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Synthesis of a fluorescein-labelled *N*-acetyllactosamine derivative for use in fluorescence polarization studies with galectins

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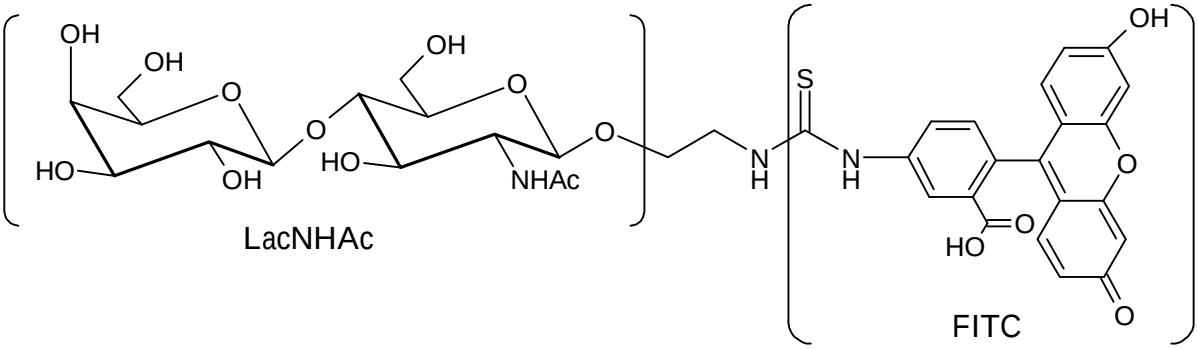
Abstract

This project is connected to a class of proteins named galectins. Galectins recognize carbohydrates and more specifically β-galactosides. Galectins have been studied for a long time and they have been suggested to be involved in tumor growth and metastasis, as well as in regulating immune responses. In this project a fluorescent derivative of *N*-acetyllactosamine (LacNHAc) has been synthesized in order to allow measurement of its binding to galectins. The fluorescent derivative was obtained by connecting the fluorophore fluorescein-5-isothiocyanate to the 2-aminoethyl glycoside of LacNHAc.



Finally, 0.58 mg of the derivative was gained. Even though this doesn't sound much, it can be used to approximately 44435 tests or 463 microwell plates, which contain 96 wells. Binding studies on galectin-1 and -3 were made by fluorescence polarization and gave values of the dissociation constant, K_d. The value of K_d was 17,936 μM for galectin-1 and 1,6915 μM for galectin-3, which means that galectin-3 has a higher affinity for the LacNHAc-FITC derivative synthesized in this master thesis.

Abbreviations

˚C	Degree Celsius
Å	Ångström, 1 Å=10 nm
Ac	Acetyl
Ac_2O	Acetic anhydride
AcCl	Acetyl chloride
aq.	Aqueous
Ar	Argon
ATP	Adenosinetriphosphate
AW	Acid washed
COSY	Correlation spectroscopy
CRD	Carbohydrate recognition domain
DMAP	*N, N'*-Dimethyl-4-aminopyridine
DMF	*N, N*'-Dimethylformamide
DMSO	Dimethylsulfoxide
e.g.	for example (exempli gratia)
eq.	Equivalent
et al.	And others (et alii)
Et	Ethyl
EtOAc	Ethyl acetate
EtOH	Ethanol
FITC	Fluorescein-5-isothiocyanate
FP	Fluorescence polarization
h	Hour
HPLC	High performance liquid chromatography
i.e.	That is (id est)
lac	lactose
LacNHAc	*N*-acetyllactoseamine
Me	Methyl
Me_4Si	Tetramethylsilane
MeOH	Methanol
mP	milli polarization units
MS	Molecular sieve
N_3	Azide
NaOMe	Sodium methoxide
NHAc	*N*-acetyl
NHS	*N*-hydroxysuccinimide
NIS	*N*-iodosuccinimide
NMR	Nuclear magnetic resonance
NTCP	*N*-Tetrachlorophtalimide
Pd/C	Palladium on activated charcoal
pg.	Page
pgs.	Pages
Ph	Phenyl
RNA	Ribonucleic acid
rt.	Room temperature
sat.	Saturated
TCP	Tetrachlorophtalimide
TfOH	Trifluoromethanesulfonic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV	Ultraviolett

Contents

1. Objectives

The objective for this project was to synthesize a new derivative of the known galectin inhibitor *N*-acetyllactosamine and couple it with fluorescein-5-isothiocyanate. This was done in order to be able to measure ligand binding by galectins.

2. Introduction

2.1 General

It is quite clear that carbohydrates play an important role inside the body. They are our main source of energy but are used in the body for much more than energy. They can for instance act as recognition molecules for antibodies in the blood. This project is connected to another type of proteins, namely galectins. Galectins are a class of proteins called lectins that recognize carbohydrates, more specifically β-galactosides. Galectins have been studied for a long time and they have been suggested to be involved in tumor growth and metastasis, as well as in regulating immune responses. In this project a fluorescent derivative of *N*-acetyllactosamine (LacNHAc) has been synthesized in order to allow measurement of its binding to galectins. The fluorescent derivative was obtained by connecting the fluorophore fluorescein-5-isothiocyanate to the 2-aminoethyl glycoside of LacNHAc.

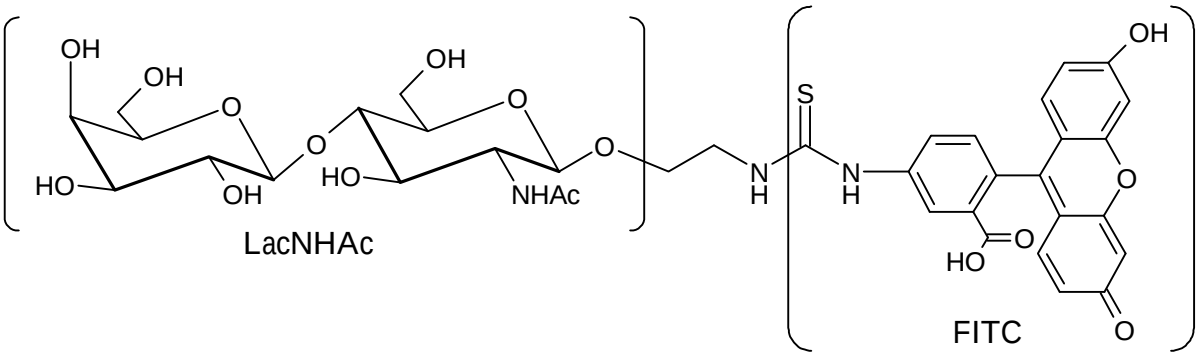


Figure 2.1 The target molecule, 2-(fluorescein-5-thiourea)-ethyl (β-D-galactopyranosyl)-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranoside) (1).

2.2 Carbohydrates

Carbohydrates received their name from early studies that revealed that these molecules often had the molecular formula $C_x(H_2O)_x$, or appeared to be hydrates of carbon [1, pg. 1046]. Carbohydrates have been known for a long time, at least since the beginning of the 19th century and at this time glucose, fructose, galactose and sorbose were already known. The three first simple sugars were discovered by the hydrolysis of ordinary table sugar or sucrose, giving fructose and glucose, respectively the milk sugar lactose, giving galactose and glucose. In 1811 Kirchhoff of St. Petersburg discovered by hydrolysis that starch was a complex structure of glucose molecules. [2, pg. 344]

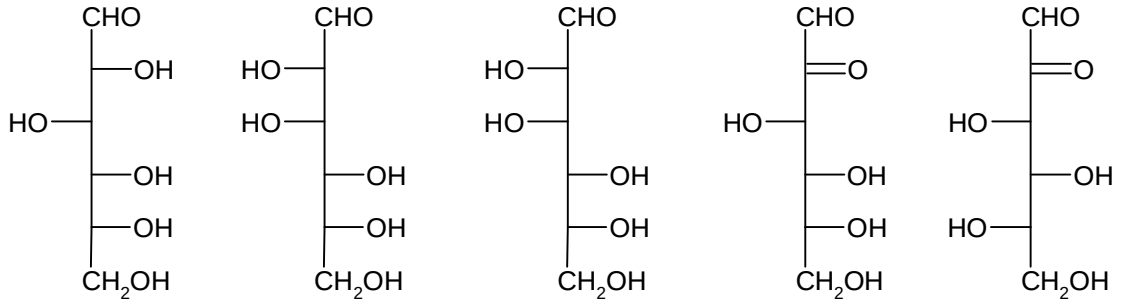


Figure 2.2 Some monosaccarides and their aldehyde structures.

In 1888 Emil Fischer started to work on the stereochemistry of D-(+)-glucose [1, pg. 1073]. To his help he used his own, recently discovered, phenylhydrazine. When it reacted with glucose, fructose and mannose, it formed the same osazone, which meant that they had to be identical below the second carbon atom. In his future work he started using the van't Hoff's rule and proposal on the carbon's tetrahedral structure and thereby could calculate that the aldehyde glucose could have 2^4=16 isomers or 8 pairs of enantiomorphs. When he attacked the problem he used the little information his predecessors had left him together with all of his own work, for instance the discovery of D-(+)-mannose. The most important among these predecessors was Killiani that worked on carbohydrates parallel to Fischer's work on the stereochemistry of glucose. To simplify the problem, Fischer decided to limit the search to the part of sugars that could be derived from D-(+)-glyceraldehyde, and it would take until 1951 before this assumption was proved to be right [1, pg. 1073]. The D in all sugar molecules comes from Fischer as well, who decided to call a substance a D-substance if the hydroxide group nearest to the primary alcohol is pointing to the right when the aldehyde group is at the top. [2, pgs. 345-354]

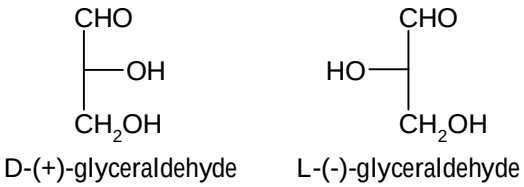


Figure 2.3 The two glyceraldehydes.

Finally in 1891 Fischer could assign glucose and some of the other sugars to the right configuration, which meant that he went on searching for new problems and two years later he discovered the first methyl glucoside. One year after this discovery, W. Alberda van Ekenstein discovered another methyl glucoside. Studies of these two methyl glucosides (α & β) suggested that hexoses must contain an oxygen-containing ring since that would create an asymmetrical fifth carbon, which would explain the two methyl glucosides. [2, pgs. 355-356]

Even though he correctly assigned the oxygen ring structure to the different methyl glucosides he failed to put those results together with glucose and did not understand that it had to do with mutarotation, first discovered in 1846 by Dubrunfaut. Mutarotation is a phenomenon, further studied by Charles Tanret, which occurs when the two different glucoses, one with a specific rotation of +113º and the other of +19º, are dissolved in water. The two solutions both change their rotation to +52,5º, which is because glucose in water is at equilibrium with the open-chain form. [2, pg. 636]

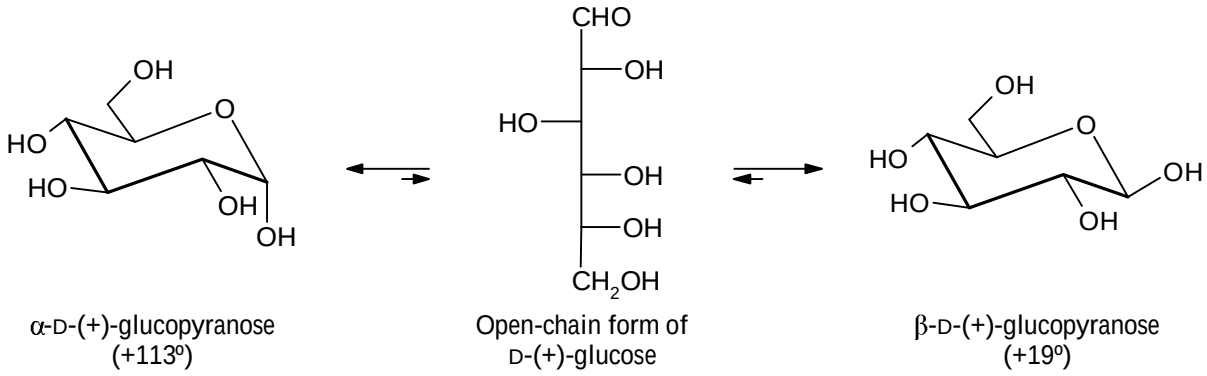


Figure 2.3 Mutarotation.

In 1903, however, Edward F. Armstrong used emulsin and maltase to produce the two different glucoses from Fischer's two methyl glucosides. Emulsin produced the high-rotating form of glucose (α) and maltase produced the low-rotating form (β). During the next thirty years the structure of the sugar molecule was studied in more detail, with special interest in the oxygen-containing ring and about 1926 Haworth and Hirst proved that the methyl glucoside commonly occurs in the pyranose form even though the furanose form also is possible. [2, pg. 636-637]

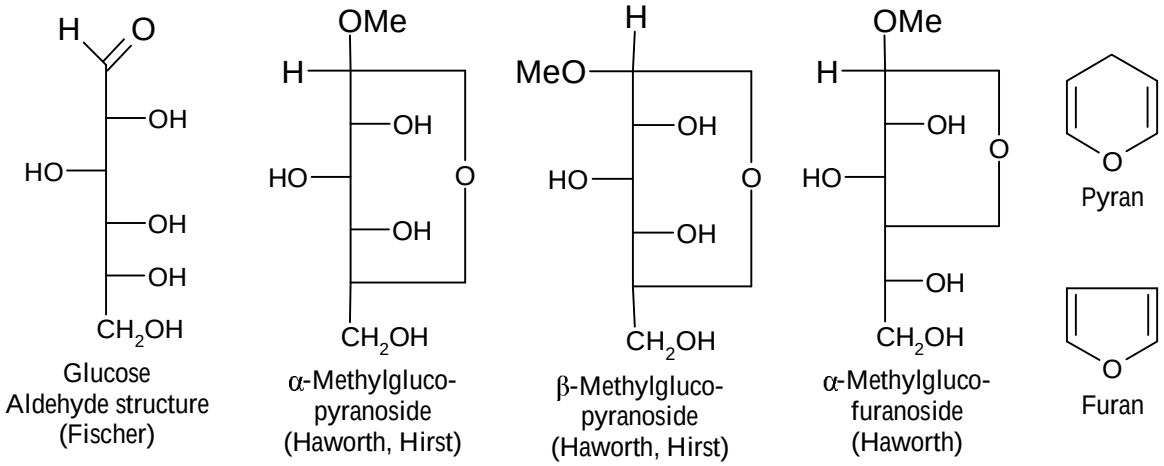


Figure 2.4 The methyl glucosides and its structures as proposed by Haworth & Hirst.

