoethyl acetate (4.0 ml, 36.1 mmol) was added dropwise. The solution was stirred for 1 h and diluted with EtOAc (200 ml), washed with H_2O (200 ml), sat. aq. NaCl (200 ml), dried ($MgSO_4$) and

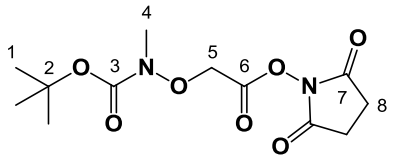
concentrated under reduced pressure. The oil was purified by flash column chromatography (silica, 6:1→4:1 Hex-EtOAc) to afford a colourless oil (3.20 g, 66%). HRMS: Found ([M+Na]$^+$) 256.1175, $C_{10}H_{19}NNaO_5$+ requires 256.1155; ^{1}H NMR (500 MHz, $CDCl_3$); δ_H = 4.44 (s, 2H, H-5), 4.22 (q, *J* 7.2 Hz, 2H, H-8), 3.20 (s, 1H, H-4), 1.48 (s, 9H, H-1), 1.29 (t, *J* 7.2 Hz, 3H, H-7); 13C NMR (126 MHz, CDCl3) δ_C = 169.3 (C-3), 157.7 (C-6), 81.9 (C-2), 72.0 (C-5), 60.9 (C-7), 38.4 (C-4), 28.1 (C-1), 14.1 (C-8).

(5.16b) (*t*-Butoxycarbonyl-*N*-methylaminooxy)acetic acid[152]



Ethyl (*t*-butoxycarbonyl-*N*-methylaminooxy)acetate (3 g, 13.4 mmol) in 3M NaOH (2 ml) and THF (15 ml) was stirred for 3h. The solution was diluted into EtOAc (100 ml), washed with 1M HCl (100 ml), H_2O (100 ml), sat. aq. NaCl (100 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by flash column chromatography (silica, 50:50:1 Hex–EtOAc–AcOH) to afford a colourless oil (1.00 g, 36%). HRMS: Found ([M+Na]$^+$) 228.0844, $C_8H_{15}NNaO_5$+ requires 228.0842; 1H NMR (500 MHz, $CDCl_3$) δ_H = 4.50 (s, 3H, H-4), 3.18 (s, 2H, H-5), 1.52 (s, 9H, H-1); 13C NMR (126 MHz, CDCl3) δ_C 176.1 (C-3), 159.3 (C-6), 84.2 (C-5), 72.8 (C-2), 37.8 (C-4), 28.2 (C-1).

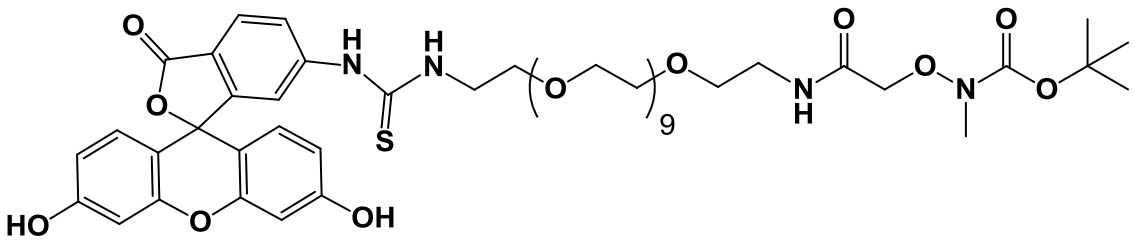
(5.17) (*t*-Butoxycarbonyl-*N'*-methylaminooxyacetyl)-*N*-hydroxysuccinimide ester[152]



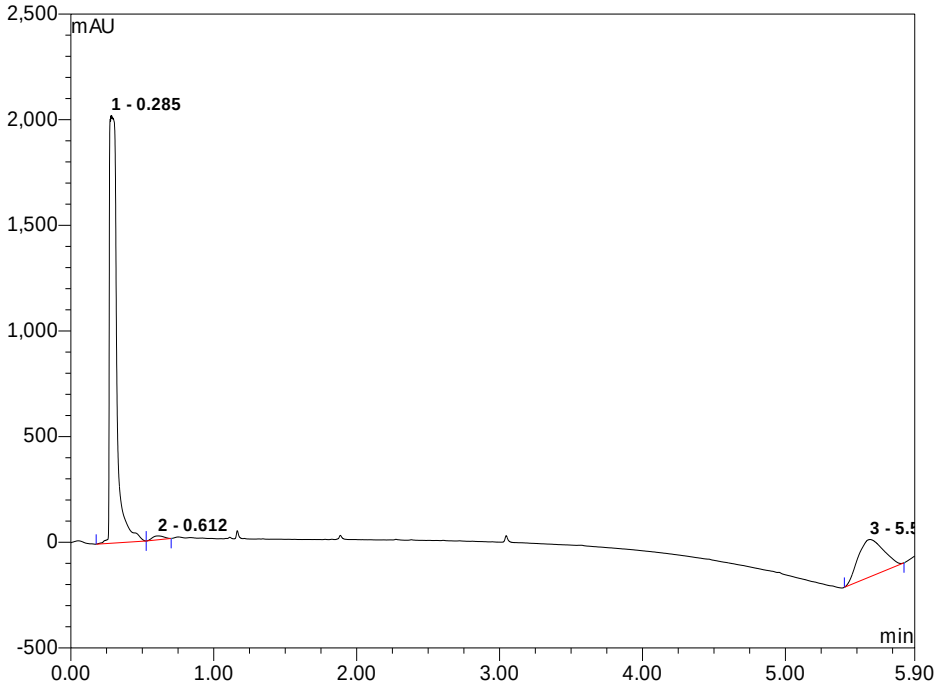
(*t*-Butoxycarbonyl-*N*-methylaminooxy)acetic acid with DCC (0.66 g, 5.85 mmol) and NHS (0.46 g, 7.32 mmol) were stirred in EtOAc (30 ml) for 2 h. The product was then washed with 1% aq. $NaHCO_3$ (30 ml), H_2O (30 ml), sat. aq. NaCl (30 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give the product as a white solid (750 mg, 51%). HRMS: Found ([M+Na]$^+$) 325.1009, $C_{12}H_{18}N_2NaO_7$+ requires 325.1006; ^{1}H NMR

(300 MHz, $CDCl_3$) δ_H 4.80 (s, 2H, H-5), 3.19 (s, 3H, H-4), 2.86 (s, 2H, H-8), 1.50 (s, 9H, H-1); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 168.6 (C-7), 165.0 (C-3), 157.7 (C-6), 82.4 (C-2), 69.8 (C-5), 38.7 (C-4), 28.1 (C-1), 25.6 (C-8).