In the thirties the most important structural problems regarding monosaccharides had been solved and the carbohydrate science was turned against glycosides and polysaccharides. Even though the last one contained a really complex problem, work continued in that direction because of the economic importance of cellulose and starch. [2, pg. 637]

2.2.1 Carbohydrate chemistry

Only two typical carbohydrate reactions will be presented, both of essential character to this project. The reactions are protective group chemistry and glycoside synthesis.

2.2.1.1 Protective groups

When a synthesis of a rather complex molecule is to be performed it can contain as much as 15-20 steps and sometimes more. In the planning of such a synthesis it is important to take notice of the different functional groups' compatibility. To prevent interference of a specific functional group it is often necessary to transform it. One way of doing so is by the use of a protective group, which is a derivative that can easily be inserted and then just as easily be removed again, without destroying the protected group's functionality. Examples are the protection of alcohols with trisubstituted silyl ethers and aldehydes as acetals. [3, pg. 677]

$$R-OH + R'_3SiX \rightarrow R-O-SiR'_3$$
$$RCH=O + R'OH \rightarrow RCH(OR')_2$$

The protective groups play a passive role in the synthesis thereby adding steps to the synthetic sequence, which means that it is desirable to keep such reactions at a minimum. When a protective group is to be chosen three different considerations are of importance:

1. The nature of the group requiring protection.
2. The reaction conditions under which the protective group must be stable.
3. The conditions that can be tolerated for removal of the protective groups.

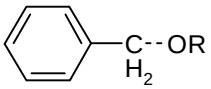
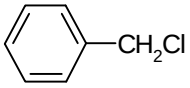
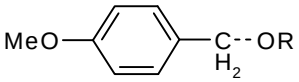
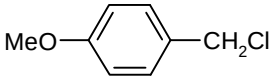
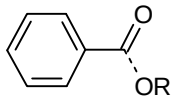
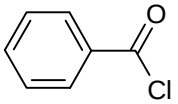
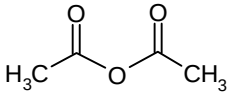
No ultimate protective groups exists but instead there have been developed a wide variety of different protective groups to fit most every need in the design of synthesis of complex molecules. [3, pgs. 677-678]

A different way of dealing with interfering functional groups is masked functionality, which means that you introduce the group in modified form. This can for example be done by introducing a vinyl ether that can be hydrolyzed to carbonyl compounds and the below Wittig reaction is one specific example of this phenomena. The vinyl ether that is produced can then be hydrolyzed to the desired carbonyl group later in the synthesis. [3, pg. 678]

$$RCH=O + Ph_3P=CHOR' \rightarrow RCH=CHOR' \xrightarrow{H^+ + H_2O} RCH_2CH=O$$

The first protective groups to consider, concerns the protection of hydroxyl groups, which is very important in carbohydrate chemistry. Some hydroxyl protective groups are shown in table 2.1. [3, pgs. 678-683]

Table 2.1 Some hydroxyl-protective groups, their introduction and removal methods.

Protective group (Including alcohol)	Reagent	Introduction	Removal
$RO--CH_2OCH_2CH_2OCH_3$ Methoxymethyl ether (MEM)	$CH_3OCH_2CH_2OCH_2Cl$ Metoxymethyl chloride	Reaction with an alkali metal salt of the alcohol	Nonaqeuos conditions with a Lewis acid (e.g. ZnBr, $TiCl_4$)
 Benzyl ether (Bz)	 Benzyl chloride	1. Williamson ether synthesis 2. Non-basic conditions, e.g. reaction with trichloroacetonitrile	a) Catalytical hydrogenolysis b) Sodium in liquid ammonia
 4-Methoxy-benzyl ether	 4-Methoxy-benzyl chloride	1. Williamson ether synthesis 2. Non-basic conditions, e.g. reaction with trichloroacetonitrile	Oxidatively with dichlorodicyanoquinone
Trimethylsilyl ether (TMS) or t-Butyldimethylsilyl ether (TBDMS)	Trimethylsilylchloride or t-Butyldimethylchloride	1. In the presence of an amine 2. By heating with hexamethyldisilazane	a) Hydrolytic conditions b) F^-, e.g. anhydrous tetra–*N*-butylammonium fluoride, aq. hydrogen fluoride or boron trifluoride
 Benzoat	 Benzoyl chloride	Pyridine or some other tertiary amine	Base-catalyzed hydrolysis
 Acetate	 Acetic anhydride	Pyridine or some other tertiary amine	Base-catalyzed hydrolysis

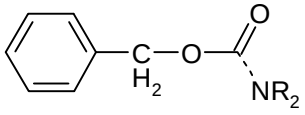
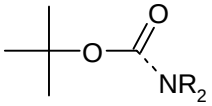
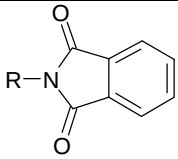
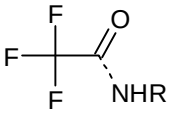
Even though most of the protective groups shown in table 2.1 are commonly used in carbohydrate chemistry, in this project only the last one were used. The hydroxyl groups were protected with acetyl and lauroyl groups.

$$R-OH+CH_3(CH_2)_{10}COCl \xrightarrow{DMAP,\ Pyridine} R-CO(CH_2)_{10}CH_3$$

Lauroyl esters were used because the hydrophobic properties of the long alkyl tail allows for simplified purifications of intermediates later in the synthesis, namely C18 separation.

Other protective groups are e.g. the ones that can protect amines. There are less amine-protective groups as compared to the hydroxyl-protecting groups. Some of them are collected in table 2.2. [3, pgs. 686-688]

Table 2.2 Some amine-protecting groups and their removal methods.

Protective group (Including amine)	Removal
 Carbobenzyloxy amine (CBz)	1. Hydrogenation, followed by spontaneous decarboxylation 2. Transfer hydrogenolysis, e.g. ammonium formate or formic acid with Pd/C catalyst
 t-Butoxycarbonyl amine (TBOC)	Acid such as trifluoroacetic acid or *p*-toluenesulfonic acid
 Phtalimide	1. Hydrazine 2. $NaBH_4$ in aq. ethanol
 Trifluoroacetamide	Mild basic conditions, e.g. $Ba(OH)_2$, 25ºC, 18 h

In this project, a modified version of the phtalimide is used. It is called tetrachlorophtalimide (NTCP) and is formed in two steps and the amine can later in the synthesis be deprotected by the use of diaminoethane in EtOH at 60-80 ºC [4, pgs. 46-47, 72-73].

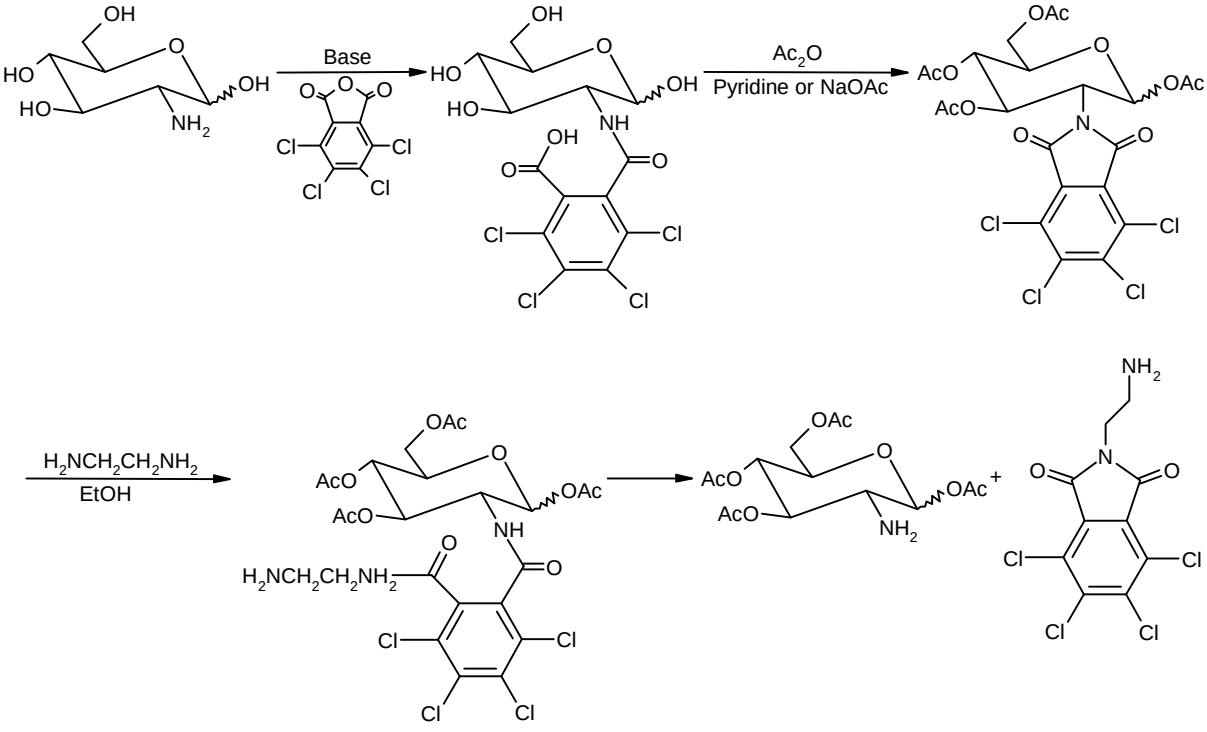


Figure 2.5 The formation and removal of the protective group tetrachlorophtalimide.

2.2.1.2 Glycoside synthesis

Just as there is no such thing as a universal protective group there is no universal method of performing a glycoside synthesis or as H. Paulsen describes it:

"Although we have now learned to synthesize oligosaccarides, it should be emphasized that each oligosaccaride synthesis remains an independent problem, whose resolution requires considerable systematic research and a good deal of know-how. There are no universal reaction conditions for oligosaccaride synthesis." [5, pg. 156]

Despite of this fact, a glycoside synthesis can only choose one of two basic pathways, which are the nucleophilic substitution reactions S_N1 and S_N2. However, in practice the S_N2 can't be used in performing a glycoside synthesis, leaving only S_N1 as the pathway of interest. A glycoside synthesis is namely run by generating a carbocation by allowing a promotor to react with the leaving group, thereby making it a good leaving group.

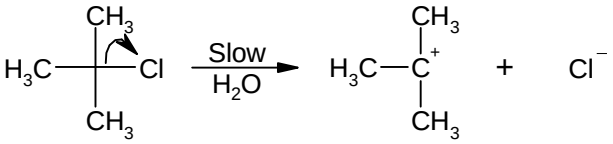
In a S_N1 reaction, the insertion of the nucleophile takes place in the three steps. In the first step, which is rate-determining, a carbocation is formed which then reacts in two steps to produce the wanted product. The carbocation that is formed must be relatively stable, which is the case of the *tert*-butyl cation. This cation is stabilized because the charge is dispersed or delocalized over the three methyl groups that are attached to it. [1, pgs 240-247]

Reaction:

$$(CH_3)_3Cl + 2H_2O \rightarrow CH_3OH + H_3O^+ + Cl^-$$

Mechanism:

Step 1:



Step 2 and 3:

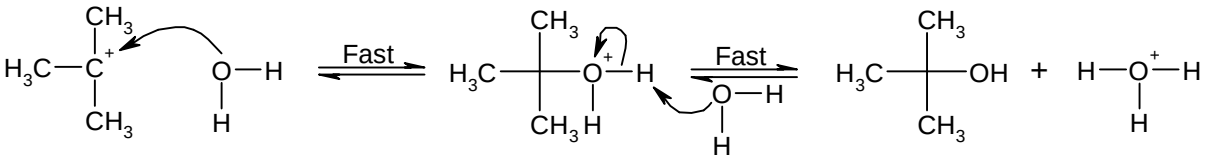


Figure 2.6 A mechanism for the S_N1 reaction.

The reaction conditions can be changed to fit the reaction and the three most important factors for the relative rate of a S_N1 reaction are listed below [1, pgs. 248-260].

1. The structure of the substrate (e.g. the *tert*-butyl chloride in figure 2.6).
2. The effect of the solvent.
3. The nature of the leaving group.

In glycoside synthesis, the most commonly used substrates, or donors as they are called in carbohydrate chemistry, are glycoside halides and such a synthesis is often catalyzed by a silver or mercury salt. When a silver salt is present it is called a Koenigs-Knorr reaction and when a mercury salt is used, the reaction is said to take place according to the Helferich procedure [6, pg. 301]. The different usually used catalysts have a reactivity as follows:

$$AgOTf\ /\ Ag_2CO_3 > AgClO_4\ /\ Ag_2CO_3 > HgBr_2 > Hg(CN)_2\ /\ HgBr_2 > Hg(CN)_2$$

Other donors used are sulfonyloxy, imidates and trichloroimidates or activated thioalkyl or thioaryl glycosides [6, pgs. 302-306]. In this project a thiophenyl acceptor was used and the thiophenyl group is directly activated by the iodonium ion generated from the promotor *N*-iodosuccinimid (NIS) and TfOH.

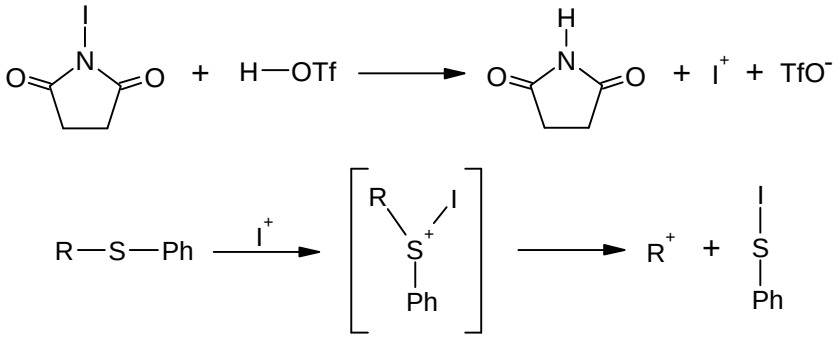


Figure 2.7 Direct activation of a thioglycoside.

The carbocation that is produced in this step is called oxocarbenium ion and if nothing would happen to this ion before the nucleophile (the acceptor) attacks the donor, a mixture of α- and β-glycosides would be the result. In this project, however, something called neighboring group participation must be taken in account. This is a phenomenon that occurs when the group on C-2 is an ester and what happens is that the oxocarbenium ion is transformed into a acyloxonium ion. The acyloxonium ion then makes it impossible for the donor to react to anything but the β-coupling.

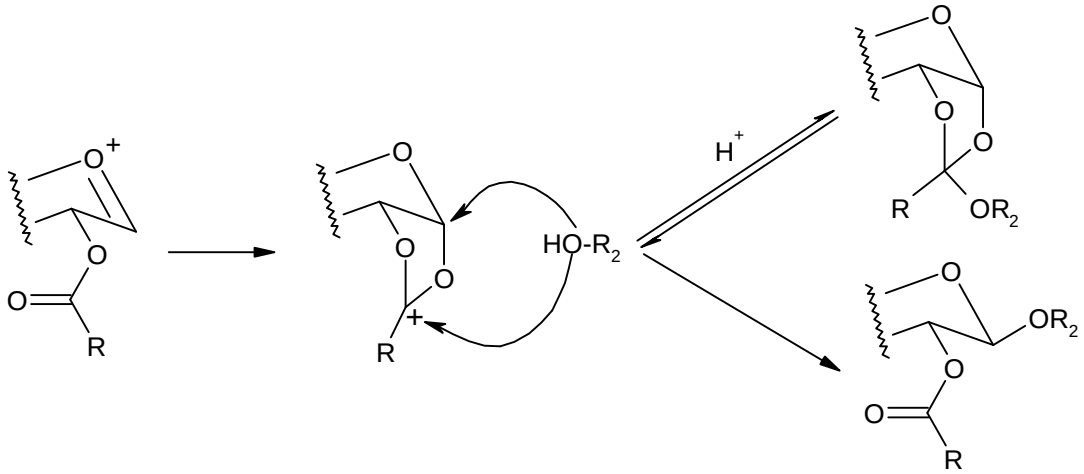
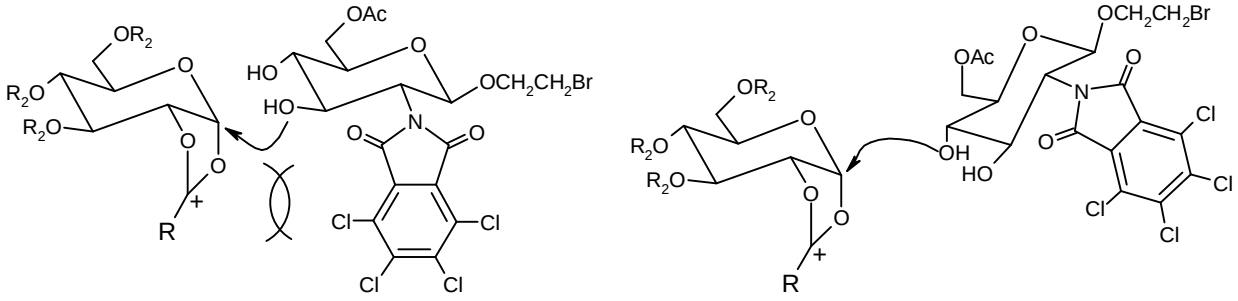


Figure 2.8 A mechanism for acyl group participation in glycoside formation.

However, the acceptor can still react at two places, either at C-1 or at the carbonyl carbon, thereby producing an ortho-ester but the formation of the ortho-ester is usually reversible under the reaction conditions. This means that even though the ortho-ester is formed during the reaction, it is only a temporary diversion from the pathway towards the glycoside. [6, pgs. 307-311]

The final step towards getting the right glycoside is to predict the regioselectivity of the glycosylation. The easiest way to do this is to have only one unprotected hydroxyl group on the acceptor, which mean that only one reaction path is possible. Of the different hydroxyl groups on a monosaccaride, the hydroxyl on C-6 is easy to protect selectively since it is a primary alcohol. Protection of the other hydroxyl groups would need more complex reaction steps, but in this project regioselectivity between HO-3 and -4 was achieved by exploiting steric hindrance [4, pg. 70].

If the acceptor is such that it can be fitted with a protective group that is quite bulky, that group could help the regioselectivity towards the wanted result. This is done in this project where the *N*-tetrachlorophtalimide group (NTCP) guides the acceptor to a (1→4) coupling by sterically blocking HO-3.



β-(1→3) coupling β-(1→4) coupling

Figure 2.9 The regioselectivity of tetrachlorophtalimide.

2.3 Carbohydrate-binding proteins

Antibodies are a class of proteins that often recognizes carbohydrates, as is the case of antibody-recognition of carbohydrates on erythrocytes. The erythrocyte membrane contains glycosylated proteins and lipids that differ from person to person. The carbohydrates thus act as antigens when exposed to another person's blood, which is the base for the dividing of the human blood into different groups, A, B, AB or O. The people in blood group A therefore have anti-B antibodies, the people in blood group B have anti-A antibodies, the people in blood group AB have neither and the people in blood group O have both. [7, pg. 718]

Enzymes are a class of proteins that act as catalytical reagents for many reactions including the metabolism of carbohydrates. One example is hexokinase that starts the glycolysis by breaking down glucose into glucose-6-phosphate by transferring a phosphoryl group from an ATP-molecule to glucose [8, pg. 486]. Another example is lactase that by hydrolysis breaks down the disaccaride molecule lactose to its monosaccaride components, galactose and glucose. Although nearly all infants and children are able to digest lactose a majority of the world's adults have a defiency of lactase, which makes them intolerant to milk. The defiency of lactase in human populations greatly varies though, for instance are 97% of the Thais deficient in lactase, compared to only 3% of Danes. [8, pg. 472] The last example is glucosyle-transferase that adds glucose to *N*-linked oligosaccarides on unfolded or misfolded glucoproteins [8, pg. 921].

Lectins are a type of carbohydrate-binding proteins that can be divided into at least three groups depending on their properties and sequence of their carbohydrate recognition domain

(CRD). These are C-type lectins, S-lac lectins, P-type lectins and those that neither belongs to C-type, S-lac or P-type lectins. The C-type lectins require calcium for carbohydrate binding and are either soluble secretory or integral membrane proteins. The S-lac lectins are lactose binding soluble vertebrate proteins that don't require any cations for carbohydrate binding and are called galectins. The P-type lectins for instance contain the mannose 6-phosphatase receptor that targets newly synthesized lysosomal hydrolases to the lysosome. The last group contains for instance a pentraxin called serum amyloid protein and this is localized to brain lesions of Alzheimer's disease. [9]

2.3.1 Galectins

Galectins are a type of cytosolic proteins that has a high affinity for β-galactosides, such as lactose [10] (i.e. S-lac lectins). Their carbohydrate-binding domains consist of about 130-140 amino acids, which are tightly folded into a sandwich of a 6-stranded and a 5-stranded β-sheet [11]. The 14 known galectins in mammals vary in size from 14-36 kDa and can be seen in figure 2.10. Three subgroups are then revealed, whereas two of them consist of dimers and the other one of monomers. All of them also contain a piece of peptide that is not included in the CRD. In galectins 4, 6, 8, 9 and 12, these *N*-terminal peptides function as a bridge between the different parts of the proteins. In galectins 3, 5, 7, 10, 11, 13 and 14 it consists of a more flexible *N*-terminal peptide and in galectin-3 this *N*-terminal peptide consists of an intervening domain, which is rich in proline, glycine and tyrosine [9].

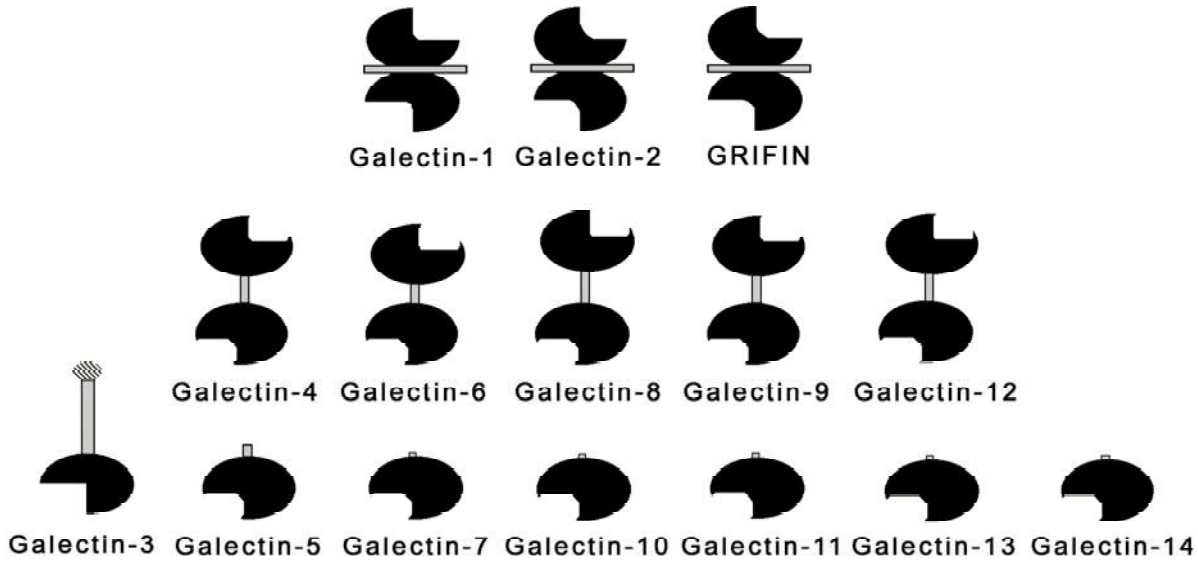


Figure 2.10 The 14 different galectins, which has been discovered so far. GRIFIN is a protein that looks like a galectin but lacks the possibility to bind carbohydrates.

Galectins are cytosolic nuclear proteins that secrete along non-classical pathways. Because they typically bind extracellular carbohydrates, most attention has been given to their actions outside the cell. Examples are binding and crosslinking glycoconjugate ligands, thereby moderating cell adhesion and cell signaling. However, galectins are also nuclear proteins, which imply that they could have intracellular actions as well, such us moderating RNA-splicing, apoptosis and the cell cycle. [10]

Although its precise mechanism is unclear there is strong evidence that galectins and specifically galectin-3 have a roll in immunity regulation, inflammation and cancer. Its proinflammatory role is based on the induction in inflammatory conditions such as effects on immune cells (oxidative burst in neutrophils, chemotaxis in monocytes) and alterations in inflammatory response (mainly in neutrophils). It has also been implicated that galectin-3 acts as a regulator when the "immunological synapse" between T-cells and antigen-presenting cells are formed. Galectin-3 has also been suggested by correlative and experimental evidence to have a cancer-promoting effect by anti-apoptotic action and by promoting angiogenesis and metastasis, this by effecting cell adhesion. Inhibition of galectins by glycoconjugates is suggested, but not proven, to decrease metastasis in mice. [10]

To find suitable inhibitors for galectin-3 its carbohydrate-binding domain can be studied. Figure 2.11 show this region, which in the case of galectin-3 is relatively large and even could bind a molecule as large as a tetrasaccaride. The protein's active site is however fitted to bind lactose or β-galactoside-containing saccarides. In lactose it is galactose that is fitted deeply inside the active site. The H-3, H-4 and H-5 atoms in the galactose unit form a hydrophobic patch that makes van der Waals contact with a tryptophan side-chain in the protein and the HO-4 and HO-6 groups binds to the protein by hydrogen bonds. Since HO-2 points directly towards the protein only HO-3 is left for extensions, which has been exploited by Pernilla Sörme et al. [10]

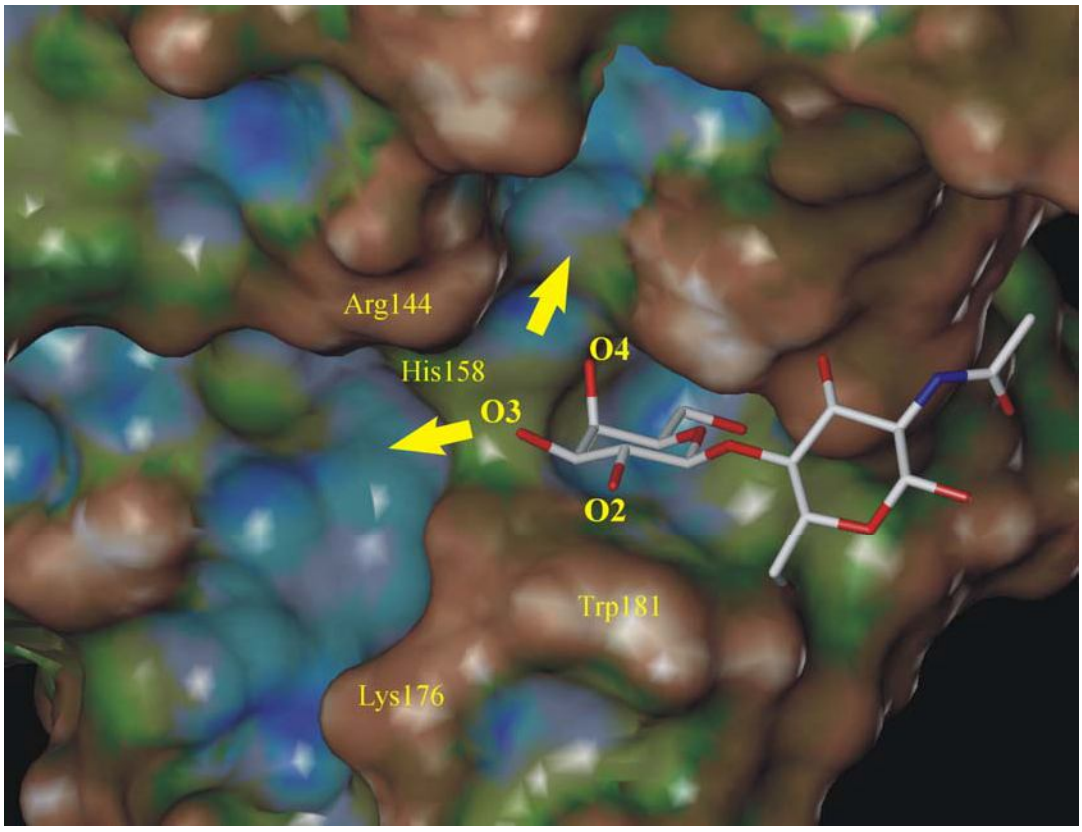


Figure 2.11 Connolly surface of the galectin-3 carbohydrate recognition domain. (Courtesy of Dr. P.-G. Nyholm, Gothenburg University)