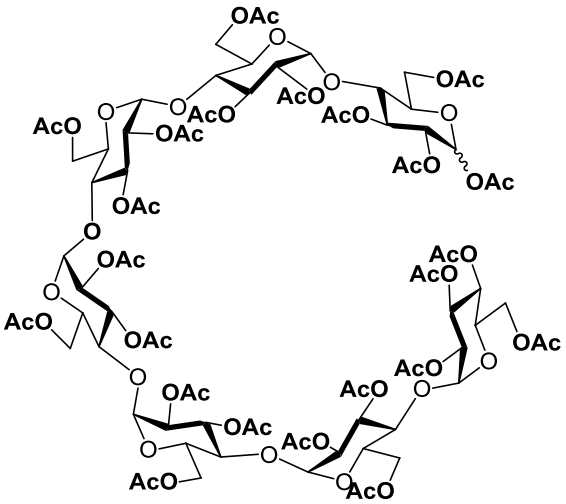
(5.23) *6*-(*O*-(2-thioureaethyl)-*O′*-(2-(*t*-Butoxycarbonyl-*N′*-methylaminooxyacetyl)-aminoethyl nonaethylene glycol) fluorescein



O-(2-(*t*-Butoxycarbonyl-*N′*-methylaminooxyacetyl)-aminoethyl)-*O′*-(2-azidoethyl)nonaethylene glycol (50 mg, 0.07 mmol) and palladium on carbon (10 wt. %, 30 mg) in MeOH (20 ml) were stirred stirred under an atmospher of H_2 at r.t.p. for 3 h. The solution was then filtered and concentrated under reduced pressure. The resulting oil was taken into sodium bicarbonate (1.2 ml, pH 8.5, 0.185 M) and FITC (12.4 mg, 0.03 mmol) in DMF (1.2 ml) was added and the solution stirred for 24 h. To purify the product it was applied directly to a size exclusion column (LH-20) yielding the product as an orange oil (26 mg, 80%). HRMS: Found ($[M+H]^+$) 1077.4597, $C_{51}H_{73}N_4O_{19}S^+$ requires 1077.4585.



(5.25) *O*-acetylmaltoheptaose[81]



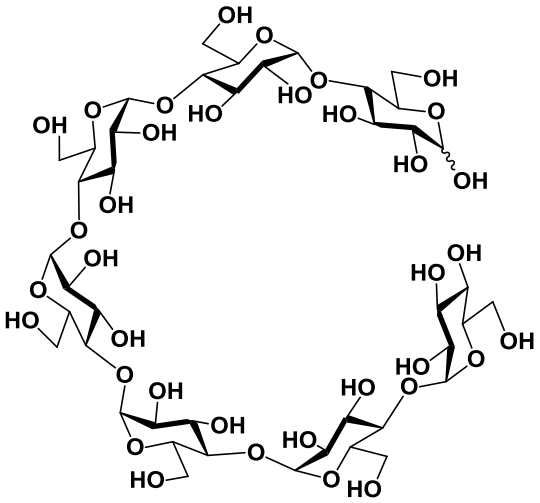
Triacetyl-β-cyclodextrin (10.0 g, 4.96 mmol) and sulfuric acid (1.2 ml, 22.5 mmol) in acetic anhydride (68.8 ml) were stirred at 60 °C o.n. The solution was quenched with pyridine (40 ml) before concentration under reduced pressure. The resulting oil was taken into EtOAc (250 ml), washed with H_2O (100 ml), sat. aq. NaCl (100 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow foam. The mixture was purified by flash column chromatography (silica, 4:1 EtOAc-Hex) to afford a white

solid (2.8 g, 28%); MS: Found ($[M+Na+NH_4]^{2+}$) 1079.9, $C_{88}H_{122}NNaO_{59}^{2+}$ requires 1079.8;

α - Major isomer: ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H 6.20 (d, 1H, $J_{1'\alpha,2'\alpha}$ 3.8 Hz, H-1'α), 5.46 (t, 1H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 10.0 Hz, H-3'), 5.40 – 5.30 (m, 7H, 5 x H-3, H-1'', H-3''), 5.30 – 5.20 (m, 5H, H-1), 5.03 (t, 1H, $J_{3'',4''}$ 10.0 Hz, $J_{4'',5''}$ 10.0 Hz , H-4''), 4.91 (dd, 1H, $J_{1'\alpha,2'\alpha}$ 3.8 Hz, $J_{2'\alpha,3'\alpha}$ 10.0 Hz, H-2'α), 4.82 (dd, 1H, $J_{1'',2''}$ 3.8 Hz, $J_{2'',3''}$ 10.0 Hz, H-2'), 4.72 – 4.64 (m, 5H, H-2), 4.51 – 4.39 (m, 7H, 5 x H-6, H-6', H-6''), 4.30 – 4.13 (m, 6H, 5 x H-6, H-5'), 4.04 – 3.85 (m, 13H, H-4, H-4', H-5, H-5''), 2.21 – 1.81 (m, 69H, 7 x $COC\underline{H}_3$); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.7, 169.7, 169.6, 169.4, 168.9, 168.8, 168.6, 168.5, 168.0 (23 x $O\underline{C}OCH_3$), 94.8, 94.7, 87.8 (C-1), 76.4, 76.0, 75.6, 72.1, 71.2, 70.7, 69.5, 69.4, 69.2, 69.0, 68.7, 68.3, 68.1, 67.9, 67.4, 66.9, 61.3, 60.3, 59.4 (C2, C3, C4, C5, C6), 20.0, 19.9, 19.7, 19.6, 19.5, 19.4, 13.2 (23 x $OCO\underline{C}H_3$).

β - Minor isomer: ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H 5.71 (d, 1H, $J_{1'\beta,2'\beta}$ 8 Hz, H-1'β), 5.46 (t, 1H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 10.0 Hz, H-3'), 5.40 – 5.30 (m, 7H, 5 x H-3, H-1'', H-3''), 5.30 – 5.20 (m, 5H, H-1), 5.03 (t, 1H, $J_{3'',4''}$ 10.0 Hz, $J_{4'',5''}$ 10.0 Hz , H-4''), 4.89 (dd, 1H, $J_{1'\beta,2'\beta}$ 8.0 Hz, $J_{2'\beta,3'\beta}$ 10.0 Hz, H-2'β), 4.82 (dd, 1H, $J_{1'',2''}$ 3.8 Hz, $J_{2'',3''}$ 10.0 Hz, H-2'), 4.72 – 4.64 (m, 5H, H-2), 4.51 – 4.39 (m, 7H, 5 x H-6, H-6', H-6''), 4.30 – 4.13 (m, 6H, 5 x H-6, H-5'), 4.04 – 3.85 (m, 13H, H-4, H-4', H-5, H-5''), 2.21 – 1.81 (m, 69H, 7 x $COC\underline{H}_3$); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.7, 169.7, 169.6, 169.4, 168.9, 168.8, 168.6, 168.5, 168.0 (23 x $O\underline{C}OCH_3$), 94.8, 94.7, 76.4, 76.0, 75.6, 72.1, 71.2, 70.7, 69.5, 69.4, 69.2, 69.0, 68.7, 68.3, 68.1, 67.9, 67.4, 66.9, 61.3, 60.3, 59.4 (C2, C3, C4, C5, C6), 20.0, 19.9, 19.7, 19.6, 19.5, 19.4, 13.2 (23 x $OCO\underline{C}H_3$).