2.4 Fluorescence polarization

The concept of fluorescence polarization (FP) was first described by Jean Perrin in 1926 but hasn't been utilized until the 1990:s. FP is based on the fact that molecules of different sizes behave differently when excited with plane-polarized light. A small fluorescent molecule rotates more rapidly in solution compared to a large molecule during its fluorescence lifetime[1], which results in a depolarization of the emitted light. If this small fluorescent molecule were to be bound to a large molecule, e.g. a protein, it would slow down the rotation thereby producing the emitted light to stay polarized. [12, pg. 1-2] This phenomena is described by the following equation:

$$\text{Equation 2.1:} \quad \textit{Polarization value} \propto \textit{Rotational relaxation time} = \frac{3\eta V}{RT}$$

It shows that a molecule's polarization value is proportional to the relaxation time, which is the time it takes to rotate 68.5º. This means that the polarization value is directly related to the molecular volume (V) if the viscosity (η) and the temperature (T) are held constant. Binding or dissociation of two or more molecules, degradation or conformational changes can result in changes in molecular volume (i.e. molecular size). [12, pg. 1-3]

Figure 2.12 shows a scheme that describes how fluorescence polarization detection works. Light from a monochromatic light source is passed through a filter that results in a vertical polarization. When the vertically polarized light hits the sample only the molecules that are vertically oriented can be excited and emit light. The emitted light is then passed through a moving polarizing filter and is then measured in both the vertical and horizontal planes.

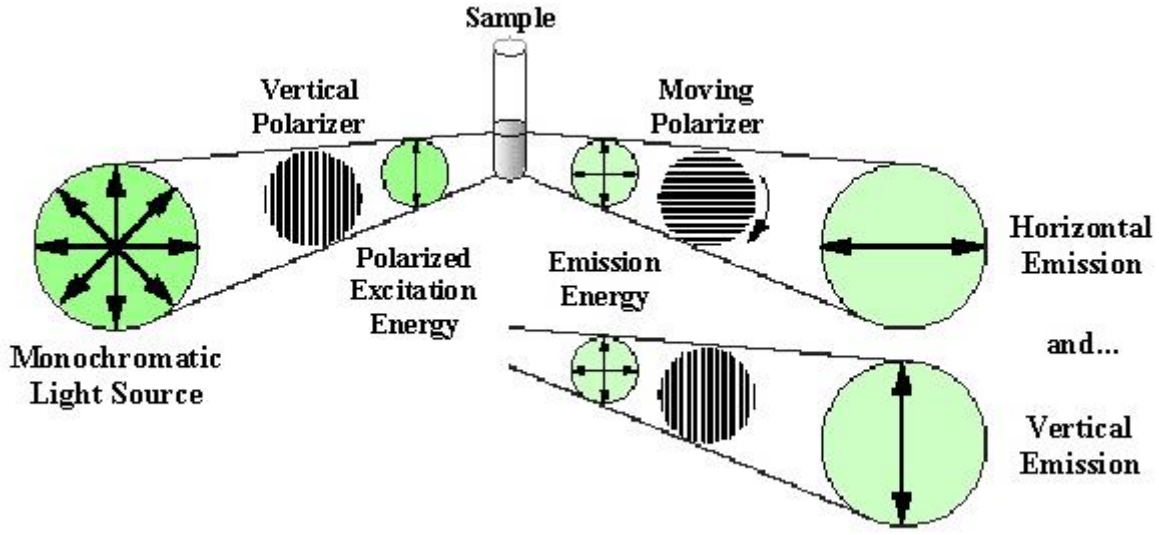


Figure 2.12 Schematic representation of FP detection [12, pg. 1-3].

[1] The time between excitation and emission.

The polarization value, which is a measure of the extent of molecular rotation during the fluorescence lifetime, can then be calculated as followed:

$$\text{Equation 2.2:} \quad \textit{Polarization value } (P) = \frac{Intensity_{vertical} - Intensity_{horizontal}}{Intensity_{vertical} + Intensity_{horizontal}}$$

Because of the fact that the polarization is given as a ratio of light intensities makes it a dimensionless number, often expressed in millipolarization units.

Illustrated examples of a small molecule respectively a large complex with high respectively low polarization values are shown in figure 2.13. The small molecule spins rapidly when excited and during emission, thereby producing a low polarization value. A large molecule, e.g. a protein-inhibitor-complex, spins slowly, thereby producing a high polarization value. [12, pgs. 1-3, 1-4]

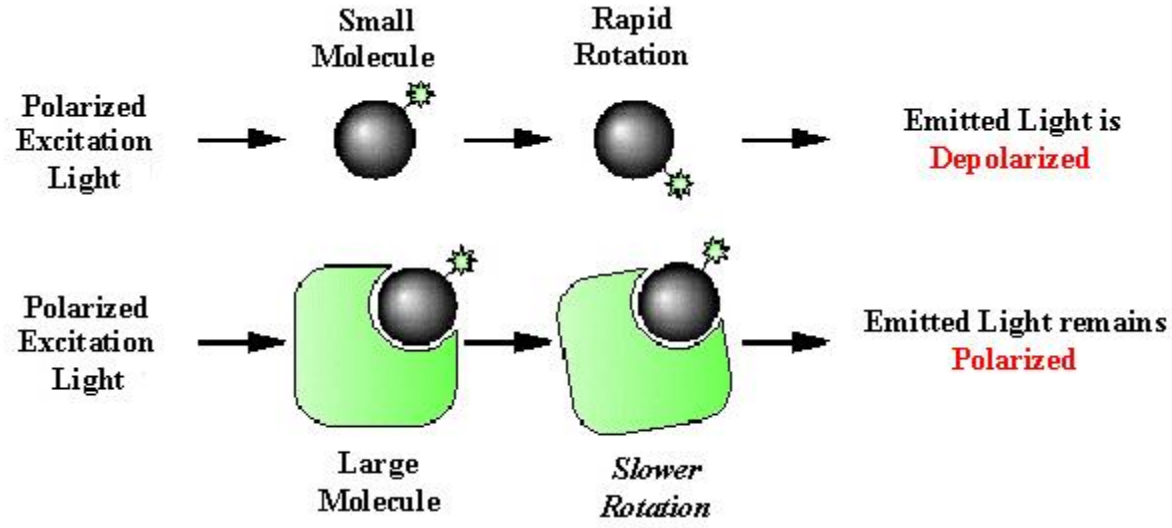


Figure 2.13 FP differences between a small molecule and a large complex [12, pg. 1-4].

Another way of representing the amount of binding that occurs is to look at the dissociation constant, K_d. This constant is an important value to get from the fluorescence polarization value. According to Clark's theory [12, pg. 7-2] the quantification of a physical constant that defines the classical ligand-receptor interaction depends on the following assumptions:

1. The interaction is irreversible. The association reaction is bimolecular while the dissociation is unimolecular.
2. All the receptor molecules are equivalent and independent.
3. The biological response is proportional to the number of occupied receptor sites.
4. The interaction and response are measured after the reaction has reached equilibrium.
5. The active chemical agent does not undergo degradation or participate in other reactions, and only exists in either a free (L_F, unbound) form or bound to the receptor (B).

The assumptions described above gives the following reaction:

$$\text{Receptor}_{\text{free}} + \text{Ligand}_{\text{free}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Receptor:Ligand complex} \xrightarrow{k_e} \text{effect}$$

where k_1 and k_{-1} are the kinetic association and dissociation constants and k_e stands for the proportionality constant between response and occupancy. However, to determine a physical constant, it is not normally necessary to measure k_e and to simplify the rest of this discussion, the focus will from now on be on the reversible reaction.

At equilibrium the dissociation constant can be written as the fraction between the reactants' and the products' concentrations or as fraction between the constants:

$$\text{Equation 2.3:} \quad K_d = \frac{[Ligand_{free}] * [\text{Re}ceptor_{free}]}{[\text{Re}ceptor : Ligand \ \ complex]} = \frac{[L_F] * [R_F]}{[B]} = \frac{k_{-1}}{k_1}$$

Equation 2.3 can then be rewritten by adding the total receptor concentration, R_T, and rearranged to show the equation for a rectangular hyperbola with horizontal asymptote corresponding to 100% saturation of R, such as [bound]=[receptor].

$$\text{Equation 2.4:} \quad K_d = \frac{[L_F] * [R_T - B]}{[B]}$$

$$\text{Equation 2.5:} \quad f = \frac{B}{R_T} = \frac{L_F}{[K_d + L_F]}$$

The curve associated with equation 2.5 is shown in figure 2.14. A new quantity has been added in equation 2.5 and it stands for the fractional occupancy. When this quantity is set to 0.5 the equation show the definition of K_d, which is defined as the concentration of free ligand at the time where 50% of the receptor sites are occupied.

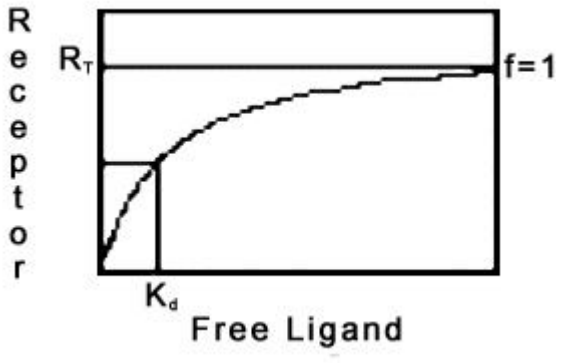


Figure 2.14 The classical hyperbolic binding curve, expressed at the fractional occupancy, f, of the receptor [12, pg. 7-3].

In the testing procedures, however, the results come in form of a diagram where the polarization value (P) in mP is plotted against the concentration of the protein, in this project galectin [G], see figure 2.15. The dissociation constant can then be gained from adapting an equation to the curve by non-linear regression:

Equation 2.6: $$P = P_0 + \frac{(P_{max} - P_0) * [G]}{K_d + [G]}$$

where P_0 is the lowest polarization value and P_{max} is the highest [13].

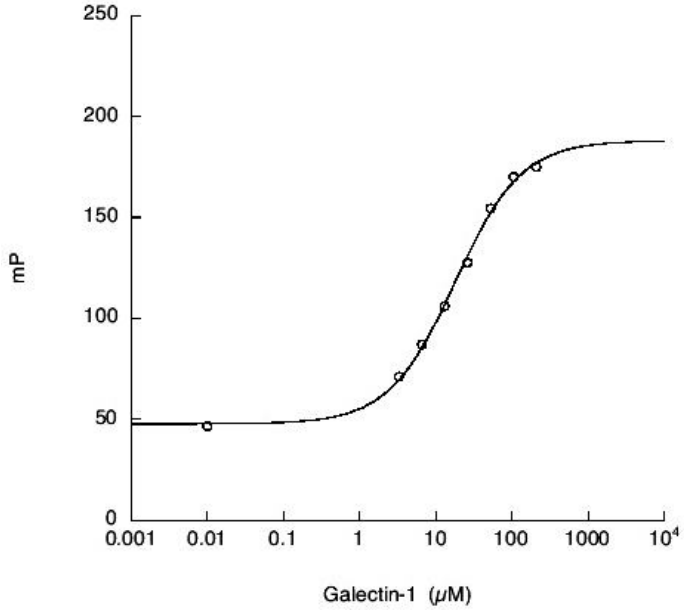


Figure 2.15 Binding study of Galectin-1.

2.5 Retro-synthetic analysis

The LacNHAc derivate coupled to FITC that was the target molecule for this project can be made from an unprotected galactose and an acetyl protected *N*-acetylglucosamine in an 8-step synthesis. The retro-synthetic analysis scheme, see figure 2.16, show the crucial steps for this synthesis.

First the hydroxyl groups on the glycoside had to be protected and this was done by lauroyl groups on the galactose part and acetyl groups on the glucose part. The NHAc had to be protected and this was done by the NTCP group. The next part offered challenges. The original idea was to synthesize the bromoethyl-substituted glycoside (8) first and then transform the bromide into the azide. This did not succeed and therefore another path had to be chosen which meant that the bromine was transformed to the azide before the glycosylation.