(4.2) Maltoheptaose

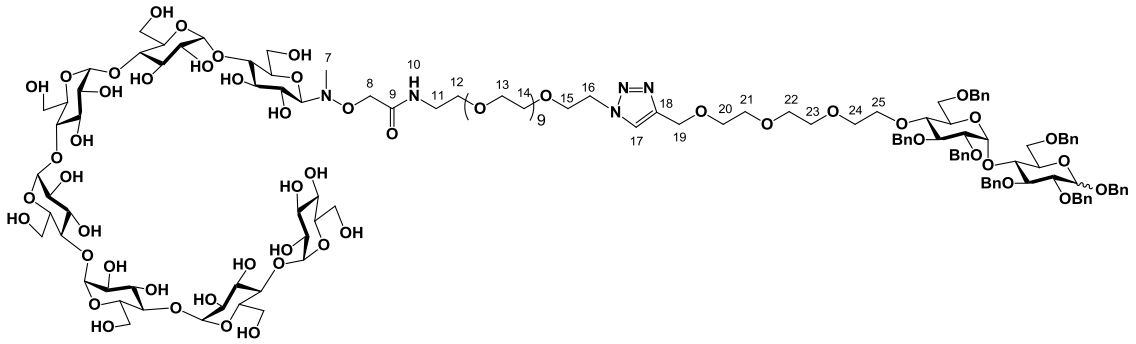


Per-acetylated maltoheptaose (2.3 g, 1.07 mmol) and sodium methoxide (230 mg, 1.17 mmol) were stirred in methanol (23 ml) for 20 h. The solution was neutralised with amberlite H-120 resin, filtered and concentrated under reduced pressure. The residue was purified by size exclusion chromatography (LH-20) to produce a white solid (100 mg, 8%). MS: Found ($[M+Na]^+$) 1175.4, $C_{42}H_{72}NaO_{36}^+$ requires 1175.4;

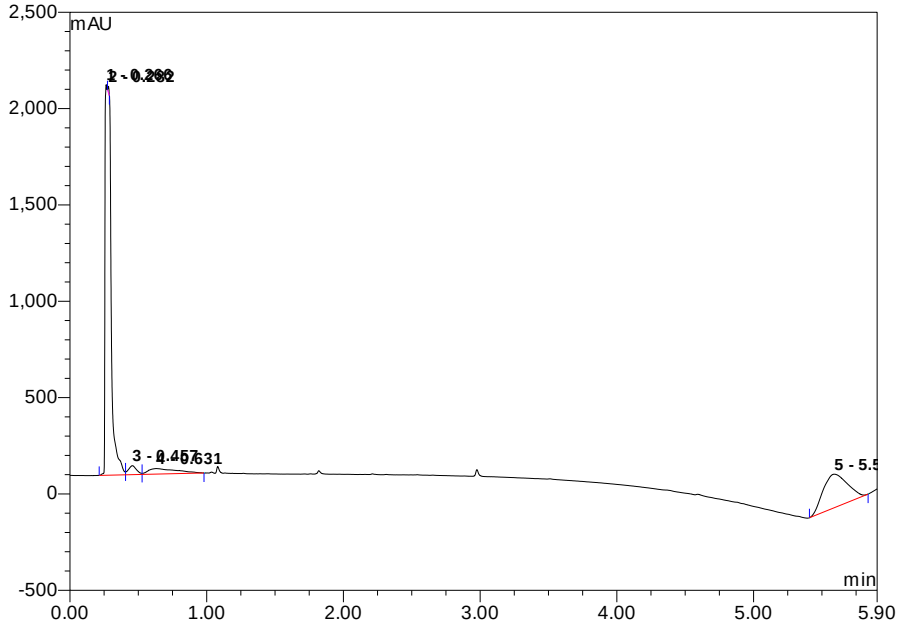
Major isomer β – ^{1}H NMR (500 MHz, D_2O) δ 5.41 (d, $J_{1',2'}$ = 3.4 Hz, 6H, H-1'), 5.24 (d, $J_{1,2}$ = 3.8 Hz, 1H, H-1α), 3.98 (m, 7H, H-3, 6 x H-3'), 3.92 – 3.75 (m, 21H, 6 x H-5', 6 x H-6a', 6 x H-6b', H-5, H-6a, H-6b), 3.65 (m, 13H, 5 x H-4', H-4, 6 x H-2', H-2α), 3.44 (t, $J_{4',3'}$ = 9.5 Hz, $J_{4',5'}$ = 9.5 Hz, 1H, H-4').

Minor isomer α – ^{1}H NMR (500 MHz, D_2O) δ 5.41 (d, $J_{1',2'}$ = 3.4 Hz, 6H, H-1'), 4.67 (d, $J_{1,2}$ = 8.0 Hz, 1H, H-1β), 3.98 (m, 7H, H-3, 6 x H-3'), 3.92 – 3.75 (m, 21H, 6 x H-5', 6 x H-6a', 6 x H-6b', H-5, H-6a, H-6b), 3.65 (m, 12H, 5 x H-4', H-4, 6 x H-2'), 3.44 (t, $J_{4',3'}$ = 9.5 Hz, $J_{4',5'}$ = 9.5 Hz, 1H, H-4'), 3.29 (t, $J_{2,1}$ = 8.0 Hz, $J_{2,3}$ = 8.0 Hz, 1H, H-2β).

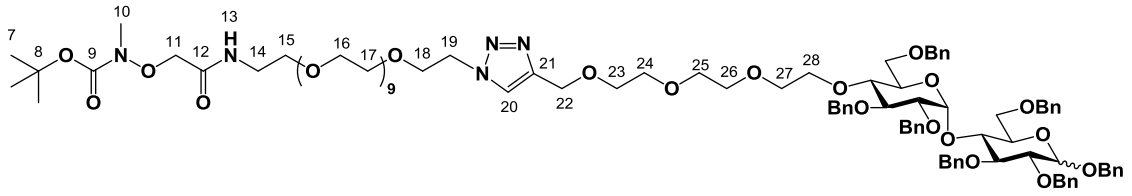
(5.26) Combined maltoheptaose and central fragments



Benzyl 2,3,6-tri-*O*-benzyl-4-2-(2-(2-(ethoxy)ethoxy)ethoxypropargyl ether)-α-D-glucopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (12.6 mg, 11.0 μmol) in DMF (600 μl) had *O*-(2-(*N*′-methylaminooxyacetyl)-aminoethyl)-*O*′-(2-azidoethyl)nonaethylene glycol maltoheptaose (16 mg, 9.2 μmol) in H_2O (10 μl) added. Copper (II) sulphate (0.37 mg, 2.3 μmol) in H_2O (23μl) was added followed by sodium-D-ascorbate (0.91 mg, 4.6 μmol) in H_2O (46 μl). The solution was stirred for 20 h and the solution purified directly by size exclusion chromatography (H_2O, LH-20) to give a colourless solid (21 mg, 79%). HRMS: Found ($[M+NH_4+ NH_4]^{2+}$) 1463.6682, $C_{137}H_{207}N_7O_{61}^{2+}$ requires 1463.6667.



(5.29) The central fragment



To a solution of Benzyl 2,3,6-tri-*O*-benzyl-4-2-(2-(2-(ethoxy)ethoxy)ethoxypropargyl ether)-α-D-glucopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (106 mg, 93 μmol) and *O*-(2-(*t*-Butoxycarbonyl-*N'*-methylaminooxyacetyl)-aminoethyl)-*O'*-(2-azidoethyl)nonaethylene glycol (66 mg, 93 μmol) in DMF (6.2 ml) was added copper (II) sulfate (3.69 mg, 23 μmol) in water (239 μl) and Na-L-Ascorbate (9.11 mg, 46 μmol) in water (460 μl). The resulting solution was stirred for 24 h before being concentrated under reduced pressure. The oil was purified by flash column chromatography (silica, 4:1 EtOAc – MeOH) to afford a colourless oil (149 mg, 83%). HRMS: Found ($[M+NH_4]^+$) 1874.9841, $C_{100}H_{141}N_6O_{28}^+$ requires 1874.9841.

Major isomer β: ^{1}H NMR (500 MHz, CD_3OD) δ_H 7.95 (s, 1H, H-20), 7.47 – 7.14 (m, 35H, Ar-H), 5.57 (d, $J_{1',2'}$ = 3.6 Hz, 1H, H-1'), 4.98 – 4.64 (m, 7H, Bn-H), 4.64 – 4.39 (m, 11H, H-1, H-22, H-19, Bn-H), 4.36 (s, 2H, H-11), 4.03 (t, $J_{4,3}$ = 8.7 Hz, $J_{4,5}$ = 8.7 Hz, 1H, H-4), 3.87 – 3.78 (m, 5H, H-3', H-4', H-5', H-18), 3.74 (t, $J_{3,2}$ = 8.7 Hz, $J_{3,4}$ = 8.7 Hz, 1H, H-3), 3.70 – 3.48 (m, 60H, H-2, H-5, H-6a, H-6b, H-6a', H-6b', H-14, H-16, H-17, H-23, H-24, H-25, H-26, H-27, H-28), 3.46 (t, *J* 5.5 Hz, 2H, H-15), 3.42 – 3.33 (m, 2H, H-2'), 3.14 (s, 3H, H-10), 1.51 (s, 9H, H-7). ^{13}C NMR (126 MHz, MeOD) δ_C 171.1 (C-9), 159.2 (C-12), 140.3, 139.8, 139.7, 139.5, 139.1 (C-21, Bn-C), 129.5, 129.4, 129.4, 129.3, 129.2, 129.1, 129.0, 129.0, 128.9, 128.8, 128.7, 128.7, 128.6, 128.3, 128.1, 128.0 (Bn-CH), 125.6 (C-20), 103.7 (C-1), 97.6 (C-1'), 85.8 (C-3), 83.7 (C-2), 80.9 (C-3'), 76.3 (C-2'), 74.9, 74.6, 74.4, 74.4, 74.2, 74.2, 72.5, 72.1, 71.8, 71.6, 71.6, 71.4, 71.3, 71.2, 71.2, 70.8, 70.5, 70.3, 70.0 (C-4, C-4', C-5, C-5', C-6, C-6', C-11, C-14, C-16, C-17, C-18, C-23, C-24, C-25, C-26, C-27, C-28, Bn-C), 65.1 (C-22), 51.4 (C-19), 40.0 (C-15), 37.9 (C-10), 28.6 (C-7).

Minor isomer α: ^{1}H NMR (500 MHz, CD_3OD) δ_H 7.95 (s, 1H, H-20), 7.47 – 7.14 (m, 35H, Ar-H), 5.61 (d, $J_{1,2}$ = 3.5 Hz, 1H, H-1), 5.57 (d, $J_{1',2'}$ = 3.6 Hz, 1H, H-1'), 4.98 – 4.64 (m, 7H, Bn-H), 4.64 – 4.39 (m, 10H, H-22, H-19, Bn-H), 4.36 (s, 2H, H-11), 3.95 – 3.86 (m, 2H, H-4', H-5), 3.87 – 3.78 (m, 6H, H-3, H-3', H-4, H-5', H-18), 3.70 – 3.48 (m, 60H, H-6a, H-6b, H-6a', H-6b', H-14, H-16, H-17, H-23, H-24, H-25, H-26, H-27, H-28), 3.46 (t, *J* 5.5 Hz, 2H, H-15), 3.42 – 3.33 (m, 2H, H-2, H-2'), 3.14 (s, 3H, H-10), 1.51 (s, 9H, H-7). ^{13}C NMR (126 MHz, MeOD) δ_C 171.1 (C-9), 159.2 (C-12), 140.3, 139.8,

139.7, 139.5, 139.1 (C-21, Bn-C), 129.5, 129.4, 129.4, 129.3, 129.2, 129.1, 129.0, 129.0, 128.9, 128.8, 128.7, 128.7, 128.6, 128.3, 128.1, 128.0 (Bn-CH), 125.6 (C-20), 97.6 (C-1, C-1'), 80.9 (C-3, C-3'), 76.3 (C-2, C-2'), 74.9, 74.6, 74.4, 74.4, 74.2, 74.2, 72.5, 72.1, 71.8, 71.6, 71.6, 71.4, 71.3, 71.2, 71.2, 70.8, 70.5, 70.3, 70.0 (C-4, C-4', C-5, C-5', C-6, C-6', C-11, C-14, C-16, C-17, C-18, C-23, C-24, C-25, C-26, C-27, C-28, (Bn-C), 65.1 (C-22), 51.4 (C-19), 40.0 (C-15), 37.9 (C-10), 28.6 (C-7).

7.2.3 SPPS

7.2.3.1 General Reagents and Equipment

All amino acids, resins and coupling reagents were purchased from Sigma Aldrich, VWR International and Cambridge Bioscience. All chemical compounds were used as received. Fritted polypropylene tubes (10 mL) purchased from Grace and Co were used as a vessel for all solid phase reactions. Agitation of the solid phase reaction mixture was achieved by rotation on a Stuart rotator which allowed multiple fritted polypropylene tubes to be spun simultaneously. H-Gly-2-ClTrt (Sigma, loading: 1.1 mmol/g) was used for solid phase peptide synthesis.

Peptides were analysed using an Agilent 1290 affinity LC system equipped with an Ascentis Express 10 cm × 2.1 mm, 2.7 µm ES-C18 peptide column (0.5 mL min−1) and ultraviolet (UV) detection at 220 nm - 280 nm. Gradient from 0.1% TFA/5% MeCN (*vol*/*vol*) in H_2O to 0.1% TFA/95% MeCN in H_2O over 5 min 40 s.

7.2.4 General procedure for solid phase peptide synthesis

Method One: Preparation of resin

The resin was swollen in DMF for 1 h. HCTU (4.9 equiv) and an Fmoc-amino acid (5 equiv) were dissolved in DMF (6 mL), followed by the addition of DIPEA (10 equiv). The solution was transferred to the swelled resin and the mixture was left to spin for 1 h

at room temperature. The resin was isolated by filtration and washed with DMF (3 × 6 mL × 2 min spins).

Method Two: Coupling reaction analysis

A small sample of beads from the isolated Fmoc-amino acid-resin were exposed to TFA (100 μl) for 2 minutes before the solution was quenched with methanol (1 mL). The solution was filtered and analysed by LCMS. If any starting material was detected method one was repeated.

Method Three: Fmoc deprotection and peptide elongation

The Fmoc-amino acid-resin was treated with piperidine (20% in DMF) (10 mL) and left to spin for 2 min at rt. This process was repeated five times before the amino acid resin was washed with DMF (5 × 10 mL × 2 min spins). The peptide sequence was elongated by repeating methods 1-3 (excluding the resin swelling step) until the correct peptide was synthesised.

Method Four: Dde-protecting group removal and Fmoc-PEG-OH addition

After elongation of the peptide to the desired length, a coupling step using a Boc-Gly-OH residue was performed. After this coupling step, the resin was treated with a solution of hydrazine in DMF (2% v/v) (5 x 10 ml x 2 min spins). The resin was then washed with DMF (5 × 10 mL × 2 min spins). Methods 1-3 were then repeated for the addition of Fmoc-PEG-OH (12 equiv) but using HCTU (11.8 equiv) and DIPEA (24 equiv).