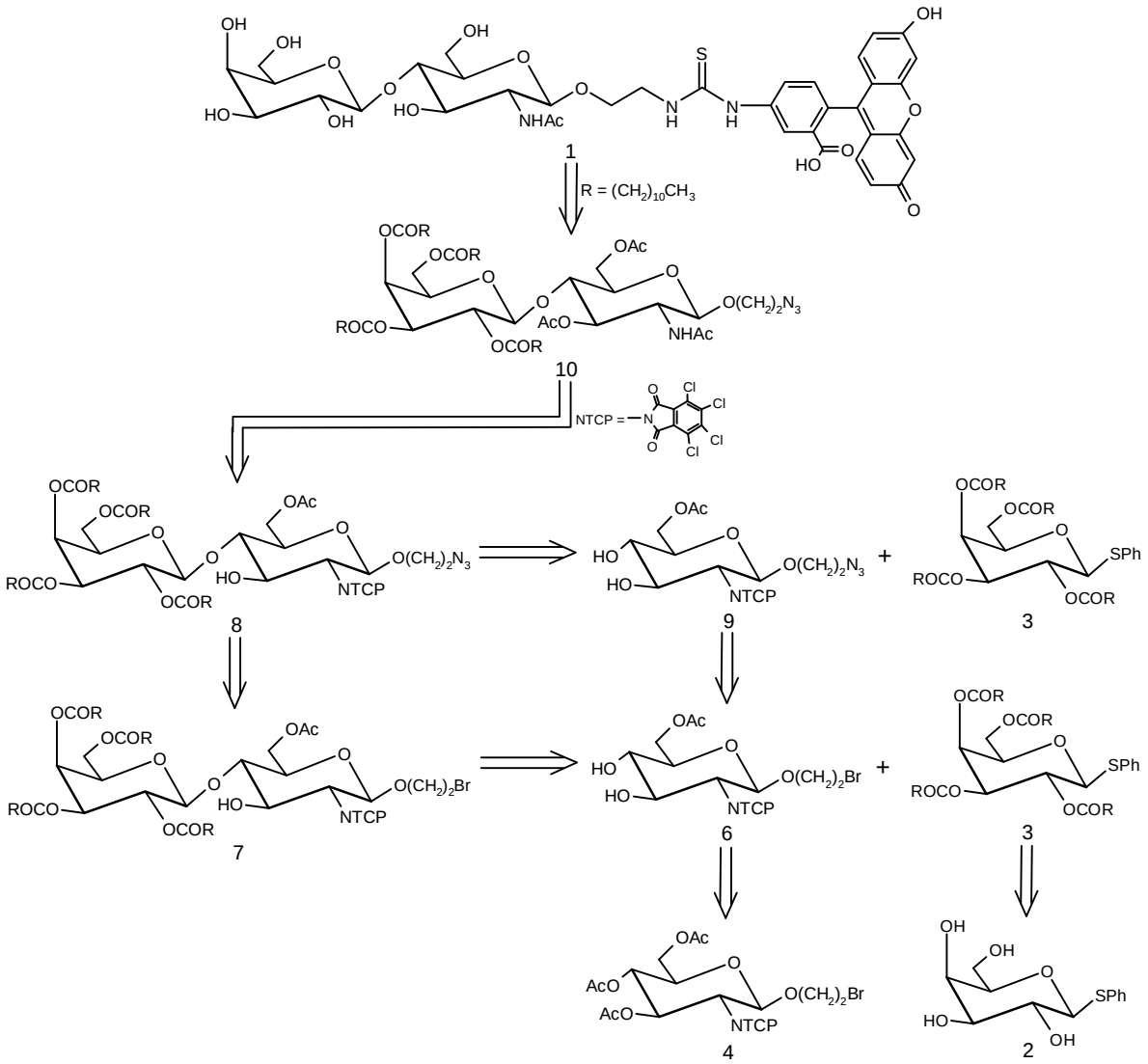
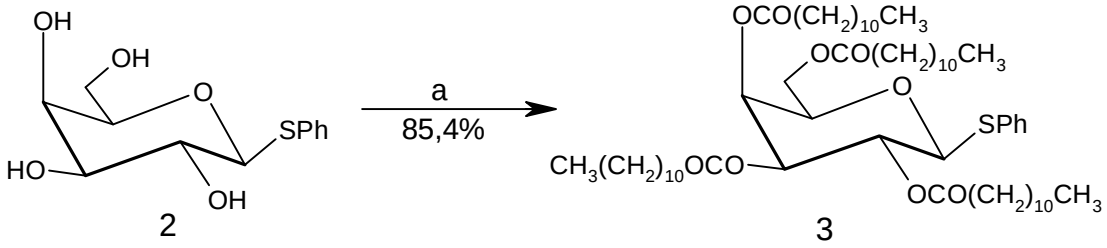


Figure 2.16 Retro-synthetic analysis scheme.

3. Results and discussion

3.1 Synthesis of the donor (3)

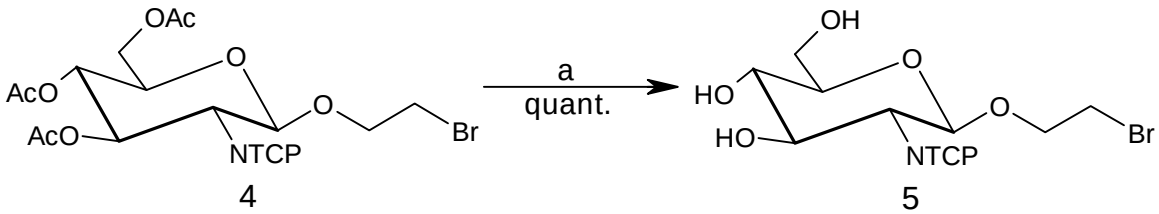
The donor was made by an acylation of phenyl 1-deoxy-1-thio-β-D-galactopyranoside (2) according to the literature [14] except for the temperature. Compound 2 and DMAP (cat.) was suspended in dry heptane at 0°C to prevent any reaction to start. After Ar atmosphere was obtained, dry pyridine and lauroylchloride were added and the reaction was allowed to reach room temperature and react for three days.



Scheme 3.1 (a) Heptane, DMAP, pyridine, lauroylchloride, 0°C→rt., 64 h.

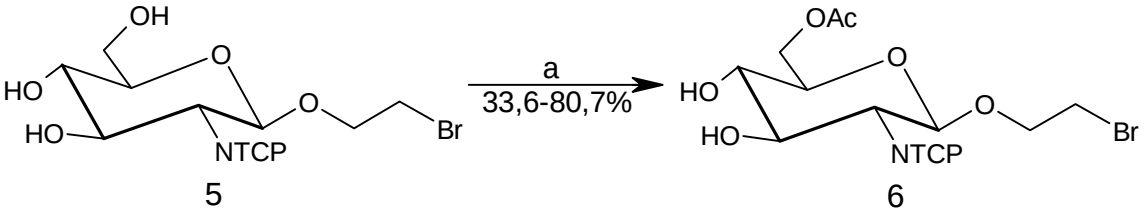
3.2 Synthesis of the acceptor (6)

To produce the acceptor (6), compound 4 first had to be deprotected to a free alcohol. This was made by letting it react with an excess of AcCl in dry MeOH. The AcCl was supposed to have been added at 0°C but unfortunately one third was added at room temperature before the reaction was lowered to 0°C and the rest was added. When all of the AcCl was added the reaction was allowed to reach room temperature and react over night. This method is a way of forming HCl in situ. When all of compound 4 had reacted, the reaction was co-concentrated with toluene.



Scheme 3.2 (a) MeOH, AcCl, rt., 18.5 h, Ar.

In the next step the acceptor (6) was made by first solving 5 in dry CH_2Cl_2 and then at -78°C add *sym*-collidine and AcCl under Ar atmosphere. The solution was then allowed to react at -26°C for 1.5 h before it was quenched (MeOH) and washed (aq. 0.5 M HCl). After the solution was dried, it was evaporated and the remaining residue was chromatographed to produce 33.6% pure 6 and 47.1% 6 with impurities.



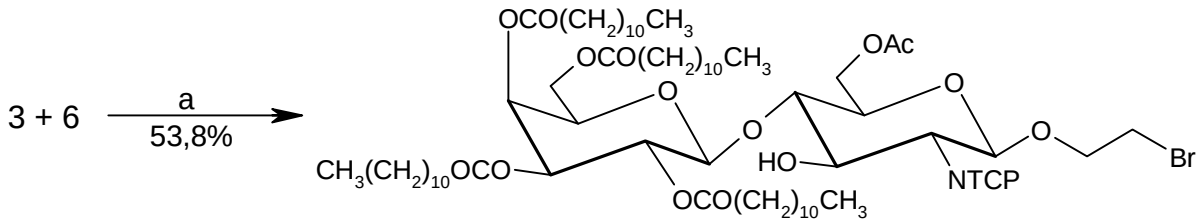
5 6

Scheme 3.3 (a) CH_2Cl_2, *sym*-collidine, AcCl, Ar, -26°C, 1.5 h.

In this step the hindered *sym*-collidine was used to react with the acylchloride to form the sterically hindered compound acylcollidinium chloride, which preferentially react with the primary alcohol on compound 5.

3.3 Glycosylation towards compound 8

3.3.1 Glycosylation 1



7

Scheme 3.4 (a) CH_2Cl_2, NIS, TfOH, 4Å MS, Ar, 2 h at -45°C, then allowed to reach rt.

At this point the first real problem occurred and the reaction in scheme 3.4 had to be remade several times before the problem could be solved by using a different TfOH batch. The TfOH that had been used unsuccessfully had a red color and when it was substituted with one that was colorless the reaction went well, which meant that the original TfOH probably had been decomposed during storage, thereby losing its activity.

The reaction was started by cannulating the distillated CH_2Cl_2 over to the reaction flask under Ar atmosphere and the temperature was lowered to -45°C. NIS was added as Ar was flushed over the reaction flask to prevent water to enter the flask and Ar atmosphere was once again obtained. Finally, TfOH was added to the flask and the solution was stirred at -45°C for 2 h before it was allowed to reach room temperature. After the reaction was quenched, washed and dried, the residue had to be chromatographed twice to produce 6 in 53.8% yield.

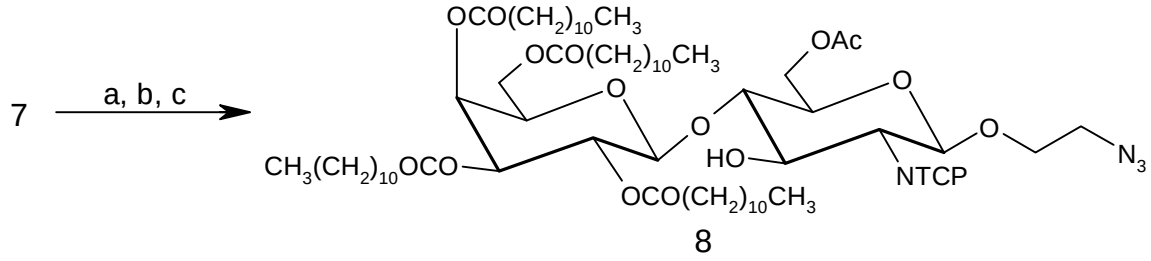
3.3.2 Azide substitution 1

This step was a regular S_N2-substitution where the bromide of 7 was supposed to change to the azide. To help the negatively charged azide to become more reactive, crown ether was used to associate with the sodium ion. Both 15-crown-5 and 18-crown-6 were tested towards finding the right crown ether for this reaction.

First the reaction was run as described in the literature [15] both in a smaller and a larger scale. It was thought that the reaction had went well especially since the reaction according to

the literature only should take 1.5 h, but the NMR showed that this wasn't the case, see appendix A, pg. A-1.

One specific peak (δ 3.14) comes from one of the hydrogens at the carbon that lies nearest to the substitution. This peak can be used to read how good the reaction has gone. If the peak shows an integral around 1 the substitution has been complete. Although these values can't be taken as absolute they give a good hint on what the absolute values are. The amount of azide was in the smaller scale 33% and in the larger scale 44% and since it wasn't even near 100%, neither of the reactions had been successful in replacing the bromide to the azide.



Scheme 3.5 (a) DMF, 15-crown-5, NaN_3.
(b) DMF, 18-crown-6, NaN_3, then THF, 18-crown-6, NaN_3.
(c) THF, 18-crown-6, NaN_3.

When this fact was discovered, parallel experiments were conducted to investigate the reaction and how it could be affected by changing the different parameters. The different parameters that can be changed are the amount of azide and amount of crown ether, which solvent and which crown ether to be used.

Therefore, two reactions was started at the same time and additions of NaN_3 and 18-crown-6 was done at three respectively four different times, see table 3.1. After each addition, the reaction was allowed to run for some time before the measurement, see table 3.1. To control how far the substitution had gone at the different measurements, NMR and the specific peak mentioned before were used, see appendix A, pgs. A-2, -3, -4 and -5.

The only difference between the two reactions was the solvent. Reaction b was started with DMF and reaction c was started with THF. However, reaction b was very slow and the solvent was therefore changed to THF after measurement number 2. Directly after this change of solvent, the substitution degree increased even though it still was much lower than reaction c, see table 3.1.

Table 3.1 Addition and measurement data from the azide substitution reactions b and c. Measurements in percent relate to the amount of azide that has been formed.

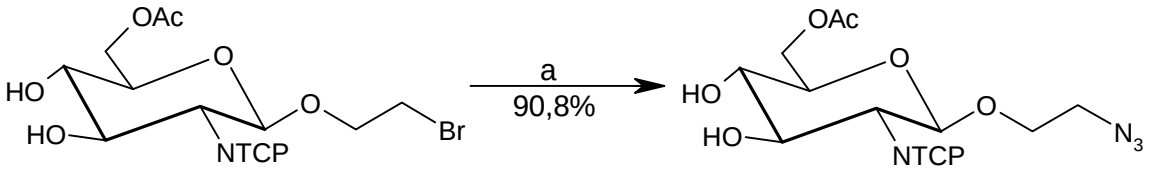
	Substitution reaction b			*Substitution reaction c*			
	Add 1	Add 2	Add 3	Add 1	Add 2	Add 3	Add 4
NaN_3 (eq.)	3	10	10	3	10	17	0
18-crown-6 (eq.)	1	1	10	1	1	3	5
Measurements (%)	26	34→43.4	48→21	53.5	70.2→71.4	73.2	67.7→63.2
	Add 1→Measure 1		*8 days*	*Add 1→Measure 1*		*8 days*	
	Measure 1→Add 2		*4 days*	*Measure 1→Add 2*		*4 days*	
	Add 2→Measure 2		*24 h*	*Add 2→Measure 2*		*24 h*	
	Measure 2→3		*23 h*	*Measure 2→3*		*23 h*	
	Measure 3→Add 3		*27 h*	*Measure 3→Add 3*		*3 h*	
	Add 3→Measure 4		*2 h*	*Add 3→Measure 4*		*22 h*	
	Measure 4→5		*20 h*	*Measure 4→Add 4*		*4 h*	
				Add 4→Measure 5		*3 days*	
				Measure 5→6		*2 days*	

Two things can be told from these experiments. First, when the amount of azide is increased, so are the yields. However, when the amount of 18-crown-6 is increased beyond a certain point, which is 10 equivalents, the yield decreases.