Method Five: Addition of Boc-Aoa-OH

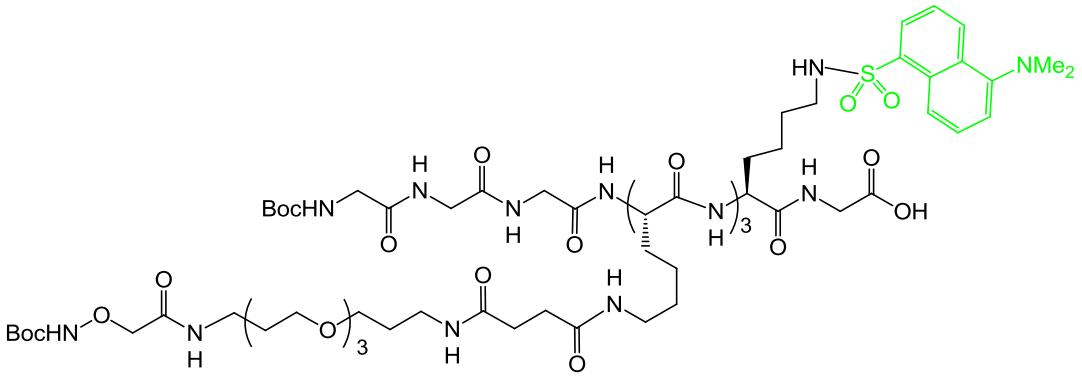
After addition of Fmoc-PEG-OH, a final coupling step using a Boc-Aoa-OH (9 equiv) residue was performed with DIPEA (18 equiv). After methods 1-2 were repeated method six was followed.

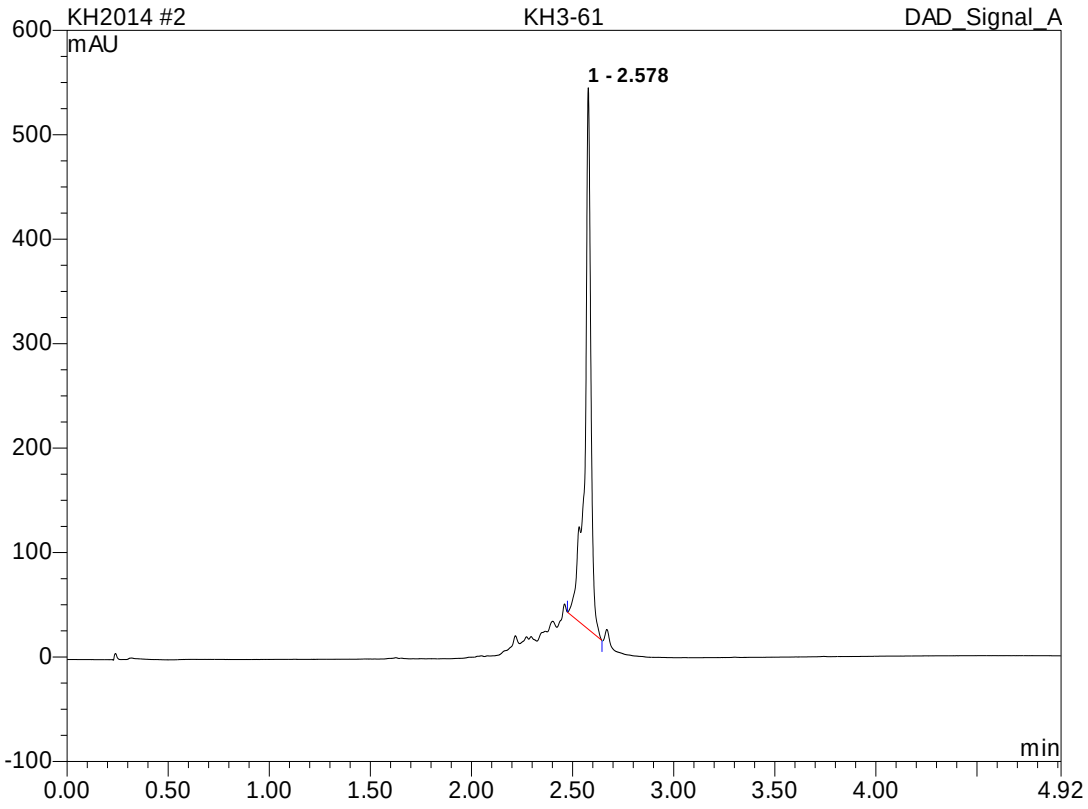
Method Six: Cleavage and isolation

The amino acid-resin was washed with DMF (3× 6 mL × 2 min spins), DCM (3 × 6 mL × 2 min spins) and methanol (3 × 6 mL × 2 min spins). The resin was isolated by filtration before being dried under high vacuum for 3 h. A cleavage cocktail consisting of HFIP (33% in H_2O) (10 mL) was added to the resin and the mixture was left to spin for 2 h at rt. The resin was filtered and the peptide was analysed by LCMS before being dissolved in the minimal amount of water and freeze dried. The peptide was purified by size exclusion chromatography (LH-20) before further purification by HPLC.

(5.10c) Trivalent ligand

Yield (18 mg, 2%). HRMS: Found ($[M+NH_4+H]^{2+}$) 1268.6920, $C_{112}H_{197}N_{23}O_{40}S^{2+}$ requires 1268.6910.





Chapter VIII: Appendix

8.1 pSAB2.2

A pSAB2.2 vector was generated in a digest of pSAB2.0 with MfeI and re-ligated (Figure 8-1) by James Ross in the Turnbull laboratory. This digestion removed the MBP gene (MalE), the CTA2 fusion protein as well as the additional terminator and promoter regions leaving the CTB gene downstream of the native tac promoter region of the pMAL-p5× plasmid. On expression, this plasmid yielded a single mRNA containing the CTB gene. This vector was produced by Dr. James Ross.

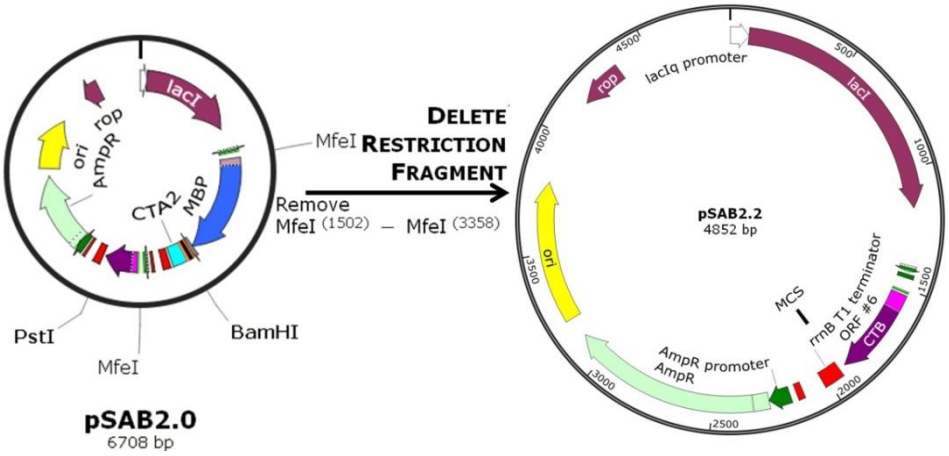


Figure 8-1 The pSAB2.2 plasmid. This plasmid is derived from the pSAB2.0 plasmid via digestion with MfeI. When induced this plasmid should promote transcription of a mRNA containing the CTB gene (purple). Figure produced with SnapGene.

The maltoporin gene was ligated into the plasmid with the use of MfeI and HIND III restriction enzymes replacing the CTB gene.

Chapter IX: References

(1) Pathem, B. K.; Claridge, S. A.; Zheng, Y. B.; Weiss, P. S. *Annu. Rev. Phys. Chem.* **2013**, *64*, 605.

(2) Lather;, A.; Lamba;, V. K.; Malik, H. *ESTIJ* **2012**, *2*, 460.

(3) Scheller, J.; Chalaris, A.; Garbers, C.; Rose-John, S. *Trends Immunol.* **2011**, *32*, 380.

(4) Marchivie, M.; Guionneau, P.; Howard, J. A.; Chastanet, G.; Létard, J.-F.; Goeta, A. E.; Chasseau, D. *J. Am. Chem. Soc.* **2002**, *124*, 194.

(5) Chen, F.; He, J.; Nuckolls, C.; Roberts, T.; Klare, J. E.; Lindsay, S. *Nano Lett.* **2005**, *5*, 503.

(6) Koshido, T.; Kawai, T.; Yoshino, K. *J. Phys. Chem.* **1995**, *99*, 6110.

(7) Toriumi, A.; Kawata, S.; Gu, M. *Opt. Lett.* **1998**, *23*, 1924.

(8) Shinkai, S.; Nakaji, T.; Nishida, Y.; Ogawa, T.; Manabe, O. *J. Am. Chem. Soc.* **1980**, *102*, 5860.

(9) Pedersen, C. J. *J. Am. Chem. Soc.* **1967**, *89*, 2495.

(10) Cram, D. J.; Cram, J. M. *Science* **1974**, *183*, 803.

(11) Anelli, P. L.; Spencer, N.; Stoddart, J. F. *J. Am. Chem. Soc.***1991**, *113*, 5131.

(12) Benniston, A. C.; Harriman, A. *Angew. Chem. Int. Ed.* **1993**, *32*, 1459.

(13) Altieri, A.; Bottari, G.; Dehez, F.; Leigh, D. A.; Wong, J. K. Y.; Zerbetto, F. *Angew. Chem. Int. Ed.* **2003**, *42*, 2296.

(14) Coutrot, F.; Busseron, E. *Chem. Eur. J.* **2008**, *14*, 4784.

(15) Romuald, C.; Arda, A.; Clavel, C.; Jimenez-Barbero, J.; Coutrot, F. *Chem. Sci.* **2012**, *3*, 1851.

(16) Jimenez-Molero, M. C.; Dietrich-Buchecker, C.; Sauvage, J.-P. *Chem. Commun.* **2003**, 1613.

(17) Zhao, Y.; Li, Y.; Li, Y.; Zheng, H.; Yin, X.; Liu, H. *Chem. Commun.* **2010**, *46*, 5698.

(18) Langton, M. J.; Beer, P. D. *Acc. Chem. Res.* **2014**, *47*, 1935.

(19) Mullaney, B. R.; Thompson, A. L.; Beer, P. D. *Angew. Chem.* **2014**, *126*, 11642.

(20) Zheng, H.; Bi, J.; Krendel, M.; Loh, S. N. *Biochemistry* **2014**, *53*, 5505.

(21) Stratton, M. M.; Mitrea, D. M.; Loh, S. N. *ACS Chem. Biol.* **2008**, *3*, 723.

(22) Guntas, G.; Mitchell, S. F.; Ostermeier, M. *Chem. Biol.* **2004**, *11*, 1483.

(23) Guntas, G.; Mansell, T. J.; Kim, J. R.; Ostermeier, M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 11224.

(24) Sacchettini, J. C.; Baum, L. G.; Brewer, C. F. *Biochemistry* **2001**, *40*, 3009.

(25) Ficko-Blean, E.; Boraston, A. B. *J. Biol. Chem.* **2006**, *281*, 37748.

(26) Benktander, J.; Ångström, J.; Breimer, M. E.; Teneberg, S. *J. Biol. Chem.* **2012**, *287*, 31712.

(27) Lowe, J. B.; Ward, P. A. *J. Clin. Invest* **1997**, *99*, 822.

(28) Zheng, C.; Page, R. C.; Das, V.; Nix, J. C.; Wigren, E.; Misra, S.; Zhang, B. *J. Biol. Chem.* **2013**, *288*, 20499.

(29) Holgersson, J.; Gustafsson, A.; Breimer, M. E. *Immunol. Cell. Biol.* **2005**, *83*, 694.

(30) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683.

(31) Navarre, N.; Amiot, N.; van Oijem, A.; Imberty, A.; Poveda, A.; Jiménez-Barbero, J.; Cooper, A.; Nutley, M. A.; Boons, G.-J. *Chem. Eur. J.* **1999**, *5*, 2281.

(32) da Silva, J. S. L.; Oliveira, M. D. L.; de Melo, C. P.; Andrade, C. A. S. *Colloids Surf., B* **2014**, *117*, 549.

(33) Volarevic, V.; Milovanovic, M.; Ljujic, B.; Pejnovic, N.; Arsenijevic, N.; Nilsson, U.; Leffler, H.; Lukic, M. L. *Hepatology* **2012**, *55*, 1954.

(34) Mandal, D. K.; Kishore, N.; Brewer, C. F. *Biochemistry* **1994**, *33*, 1149.

(35) Betton, G. R. *Int. J. Cancer* **2006**, *18*, 687.

(36) Goldstein, I. J.; Reichert, C. M.; Misaki, A. *Ann. NY Acad. Sci.* **1974**, *234*, 283.

(37) Moothoo, D. N.; Canan, B.; Field, R. A.; Naismith, J. H. *Glycobiol.* **1999**, *9*, 539.

(38) Maupin, K. A.; Liden, D.; Haab, B. B. *Glycobiol.* **2012**, *22*, 160.

(39) Bouwman, L. H.; Roep, B. O.; Roos, A. *Human Immunol.*, *67*, 247.

(40) Kasperkiewicz, K.; Swierzko, A. S.; Bartlomiejczyk, M. A.; Cedzynski, M.; Noszczynska, M.; Duda, K. A.; Michalski, M.; Skurnik, M. *Int. J. Med. Microbiol.* **2015**, *305*, 544.

(41) Barre, A.; Van Damme, E. J.; Peumans, W. J.; Rouge, P. *Plant Physiol.* **1996**, *112*, 1531.

(42) Ji, X.; Gewurz, H.; Spear, G. T. *Mol. Immunol.* **2004**, *42*, 145.

(43) Granell, M.; Urbano-Ispizua, A.; Suarez, B.; Rovira, M.; Fernandez-Aviles, F.; Martinez, C.; Ortega, M.; Uriburu, C.; Gaya, A.; Roncero, J. M.; Navarro, A.; Carreras, E.; Mensa, J.; Vives, J.; Rozman, C.; Montserrat, E.; Lozano, F. *Exp. Hematol.* **2006**, *34*, 1435.

(44) Rothfuchs, A. G.; Roffê, E.; Gibson, A.; Cheever, A. W.; Ezekowitz, R. A. B.; Takahashi, K.; Steindel, M.; Sher, A.; Báfica, A. *PLOS ONE* **2012**, *7*, e47835.

(45) Sandvig, K.; van Deurs, B. *Physiol. Rev.* **1996**, *76*, 949.

(46) Sandvig, K. *Toxicon* **2001**, *39*, 1629.

(47) Spangler, B. D. *Microbiol. Rev.* **1992**, *56*, 622.

(48) Ling, H.; Boodhoo, A.; Hazes, B.; Cummings, M. D.; Armstrong, G. D.; Brunton, J. L.; Read, R. J. *Biochemistry* **1998**, *37*, 1777.

(49) Dam, T. K.; Brewer, C. F. *Biochemistry* **2008**, *47*, 8470.

(50) Lee, R. T.; Ichikawa, Y.; Fay, M.; Drickamer, K.; Shao, M. C.; Lee, Y. C. *J. Biol. Chem.* **1991**, *266*, 4810.

(51) Dam, T. K., Gabius, H. J., Andre, S., Kaltner, H., Lensch, M. and Brewer, C. F. *Biochemistry* **2005**, *41*, 1351.