Even though a high degree of substitution could be achieved in reaction c, 100% azide was never obtained this way. One reason to this could lie in the fact that there have been problems before concerning the azide because of the NTCP group. This group is sensitive to heat and will fall apart if it is heated to the temperature in the literature. A different approach therefore had to be considered.

3.3.3 Azide substitution 2 (Synthesis of an azide-acceptor)

To find a solution to the azide problem two test reactions were made, one on compound 4 and one on compound 6. Both reactions were made according to the original reaction with DMF and 15-crown-5.



6 9

Scheme 3.6 (a) DMF, 15-crown-5, NaN_3, 24 h.

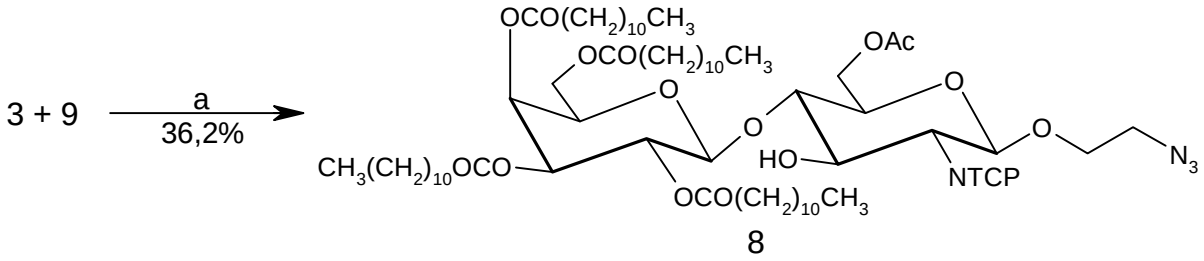
Both of these reactions went very well and the final products contained only the azide. However, the overall yield in the reaction with 6 was much better than in the other reaction. Thus, the reaction with 6 was made in a larger scale to produce an azide acceptor 9 in a high yield (90.8%). After this reaction was complete the solvents was evaporated.

The high yield in this reaction reveals a new fact regarding the NTCP group. It doesn't interfere with S_N2 reaction at all as believed after azide substitution 1. A better explanation probably lies in the long lauroyl groups and the best explanation is that they neither solve in DMF nor in THF.

The only mystery in this reaction was that the crystals had a yellow color. The most probable reason is that the product contains a small amount of a very colorful byproduct, which is hard or impossible to separate from the product. This conclusion is based upon the NMR of the product. It is very clean and doesn't reveal any major impurities. However, the spectra show minor impurities and these don't disappear until after the *O*-lauroyl groups are removed.

3.3.4 Glycosylation 2

This reaction was performed just as glycosylation 1 with the only major difference that a new acceptor 9 was used. The other differences was that the dry CH_2Cl_2 wasn't cannulated to the reaction flask since the amount (10 ml) could be added with a syringe and that the organic phase was washed six times with 10% aq. Na_2SO_4. Thus, glycosylation of 3 with 9 gave the desired disaccharide 8 as a colored oil in 36,2%.



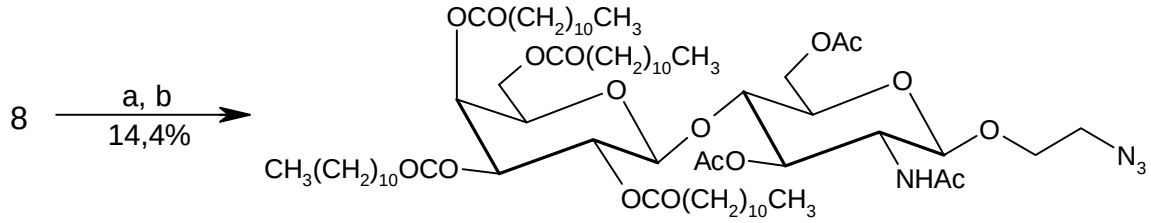
8

Scheme 3.7 (a) CH_2Cl_2, NIS, TfOH, 4Å MS, Ar, 2 h at -45°C, then allowed to reach rt.

3.4 Fluorescein conjugation

3.4.1 Removal of the NTCP group

A suitable method for the synthesis of compound 10 was found in the literature [16, 17] (2 eq. 1,2-diaminoethane). The difference was that the reaction was made at rt. instead of 60-80°C and that the acetylation conditions were changed from MeOH:Ac_2O:H_2O to Pyridine:Ac_2O. The only problem by making the reaction at rt. was that compound 8 didn't solve in EtOH, but since the product was expected to be soluble the reaction was run anyhow.



10

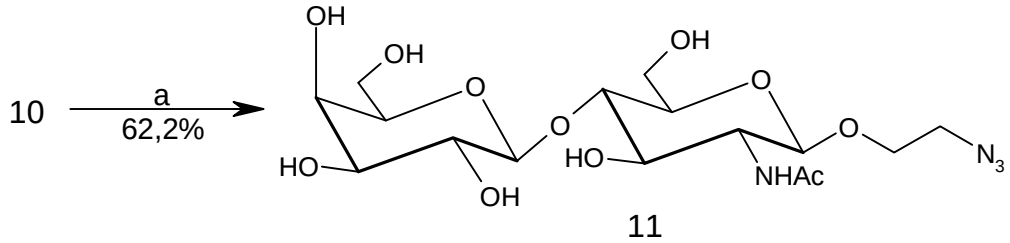
Scheme 3.8 (a) EtOH, 1,2-diaminoethane, Ar, 25 h, then Pyridine:Ac_2O, 23.5 h.
(b) EtOH, 1,2-diaminoethane, Ar, 21 h at 74°C, then Pyridine:Ac_2O, 24 h.

TLC of the reaction run at rt. showed that something else than 10 and the known byproducts had formed. The NTCP group had presumably only opened halfway and therefore the intermediate amine had never been formed [18]. To be able to see if this was the case the reaction was remade at 74°C with 5 eq. 1,2-diaminoethane. After 1.5 h the reaction flask turned opaque, indicating that the TCP-2-aminoethane adduct started to form [18]. Therefore the reaction was left over night to let it react to completion.

The crude intermediate amine was then acetylated in MeOH:Ac_2O:H_2O (10:3:2 ml) but since the residue didn't solve, conditions were changed to Pyridine:Ac_2O (20:10 ml). However, TLC analysis showed at least 6 products and pure 10 could only be obtained after repeated chromatography.

3.4.2 Removal of all protection groups

All remaining protection groups were esters and could therefore be removed in one step by treatment of metanolic sodium methoxide.



11

Scheme 3.9 (a) MeOH, MeONa-MeOH, over night, then Duolite C436.

The residue was purified using a solid-phase extraction method on C18 reverse-phase silica [14]. The silica in the method contains chains that are 18 carbons long and because of these, a molecule that contain a long carbon chain can be separated from those who doesn't. The solvents used in this method can be selected freely to fit the separation need.

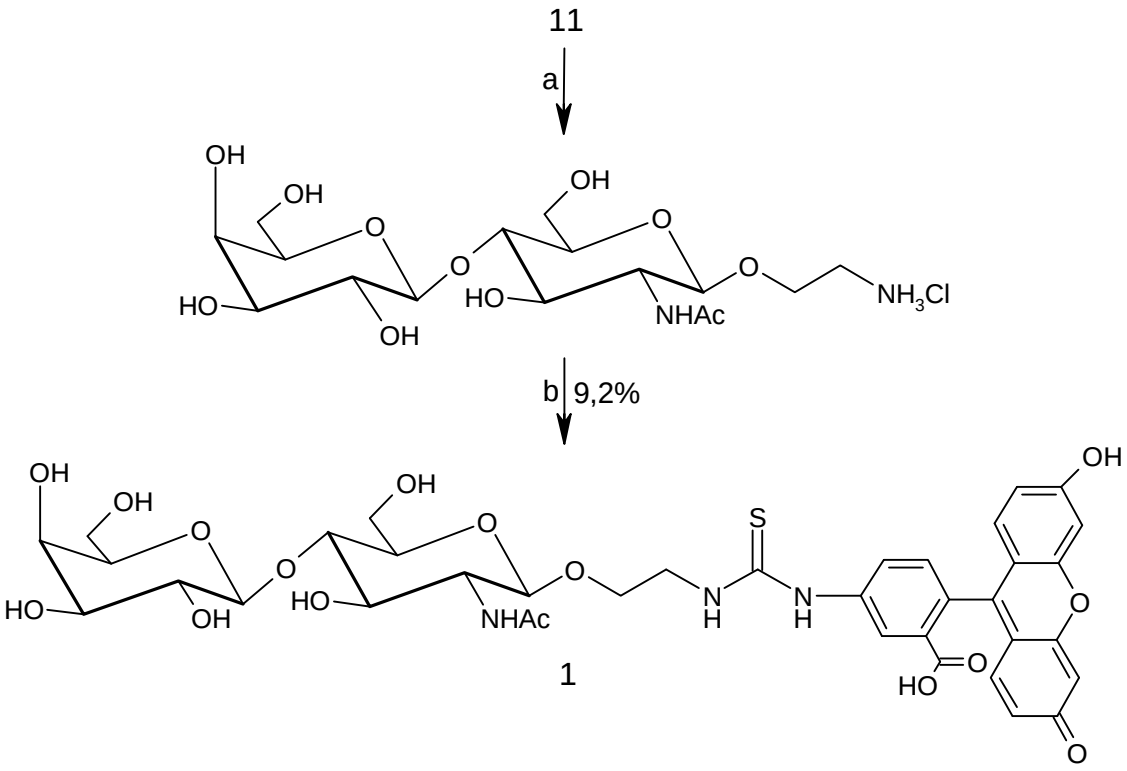
Table 3.2 The separation of compound 10 with C18 solid phase extraction.

Fraction	Volume (ml)	Amount H_2O (%)	Amount MeOH (%)
1-2	10	100	0
3	5	90	10
4	5	80	20
5	5	70	30
6	5	60	40
7	5	40	60
8	5	20	80
9-10	10	0	100

In the present work water and methanol were used as solvents, see table 3.2, and the product came in fraction 5 and to some extent in fraction 6. This means that approximetly 30% MeOH is needed to elute compound 11.

3.4.3 Coupling of FITC to the LacNHAc derivative (11)

To be able to couple the fluorophore FITC to compound 11, it first had to be transformed into an amine. This was made by catalytically hydrogenating the azide with 10% palladium on activated charcoal and hydrogen gas at low pressure and room temperature. The produced amine was then coupled with FITC by allowing it to react in a buffer containing aq. 0.1 M $NaHCO_3$ with 0.9% NaCl (pH 9.3), conditions similar to those described in the literature [19]. FITC had to be solved in DMSO prior to the addition to 11 in buffer.



Scheme 3.10 (a) EtOH, HCl, Pd/C, H_2, 2 h, then filtered through Celite.
(b) Aq. 0.1 M $NaHCO_3$ with 0.9% NaCl (pH 9.3), FITC-DMSO.

Since the FITC that was used in this reaction was bought as a mixed isomer, which contained mostly flurescein-5-isothiocyanate, the LacNHAc derivative was produced as a mixed isomer. However in the scheme 3.10 above only the product that is produced with flurescein-5-isothiocyanate is drawn.

The low yield has two explanations, where the problem with having two isomers is the first, but the most important is the many separation methods that were run before the pure product was obtained. The first separation method was a regular column chromatography with silica where the remaining FITC came in fractions 1-18 and the product in 7-23. This meant no separation at all and a C18-separation was prepared and run on the fractions that contained the product. But just before the separation was to be started the flask was dropped and it broke. To be able to get as much of the product over to the C18-column as possible, the pieces was collected and washed with water, thereby collecting all of the fluorescent products. Then the C18-separation was run according to the table below.

Table 3.3 The separation of compound 10 with C18 solid phase extraction.

Fraction	Amount H_2O (%)	Amount MeOH (%)
1-2	100	0
3	95	5
4	90	10
5	85	15
6	80	20
7	60	40
8	30	70
9-10	0	100

Compound 1 came in fraction 6-8 and FITC came in 7-8, which meant very little or no separation in this case either so fraction 6-8 was freeze-dried from water. When the flask was weighed it was clear that the weight had dropped from 14.6 mg just after the flash chromatography to 0.9 mg now, which meant that the weight had been reduced by 93.8%. Just as this fact wasn't enough it was discovered that the ice trap contained something that responded to long-wave UV.

However, the remaining 1 was separated by HPLC with a gradient between acetonitrile and water, going from 0→25% acetonitrile in 15 min., then 25→30% acetonitrile in 60 min. and finally 30→100% acetonitrile in 15 min, see appendix B for more details concerning the HPLC-separation. The program was aborted after approximately 35 min., since all of the interesting material had passed the column.

Table 3.4 The interesting peaks in the HPLC separation.

Peak number	Retention time	Peak area	Area percent
7	19,887	195,63246	16,4
14	21,840	412,25412	34,6
15	21,910	276,27487	23,2
16	22,017	307,66806	25,8
Totals:	-	1191,82951	100,0

This time everything separated and compound 1 and its regioisomer with fluorescein-6-isothiocyanate were collected separately. Compound 1 came as a wide peak that the UV-detector divided into three peaks, see table 3.4, and were 83,6% of the main product.

The fraction that contained compound 1 was freeze-dried and transferred to a small preparation flask and weighed and 0.58 mg (9.2%) of the interesting compound 1 had been produced.

To be able to say that the product gained in the HPLC separation was indeed compound 1, comparative studies had to be done. My original NMR & COSY in D_2O, were compared to NMR & COSY in MeOD of the compound prepared later by Pernilla Sörme, which showed that compound 1 indeed had been obtained.

3.5 Fluorescence measurements

Even though the amount of compound 1 that had been gained from the last reaction was very small this amount will last a long time because the amount needed for an experiment is very small. Approximately 16 pmole/well in the microwell plate is needed which means that 0.58 mg of compound 1 is enough to do about 44435 tests or about 463 microwell plates.

Compound 1, later prepared on a larger scale by Pernilla Sörme, was tested against galectin-1 and -3 and the resulting curves can be seen in figure 3.1. The regression results provide the dissociation constant K_d (K_d=m3).

$$K_d(Galectin-1) = 17{,}936\mu M$$
$$K_d(Galectin-3) = 1{,}6915\mu M$$

These results show that galectin-3 (K_d=1.69 µM) has a higher affinity for the fluorescein-labelled LacNHAc, 2-(fluorescein-5-thiourea)-ethyl (β-D-galactopyranosyl)-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranoside) (1), than galectin-1 (K_d=17,9 µM).

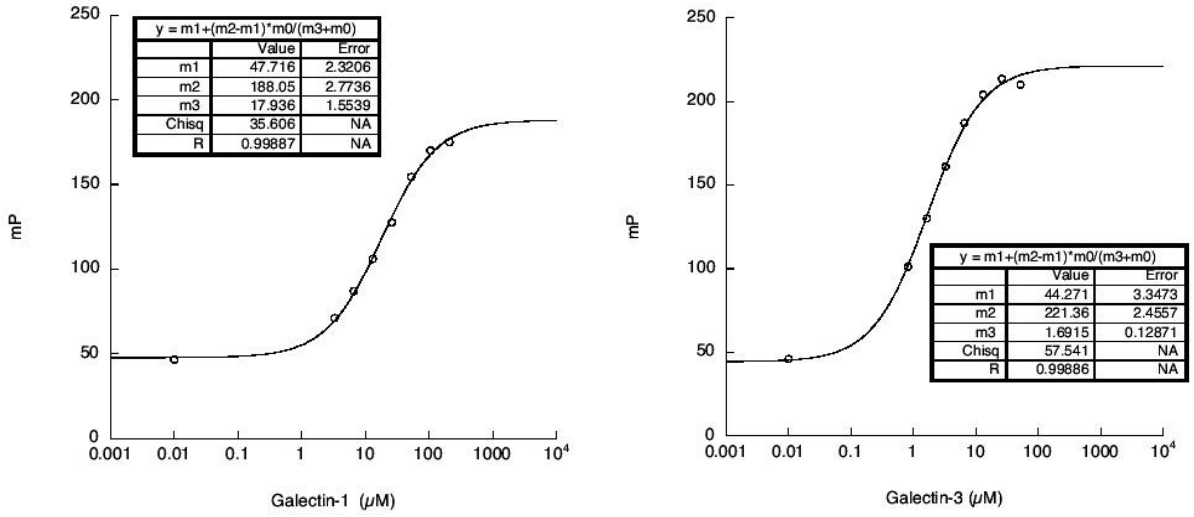


Figure 3.1 Fluorescence polarization measurements with compound 1 against galectin-1 and 3.

In conclusion, although several synthetic and practical problems were encountered, the objectives of the project were met and a fluorescence-labelled ligand was obtained and could indeed be used for monitoring galectin-1 and -3 binding activities.

4. Future aspects

There are three different areas of interest for future aspects:

1. Varying the carbohydrate ligand.
2. Varying the coupling between the fluorophore and the carbohydrate.
3. Varying the fluorophore.

Different ways of varying the carbohydrate ligand has been done by Pernilla Sörme by adding groups to the C-3 on the galactose unit of the LacNHAc. Another thing that could be interesting to do is to test different saccharides like mono-, di-, tri- and even tetrasaccharides since the galectins' CRD are large enough to bind a tetrasaccaride.

Different ways of varying the length of the coupling is another interesting thing to do. Since it has been discovered that FITC-coupled carbohydrates have a tendency to break down over time and it is the thiourea coupling that is responsible for this behavior, other coupling methods have been tested. NHS-fluorescein is one and another is a triazine derivative.

Didecyl squarate can be used to make the LacNHAc derivative 11 amine-reactive, which allow for conjugation with different amine-functionalised fluorophores of interest, see figure 5.1.

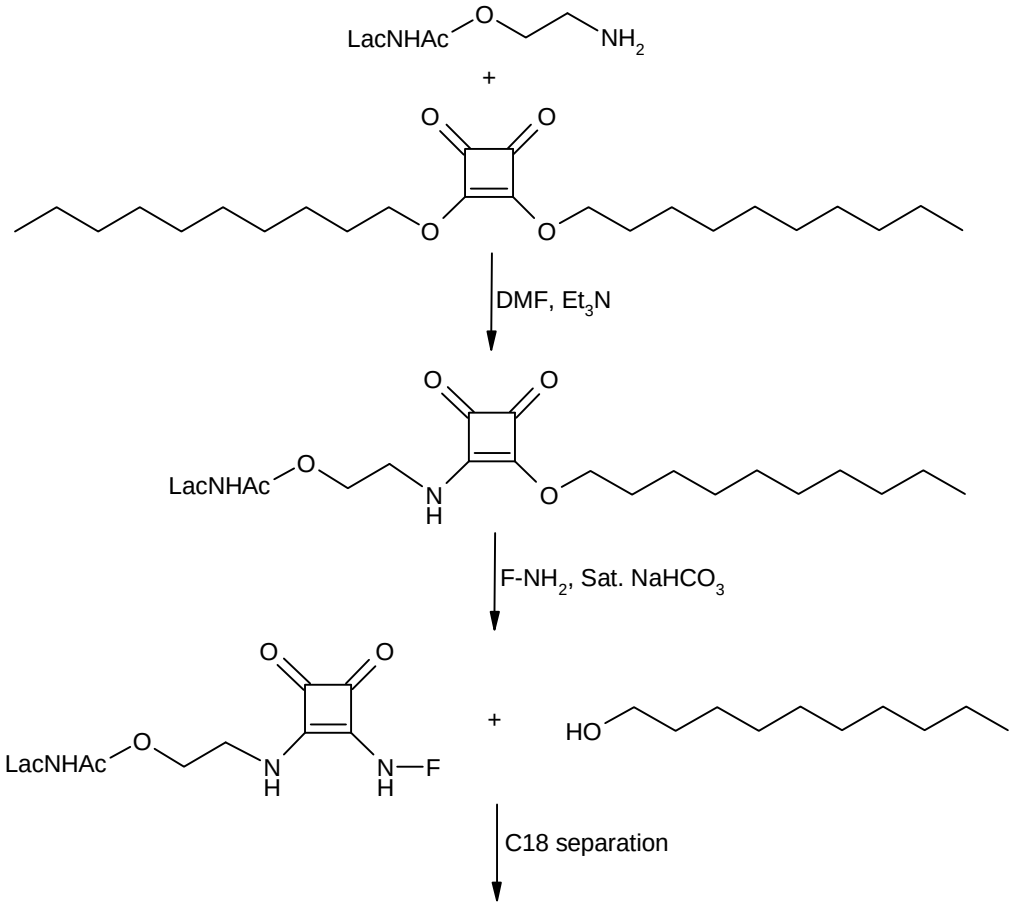
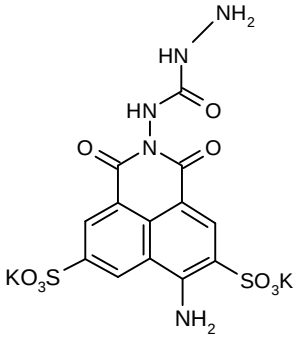


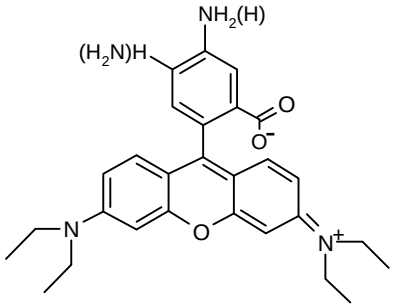
Figure 5.1 Fluorophore (F) coupling via didecyl squarate.

Finally, it would be interesting to couple other fluorophores than fluorescein to LacNHAc. Even though fluorescein and more specifically FITC have been used for many years and therefore have been thoroughly investigated, there still exist problems with it. One of these problems is that fluorescein has a short fluorescence lifetime, about 4 ns. This means that if we have a small molecule (<10 kDa) that binds to a larger molecule, the fluorescence will remain polarized. However, a lifetime of 4 ns is not optimum for binding studies of a large protein (>30 kDa) to other proteins because the fluorescence is already highly polarized [12, pg. 1-7].

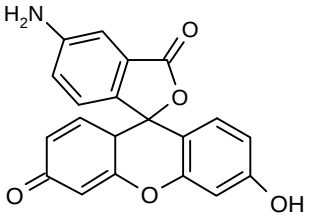
Other fluorophores that could have a better fluorescence lifetime or at least be a better fluorophore for LacNHAc is shown in figure 5.2. The only requirement was that it had to be able to be coupled to the inhibitor via an amine. The interesting fluorophores that can be seen in figure 5.2 has been found and purchased after going through the literature [20] and comparing with what could be bought to a reasonable price.



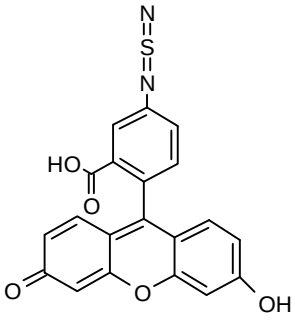
Lucifer Yellow CH Dipotassiumsalt
Excitation (H_2O): 428 nm
Emission (H_2O): 540 nm (Lit.)
Solubility: H_2O



Rhodamine amine, mixed isomers
Excitation (MeOH): 544 nm
Emission (MeOH): 574 nm (Lit.)
Solubility: H_2O (50 mg/ml), EtOH (3 mg/ml), 2-metoxyetanol (100 mg/ml) (Lit.)



Fluoresceinamine, isomer I
Excitation (pH 9): 488 nm
Emission (H_2O): 518 nm (Lit.)
Solubility: pH≥6



FITC, (Fluorescein-5-isothiocyanate)
Excitation (pH 9): 495 nm
Emission (H_2O): 520 nm (Lit.)
Solubility: DMF, pH≥9 (Lit.)

Figure 5.2 Fluorophores and its excitation, emission and solubility data. The data comes from the FLUKA catalogue Fluorescent Probes. FITC is used as a reference fluorophore.

5. Experimental section

5.1 General methods

NMR-spectra were recorded either on a Bruker ARX-300 or on a Bruker DRX-400 instrument. Chemical shifts and coupling constants were obtained from ^{1}H-NMR and COSY was used to assign the proton resonances. Chemical shifts are given in ppm downfield from the signal for Me_4Si, with reference to internal $CDCl_3$ (7.26 ppm), D_2O (4.70 ppm) or MeOD (3.32, 4.85 ppm). Column chromatography was performed on SiO_2 (Matrex, 60 Å, 35-70 μm, Grace Amicon) and TLC was carried out on aluminum sheets coated with silica gel 60 F254 (Merck) and visualized using UV-light and by charring with H_2SO_4 (aq. 10% solution). Dichloromethane was dried either by 4Å MS or by distillation from CaH_2. Heptane and pyridine was dried over 4Å MS and MeOH and EtOH was dried over 3Å MS. DMF and 1, 2-diaminoethane was distilled before use. All solvents were of PA-quality. The reagents 3, 6, 9 and *N*-iodosuccinimide (NIS) were dried with vacuum overnight before the glycosylation reaction. Washing the column with first large amounts of MeOH and then large amounts of water started every C18-separation. Then the sample was applied and eluted with a gradient that started on 100% water and ended at 100% MeOH. High-pressure liquid chromatography (HPLC) was performed on Beckman System Gold with fraction collector (Pharmacia LKB-FRAC-100) and the system was monitored by a UV-detector. The column used was Supelcosil SPLC-18-DB (l=25.0 cm, d=10.0 mm, d_P=5 μm).

5.2 Reactions

Phenyl 1-deoxy-2, 3, 4, 6-tetra-*O*-lauroyl-1-thio-β-D-galactopyranoside (3). Phenyl 1-deoxy-1-thio-β-D-galactopyranoside (2, 4.0 g, 14.7 mmol) and a catalytic amount of *N, N'*-dimethyl-4-aminopyridine were dissolved in dry heptane (103.2 mL) at 0ºC under argon atmosphere. Dry pyridine (32.0 mL) and lauroylchloride (31.5 mL, 132.8 mmol) were added. The mixture was allowed to reach room temperature, stirred for 64 h and concentrated. The residue was first dry flashed (SiO_2, 20:1→10:1 petroleum ether-EtOAc) and then chromatographed (SiO_2, 20:1 petroleum ether-EtOAc) to give 3 (12.6 g, 85.4%);

^{1}H-NMR ($CDCl_3$): δ 7.50 (m, 2 H, SPh), 7.30 (m, 3 H, SPh), 5.43 (m, 1 H, H-4), 5.24 (t, 1 H, *J* 9.94 Hz, H-2), 5.07 (dd, 1 H, *J* 9.95, 3.3 Hz, H-3), 4.72 (d, 1 H, *J* 9.95 Hz, H-1), 4.19 (dd, 1 H, *J* 11.3 Hz, H-6), 4.08 (dd, 1 H, *J* 11.3 Hz, H-6), 3.95 (t, 1 H, *J* 6.8 Hz, H-5), 2.23-2.40 (m, 6 H, -$OCOCH_2$-), 2.12-2.21 (m, 2 H, -$OCOCH_2$-), 1.43-1.70 (m, 8 H, -$OCOCH_2C\underline{H_2}$-), 1.14-1.40 (m, 64 H, -CH_2-), 0.80-0.95 (m, 12 H, -CH_3).