(52) Wong, J. E. M. M.; Alsarraf, H. M. A. B.; Kaspersen, J. D.; Pedersen, J. S.; Stougaard, J.; Thirup, S.; Blaise, M. *FEBS Journal* **2014**, *281*, 1196.

(53) Renz, M.; Daniels, B. R.; Vámosi, G.; Arias, I. M.; Lippincott-Schwartz, J. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, E2989.

(54) Zacco, E.; Hütter, J.; Heier, J. L.; Mortier, J.; Seeberger, P. H.; Lepenies, B.; Koksch, B. *ACS Chem. Biol.* **2015**.

(55) Kitov, P. I.; Sadowska, J. M.; Mulvey, G.; Armstrong, G. D.; Ling, H.; Pannu, N. S.; Read, R. J.; Bundle, D. R. *Nature* **2000**, *403*, 669.

(56) Dam, T. K., Roy, R., Das, S. K., Oscarson, S. and Brewer, C. F. *J. Biol. Chem.* **2000**, *275*, 14223.

(57) Dvir, A.; Peterson, S. R.; Knuth, M. W.; Lu, H.; Dynan, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11920.

(58) Kozak, M. *Cell* **1980**, *22*, 7.

(59) Blainey, P. C., Van Oijen, A. M., Banerjee, A., Verdine, G. L. and Xie, X. S. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 5752.

(60) Dam, T. K., Gerken, T. A., Cavada, B. S., Nascimento, K. S., Moura, T. R. and Brewer, C. F. *J. Biol. Chem.* **2007**, *282*, 28256.

(61) Dam, T. K.; Gerken, T. A.; Brewer, C. F. *Biochemistry* **2009**, *48*, 3822.

(62) Lehotzky, R. E.; Partch, C. L.; Mukherjee, S.; Cash, H. L.; Goldman, W. E.; Gardner, K. H.; Hooper, L. V. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 7722.

(63) Randall-Hazelbauer, L. a. S., M. *J. Bacteriol.* **1973**, *116*, 1436.

(64) Meyer, J. E., Hofnung, M. and Schulz, G. E. *J. Mol. Biol.* **1997**, *266*, 761.

(65) Dutzler, R., Wang, Y-F., Rizkallah, P. J., Rosenbusch, J. P. and Schirmer, T. *Structure* **1996**, *4*, 127.

(66) Quiocho, F. A.; Spurlino, J. C.; Rodseth, L. E. *Structure* **1997**, *5*, 997.

(67) Schirmer, T.; Keller, T. A.; Wang, Y.-F.; Rosenbusch, J. P. *Science* **1995**, *267*, 512.

(68) Bezrukov, S. M.; Kullman, L.; Winterhalter, M. *FEBS Lett.* **2000**, *476*, 224.

(69) Jordy, M.; Andersen, C.; Schulein, K.; Ferenci, T.; Benz, R. *J. Mol. Biol.* **1996**, *259*, 666.

(70) Benz, R.; Schmid, A.; Vos-Scheperkeuter, G. *J.Membr. Biol.* **1987**, *100*, 21.

(71) Hall, J. A.; Ganesan, A. K.; Chen, J.; Kikaido, H. *J. Biol. Chem.* **1997**, *272*, 17615.

(72) Sharff, A. J.; Rodseth, L. E.; Spurlino, J. C.; Quiocho, F. A. *Biochemistry* **1992**, *31*, 10657.

(73) Bucher, D.; Grant, B. J.; Markwick, P. R.; McCammon, J. A. *PLoS Comput. Biol.* **2011**, *7*, e1002034.

(74) Mascarenhas, N. M.; Kästner, J. *Proteins* **2013**, *81*, 185.

(75) Spurlino, J. C.; Lu, G. Y.; Quiocho, F. A. *J. Biol. Chem.* **1991**, *266*, 5202.

(76) Duan, X.; Hall, J. A.; Nikaido, H.; Quiocho, F. A. *J. Mol. Biol.* **2001**, *306*, 1115.

(77) Boos, W.; Shuman, W. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 204.

(78) Oldham, M. L.; Chen, S.; Chen, J. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 18132.

(79) Brunkhorst, C.; Andersen, C.; Schneider, E. *J. Bacteriol.* **1999**, *181*, 2612.

(80) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

(81) Haddleton, D. M.; Ohno, K. *Biomacromolecules* **2000**, *1*, 152.

(82) Rye, C. S.; Withers, S. G. *Curr. Opin. Chem. Biol.* **2000**, *4*, 573.

(83) Wu, P.; Feldman, A. K.; Nugent, A. K.; Hawker, C. J.; Scheel, A.; Voit, B.; Pyun, J.; Fréchet, J. M. J.; Sharpless, K. B.; Fokin, V. V. *Angew. Chem. Int. Ed.* **2004**, *43*, 3928.

(84) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem.* **2002**, *114*, 2708.

(85) Dedola, S.; Nepogodiev, S. A.; Field, R. A. *Org. Biomol. Chem.* **2007**, *5*, 1006.

(86) Greffe, L.; Driguez, H.; CNRS. **2006**, Patent number: 7087746 2003.

(87) Kullman, L.; Winterhalter, M.; Bezrukov, S. M. *Biophys. J.* **2002**, *82*, 803.

(88) Cross, A. J.; Fleming, G. R. *Biophys. J.* **1984**, *46*, 45.

(89) Weatherman, R. V.; Kiessling, L. L. *J. Org. Chem.* **1996**, *61*, 534.

(90) Ranquin, A. a. V. G., P. *Res. Microbiol.* **2004**, *155*, 611.

(91) Dumas, F.; Koebnik, R.; Winterhalter, M.; Van Gelder, P. *J. Biol. Chem.* **2000**, *275*, 19747.

(92) Nallamsetty, S.; Waugh, D. S. *Nat. Protocols* **2007**, *2*, 383.

(93) Meyer, J. E. W.; Hofnung, M.; Schulz, G. E. *J. Mol. Biol.* **1997**, *266*, 761.

(94) Brzostek, K.; Heleszko, H.; Hrebenda, J. *J. Gen. Microbiol.* **1993**, *139*, 195.

(95) Stauffer, K. A.; Page, M. G. P.; Hardmeyer, A.; Keller, T. A.; Pauptit, R. A. *J. Mol. Biol.* **1990**, *211*, 297.

(96) Van Gelder, P.; Dumas, F.; Bartoldus, I.; Saint, N.; Prilipov, A.; Winterhalter, M.; Wang, Y.; Philippsen, A.; Rosenbusch, J. P.; Schirmer, T. *J. Bacteriol.* **2002**, *184*, 2994.

(97) Orlik, F.; Andersen, C.; Benz, R. *Biophys. J.* **2002**, *83*, 309.

(98) Bannwarth, M.; Schulz, G. E. *Biochim. Biophys. Acta - Biomembranes* **2003**, *1610*, 37.

(99) Baldwin, V.; Bhatia, M.; Luckey, M. *Biochim. Biophys. Acta - Biomembranes* **2011**, *1808*, 2206.

(100) Reimann, S. A.; Wolfe, A. J. *J. Bacteriol.* **2011**, *193*, 842.

(101) Prilipov, A.; Phale, P. S.; Van Gelder, P.; Rosenbusch, J. P.; Koebnik, R. *FEMS Microbiol. Lett.* **1998**, *163*, 65.

(102) Jaffe;, A.; Chabbert;, Y. A.; Semonin;, O. *Antimicrob. Agents Chemother.* **1982**, *22*, 942.