2-Bromoethyl 2-deoxy-2-tetrachlorophtalimido-β-D-glucopyranoside (5). 2-Bromoethyl 3, 4, 6-tri-*O*-acetyl-2-deoxy-2-tetrachlorophtalimido-β-D-glucopyranoside (4, 9.74 g, 14.3 mmol) was dissolved in dry MeOH (310 mL) and then AcCl (10 mL, 140.6 mmol) was added at room temperature. The reaction was then cooled to 0°C and an additional amount of AcCl (18 mL, 253.2 mmol) were added. The reaction was allowed to reach room temperature and was stirred for 18.5 h. It was co-concentrated with toluene and chromatographed (SiO_2, 20:1 EtOAc-MeOH) to give 5 (8.27 g, quant.);

^{1}H-NMR ($CDCl_3$): δ 5.26 (d, 1 H, *J* 8.4 Hz, H-1), 4.34 (dd, 1 H, *J* 10.9, 8.8 Hz, H-3), 4.02-4.20 (m, 2 H, H-2, CH), 3.96 (d, 2 H, *J* 3.0 Hz, H-6), 3.69-3.83 (m, 2 H, H-4, CH), 3.31-3.45 (m, 2 H, CH).

2-Bromoethyl 6-*O*-acetyl-2-deoxy-2-tetrachlorophtalimido-β-D-glucopyranoside (6). Compound 5 (8.23 g, 14.9 mmol) was dissolved in dry CH_2Cl_2 (262 mL) and cooled to -78°C. *Sym*-collidine (10.0 mL, 75.4 mmol) and AcCl (1.09 mL, 16.5 mmol) were added under argon atmosphere and the reaction was kept at -26°C for 1.75 h and then an additional amount of *sym*-collidine (3.76 mL, 28.4 mmol) and AcCl (0.5 mL, 7.6 mmol) were added. The reaction was kept at -26°C for 1.5 h and then it was quenched with MeOH, diluted with aq. 0.5 M HCl, washed twice with aq. 0.5 M HCl, twice with sat. aq. $NaHCO_3$, dried over Na_2SO_4 and concentrated. The residue was then chromatographed (SiO_2, 20:1 CH_2Cl_2-MeOH) to give 6 (2.98 g, 33.6%);

^{1}H-NMR ($CDCl_3$): δ 5.22 (d, 1 H, *J* 8.3 Hz, H-1), 4.60 (dd, 1 H, *J* 12.4, 3.9 Hz, H-4), 4.25-4.34 (m, 2 H, H-3, 6), 4.07-4.18 (m, 2 H, H-2, CH), 3.75 (ddd, 1 H, *J* 7.6, 11.5, 5.6 Hz, CH), 3.59 (ddd, 1 H, *J* 9.8, 3.8, 2.3 Hz, H-5), 3.28-3.45 (m, 3 H, H-6, CH), 2.17 (s, 3 H, OAc).

2-Bromoethyl (2, 3, 4, 6-tetra-*O*-lauroyl-β-D-galactopyranosyl)-(1→4)-(6-*O*-acetyl-2-deoxy-2-tetrachlorophtalimido-β-D-glucopyranoside) (7). To a flask with compound 3 (2.335 g, 2.351 mmol) and 6 (1.000 g, 1.678 mmol), activated MS (4Å, 1.72 g) was added under argon atmosphere. Dry dichloromethane (54 ml) was cannulated to the reaction flask, the temperature was lowered to -45°C and NIS (0.5665 g, 2.518 mmol) was added under argon flushing. TfOH (105 μl, 1.187 mmol) was added under argon atmosphere and the reaction was stirred for 2 h at -45°C and then allowed to reach room temperature. The reaction was quenched with 10% aq. Na_2SO_4 and CH_2Cl_2 washed four times with 10% aq. Na_2SO_4. The organic phase was dried over $MgSO_4$ and concentrated. The residue was chromatographed twice (SiO_2, 5:1 petroleum ether-EtOAc) to give 7 (1.343 g, 53,8%);

^{1}H-NMR ($CDCl_3$): δ 5.38 (d, 1 H, *J* 3.4 Hz, H-4'), 5.251 (d, 1 H, *J* 8.5 Hz, H-1), 5.248 (dd, 1 H, *J* 10.5, 7.9 Hz, H-2'), 5.01 (dd, 1 H, *J* 10.5, 3.4 Hz, H-3'), 4.54 (d, 1 H, *J* 8.1 Hz, H-1'), 4.35-4.41 (m, 2 H, H-3, 6), 4.16 (dd, 1 H, *J* 10.8, 8.5 Hz, H-2), 3.96-4.13 (m, 5 H, H-5', 6, 6', CH), 3.67-3.80 (m, 2 H, H-5, CH), 3.56 (dd, 1 H, *J* 9.8, 7.9 Hz, H-4), 3.30-3.42 (m, 2 H, CH_2), 2.36-2.43 (m, 2 H, -$OCOCH_2$-), 2.11-2.36 (m, 6 H, -$OCOCH_2$-), 2.17 (m, 3 H, OAc), 1.40-1.70 (m, 8 H, -$OCOCH_2C\underline{H}_2$-), 1.08-1.38 (m, 64 H, -CH_2-), 0.82-0.94 (m,12 H, -CH_3).

2-Azidoethyl 6-*O*-acetyl-2-deoxy-2-tetrachlorophtalimido-β-D-glucopyranoside (9). Compound 6 (401.8 mg, 0.674 mmol) was dissolved in distilled DMF (10 mL). Sodium azide (218.6 mg, 3.36 mmol) and 15-crown-5 (140 (L, 0.705 mmol) were added and the reaction was stirred for 24 h. DMF and 15-crown-5 were removed by vacuum pumping and the residue was chromatographed (SiO_2, 1:1 toluene-EtOAc) to give 9 (341.8 mg, 90.8%);

^{1}H-NMR ($CDCl_3$): δ 5.27 (dd, 1 H, *J* 8.3, 2.5 Hz, H-1), 4.63 (dd, 1 H, *J* 12.4, 3.6 Hz, H-4), 4.22-4.35 (m, 2 H, H-3, 6), 4.08-4.16 (m, 1 H, H-2), 4.02-4.09 (m, 1 H, CH), 3.60-3.69 (m, 1 H, CH), 3.54-3.62 (m, 1 H, H-5), 3.34-3.49 (m, 2 H, H-6, CH), 3.31 (ws, 1 H, 4-OH), 3.14 (ddd, 1 H, J 13.5, 4.4, 2.9 Hz, CH), 2.67 (ws, 1 H, 3-OH), 2.17 (s, 3 H, OAc).

2-Azidoethyl (2, 3, 4, 6-tetra-*O*-lauroyl-β-D-galactopyranosyl)-(1→4)-(6-*O*-acetyl-2-deoxy-2-tetrachlorophtalimido-β-D-glucopyranoside) (8). To a flask containing compound 3 (654.5 mg, 0.6535 mmol), 9 (227.8 mg, 0.4081 mmol) and activated MS (4Å, 591.5 mg), dry CH_2Cl_2 (10 ml) was added under argon atmosphere and the reaction was lowered to -48°C. NIS (137.9 mg, 0.6129 mmol) and TfOH (26 μl, 0.29 mmol) were added. The reaction was stirred for 2 h, allowed to reach room temperature, quenched with 10% aq. Na_2SO_4 and washed six times with 10% aq. Na_2SO_4. The organic phase was dried over $MgSO_4$ and concentrated. The residue was chromatographed (SiO_2, 6:1 petroleum ether-EtOAc) to give 8 (214.1 mg, 36.2%);

^{1}H-NMR ($CDCl_3$): δ 5.38 (d, 1 H, *J* 3.2 Hz, H-4'), 5.20-5.29 (m, 2 H, H-1, 2'), 5.01 (dd, 1 H, *J* 10.5, 3.3 Hz, H-3'), 4.54 (d, 1 H, *J* 8.0 Hz, H-1'), 4.30-4.39 (m, 2 H, H-3, 6), 3.95-4.18 (m, 6 H, H-2, 5', 6, 6', CH, CH), 3.60-3.76 (m, 2 H, H-5, CH), 3.56 (t, 1 H, *J* 8.9 Hz, H-4), 3.40-3.49 (m, 1 H, CH), 3.08-3.18 (m, 1 H, CH), 2.13-2.43 (wm, 8 H, -$OCOCH_2$-), 2.12 (s, 1 H, OAc), 1.40-1.70 (m, 8 H, -$OCOCH_2C\underline{H}_2$-), 1.11-1.37 (m, 64 H, -CH_2-), 0.88 (t, 12 H, J 6.6 Hz, -CH_3).

2-Azidoethyl (2, 3, 4, 6-tetra-*O*-lauroyl-β-D-galactopyranosyl)-(1→4)-(2-acetamido-3, 6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside) (10). Compound 8 (173.0 mg, 0.119 mmol) was partly dissolved in dry EtOH (17.6 mL) with ultrasound and distilled 1,2-diaminoethane (16 μL, 0.239 mmol) was added. The mixture was stirred for 25 h and was co-concentrated with toluene. The residue was dissolved in pyridine-Ac_2O (8:4 mL) and the mixture was stirred for 23.5 h and co-concentrated with toluene. Dry EtOH (7 mL) and 1,2-diaminoethane (40.0 (L, 0.598 mmol) were added and the mixture was heated to 74°C. The residue dissolved and the mixture was stirred for 21 h at 74°C and was co-concentrated with toluene. The residue was dissolved in pyridine-Ac_2O (20:10 mL), stirred for 24 h, co-concentrated with toluene and chromatographed (SiO2, heptane:EtOAc 1:3) twice to give 10 (21.7 mg, 14.4%);

^{1}H-NMR ($CDCl_3$): δ 5.63 (d, 1 H, *J* 9.3 Hz, NH), 5.37 (d, 1 H, *J* 3.2 Hz, H-4'), 5.13 (dd, 1 H, *J* 10.5, 7.8 Hz, H-2'), 5.11 (dd, 1 H, *J* 9.3, 8.0 Hz, H-3), 4.98 (dd, 1 H, *J* 10.5, 3.4 Hz, H-3'), 4.48-4.58 (m, 3 H, H-1, 1', 6), 4.09-4.19 (m, 2 H, H-6, 6'), 3.94-4.09 (m, 3 H, H-2, 6', CH), 3.88 (t, 1 H, *J* 6.8 Hz, H-5'), 3.79 (t, 1 H, *J* 8.2 Hz, H-4), 3.58-3.68 (m, 2 H, H-5, CH), 3.47 (ddd, 1 H, *J* 13.2, 8.3, 3.4 Hz, CH), 3.20-3.31 (m, 1 H, CH), 2.35-2.42 (m, 2 H, -$OCOCH_2$-), 2.21-2.33 (m, 4 H, -$OCOCH_2$-), 2.10-2.20 (m, 2 H, -$OCOCH_2$-), 2.11 (s, 3 H, 6-OAc), 2.07 (s, 3 H, 4-OAc), 1.97 (s, 3 H, NAc), 1.40-1.70 (wm, 8 H, -$OCOCH_2C\underline{H}_2$-), 1.14-1.40 (ws, 64 H, -CH_2-), 0.88 (t, 8 H, *J* 6.5 Hz, -CH_3).

2-Azidoethyl (β-D-galactopyranosyl)-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranoside) (11). Compound 10 (20.8 mg, 0.0164 mmol) was dissolved in dry MeOH (2.4 mL) and NaOMe-MeOH (1 M, 12 μL) was added. The mixture was stirred over night and neutralized with Duolite C436 and concentrated. The residue was separated on C18-column (5 g, 100:0→0:100% H_2O:MeOH) to give 11 (4.6 mg, 63.9%);

^{1}H-NMR (D_2O): δ 4.55 (d, 1 H, *J* 7.9 Hz, H-1), 4.42 (d, 1 H, *J* 7.7 Hz, H-1'), 3.90-4.04 (m, 2 H, H-3, CH), 3.87 (d, 1 H, *J* 3.3 Hz, H-4'), 3.48 (dd, 1 H, *J* 10.0, 7.7 Hz, H-2'), 3.52-3.58 (m, 1 H, CH), 3.36-3.45 (m, 1 H, CH), 3.31-3.83 (wm, 11 H, H-2, 3', 4, 5, 5', 6, 6', CH, NH), 1.99 (s, 3 H, NAc).

2-(Fluorescein-5-thiourea)-ethyl (β-D-galactopyranosyl)-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranoside) (1). Compound 11 was dissolved in EtOH (15.1 mL) and the mixture was degassed. Aqueous HCl (1 M, 82 μL) was added and the mixture was catalytically reduced (H_2, 1 atm, 10% Pd/C, 9.0 mg) for 2 h. The mixture was filtered through Celite and concentrated. The residue was dissolved in aq. 0.1 M $NaHCO_3$ with 0.9% NaCl (pH 9.3, 1.9 ml). A mixture of FITC (3.3 mg, 0.00847 mmol) in DMSO (150 μL) was added and the mixture was kept at 2°C over night. The mixture was freeze-dried and first chromatographed (SiO_2, 65:35:5 CH_2Cl_2:MeOH:H_2O), then separated on C18-column (5 g, 100:0→0:100% H_2O:MeOH) and finally chromatographed on HPLC (0:100→100:0% acetonitrile-H_2O) to give 1 (0.58 mg, 9.2%);

^{1}H-NMR (MeOD): δ 8.10-8.13 (m, 1 H, Ar-H), 7.83 (dd, 1 H, *J* 8.4, 2.0, Ar-H), 7.20 (d, 1 H, *J* 8.2, Ar-H), 6.87-6.92 (m, 2 H, Ar-H), 6.69 (d, 2 H, *J* 2.4, Ar-H), 6.61 (dd, 2 H, *J* 9.2, 2.2, Ar-H), 4.50 (d, 1 H, *J* 8.6, H-1), 4.36 (d, 1 H, *J* 7.5, H-1'), 3.81 (m, 1 H, H-4'), 3.48 (d, 1 H, *J* 9.7, H-3'), 4.36 (wm, 15 H, H-2, 2', 3, 4, 5, 5', 6, 6', NH, CH), 1.98 (s, 3 H, NAc).

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