(103) Yu, F.; Mizushima, S. *J. Bacteriol.* **1982**, *151*, 718.

(104) Wang, Y. *Biochem. Biophys. Res. Commun.* **2002**, *292*, 396.

(105) Bredin, J.; Simonet, V.; Iyer, R.; Delcour, A. H.; PagÈS, J.-M. *Biochem. J.* **2003**, *376*, 245.

(106) Masi, M.; Pagès, J.-M. *Open Microbiol. J.* **2013**, *7*, 22.

(107) Prilipov, A.; Phale, P. S.; Koebnik, R.; Widmer, C.; Rosenbusch, J. P. *J. Bacteriol.* **1998**, *180*, 3388.

(108) Wang, D.-N.; Safferling, M.; Lemieux, M. J.; Griffith, H.; Chen, Y.; Li, X.-D. *Biochim. Biophys. Acta - Biomembranes* **2003**, *1610*, 23.

(109) Buchanan, S. K. *Curr. Opin. Struct. Biol.* **1999**, *9*, 455.

(110) Visudtiphole, V.; Thomas, M. B.; Chalton, D. A.; Lakey, J. H. *Biochem. J.* **2005**, *392*, 375.

(111) Francis, G.; Brennan, L.; Ferenci, T. *Biochim. Biophys. Acta - Biomembranes* **1991**, *1067*, 89.

(112) Wallace, B. A.; Lees, J. G.; Orry, A. J. W.; Lobley, A.; Janes, R. W. *Prot. Sci.* **2003**, *12*, 875.

(113) Jerabek-Willemsen, M.; Wienken, C. J.; Braun, D.; Baaske, P.; Duhr, S. *Assay Drug Dev. Technol.* **2011**, *9*, 342.

(114) Tribet, C.; Audebert, R.; Popot, J.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 15047.

(115) Luckey, M.; Ling, R.; Dose, A.; Malloy, B. *J. Biol. Chem.* **1991**, *266*, 1866.

(116) Giannis, A.; Sandhoff, K. *Tet. Lett.* **1985**, *26*, 1479.

(117) Alberti, A.; Bertini, S.; Comoli, M.; Guerrini, M.; Mele, A.; Vismara, E. *Tetrahedron* **2000**, *56*, 6291.

(118) Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 2928.

(119) Toshima, K.; Tatsuta, K. *Chem. Rev.* **1993**, *93*, 1503.

(120) Demchenko, A. V. In *Handbook of Chemical Glycosylation*; Wiley-VCH Verlag GmbH & Co. KGaA: **2008**, p 1.

(121) Cheaib, R.; Listkowski, A.; Chambert, S.; Doutheau, A.; Queneau, Y. *Tetrahedron-Asymmetry* **2008**, *19*, 1919.

(122) Van Gelder, P.; Dutzler, R.; Dumas, F.; Koebnik, R.; Schirmer, T. *Protein Eng.* **2001**, *14*, 943.

(123) Nikaido, H.; Rosenberg, E. Y. *J. Bacteriol.* **1983**, *153*, 241.

(124) Van Gelder, P.; Dutzler, R.; Dumas, F.; Koebnik, R.; Schirmer, T. *Protein Eng.* **2001**, *14*, 943.

(125) Park, J.; Rader, L. H.; Thomas, G. B.; Danoff, E. J.; English, D. S.; DeShong, P. *Soft Matter* **2008**, *4*, 1916.

(126) Panchadhayee, R.; Misra, A. K. *Synlett.* **2010**, *2010*, 1193.

(127) Williamson, D. J.; Fascione, M. A.; Webb, M. E.; Turnbull, W. B. *Angew. Chem. Int. Ed.* **2012**, *51*, 9377.

(128) Sekar, R. B.; Periasamy, A. *J. Cell Biol.* **2003**, *160*, 629.

(129) Heaney, H. In *Encyclopedia of Reagents for Organic Synthesis*; John Wiley & Sons, Ltd: **2001**.

(130) Niemietz, M.; Perkams, L.; Hoffman, J.; Eller, S.; Unverzagt, C. *Chem. Commun.* **2011**, *47*, 10485.

(131) Carrasco, M. R.; Nguyen, M. J.; Burnell, D. R.; MacLaren, M. D.; Hengel, S. M. *Tetrahedron Letters* **2002**, *43*, 5727.

(132) Rashidian, M.; Mahmoodi, M. M.; Shah, R.; Dozier, J. K.; Wagner, C. R.; Distefano, M. D. *Bioconjugate Chem.* **2013**, *24*, 333.

(133) Angyal, S. J. In *Advances in Carbohydrate Chemistry and Biochemistry*; Tipson, R. S., Derek, H., Eds.; Academic Press: **1984**; Vol. 42, p 15.

(134) Angyal, S. J. In *Advances in Carbohydrate Chemistry and Biochemistry*; Derek, H., Ed.; Academic Press: **1991**; Vol. 49, p 19.

(135) Rashidian, M.; Dozier, J. K.; Lenevich, S.; Distefano, M. D. *Chemical Commun.* **2010**, *46*, 8998.

(136) Moyle, P. M.; Muir, T. W. *J. Am. Chem. Soc.* **2010**, *132*, 15878.

(137) Mower, L. *Science* **1965**, *148*, 354.

(138) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131.

(139) Bycroft, B. W.; Chan, W. C.; Chhabra, S. R.; Hone, N. D. *J. Chem. Soc., Chem. Commun.* **1993**, 778.

(140) Patil, B. S.; Babu, V. S. *Chemistry* **2004**, *43*, 1288.

(141) Mani, N. S.; Kanakamma, P. P. *Synth. Commun.* **1992**, *22*, 2175.

(142) Watanabe, K.; Itoh, K.; Araki, Y.; Ishido, Y. *Carbohydr. Res.* **1986**, *154*, 165.

(143) Cheaib, R., Listkowski, A., Chambert, S., Doutheau, A., Queneau, Y. *Tetrahedron: Asymmetry* **2008**, *19*, 1919.

(144) Lopez, M.; Trajkovic, J.; Bornaghi, L. F.; Innocenti, A.; Vullo, D.; Supuran, C. T.; Poulsen, S.-A. *J. Med. Chem.* **2011**, *54*, 1481.

(145) Takeo, K.; Shinmitsu, K. *Carbohydr. Res.* **1984**, *133*, 135.

(146) Blanc-Muesser, M.; Driguez, H.; Lehmann, J.; Steck, J. *Carbohydr. Res.* **1992**, *223*, 129.

(147) Weng, S.-S.; Lin, Y.-D.; Chen, C.-T. *Org. Lett.* **2006**, *8*, 5633.

(148) Pearce, A. J.; Sollogoub, M.; Mallet, J.-M.; Sinaÿ, P. *Eur. J. Org. Chem* **1999**, *1999*, 2103.

(149) Ogawa, Y.; Hinou, H.; Matsuoka, K.; Terunuma, D. *Tet. Lett.* **1998**, *39*, 5789.

(150) Chen, Y.-X.; Koch, S.; Uhlenbrock, K.; Weise, K.; Das, D.; Gremer, L.; Brunsveld, L.; Wittinghofer, A.; Winter, R.; Triola, G.; Waldmann, H. *Angew. Chem. Int. Ed.* **2010**, *49*, 6090.

(151) Foillard, S.; Rasmussen, M. O.; Razkin, J.; Boturyn, D.; Dumy, P. *J. Org. Chem.* **2008**, *73*, 983.

(152) Gudmundsdottir, A. V.; Paul, C. E.; Nitz, M. *Carbohydr. Res.* **2009**, *344*, 278.