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DOCTORAL THESIS

Transesterification of Monosaccharides with *Candida Antarctica* Lipase-B and Their Use in the Synthesis of Human Milk Oligosaccharides

Kaarel Erik Hunt

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Kaarel Erik Hunt

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TALLINNA TEHNIKAÜLIKOOL
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Monosahhariidide ümberesterdamine *Candida antarctica* lipaas-B abil ja nende kasutamine rinnapiima oligosahhariidide sünteesis

KAAREL ERIK HUNT

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Contents

List of publications

The list of author's publications, on the basis of which the thesis has been prepared:

I Hunt, K. E.; García-Sosa, A. T.; Shalima, T.; Maran, U.; Vilu, R.; Kanger, T. Synthesis of 6'-Galactosyllactose, a Deviant Human Milk Oligosaccharide, with the Aid of *Candida Antarctica* Lipase-B. *Org. Biomol. Chem.* **2022**, *20* (23), 4724–4735. https://doi.org/10.1039/D2OB00550F.

II Hunt, K. E.; Miller, A.; Liias, K.; Jarg, T.; Kriis, K.; Kanger, T. Interplay of Monosaccharide Configurations on the Deacetylation with *Candida Antarctica* Lipase-B. *J. Org. Chem.* **2025**, *90* (1), 663–671. https://doi.org/10.1021/acs.joc.4c02582.

III Hunt, K. E.; Miller, A.; Jarg, T.; Kriis, K.; Kanger, T. Selective acetylation of unprotected thioglycosides and fully unprotected monosaccharides with *Candida antarctica* lipase-B. *ACS Omega*, **2025**, *10* (19), 20047–20053. https://doi.org/10.1021/acsomega.5c02467.

Author's contribution to the publications

Contribution to the papers in this thesis are:

I The author had a major role in the development of the methodologies, synthetic preparation and characterisation of compounds used in the study. The author wrote the manuscript, with contributions from the co-authors, and compiled the supporting information.

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Introduction

Breastfeeding rates in the European region are the lowest in the world. Although there are many reasons why, the outcome is the same – breast milk has been substituted with infant formulas. Unfortunately, not all the compounds found in breast milk have been transferred to infant formulas. Most complex natural oligosaccharides are currently too expensive to be included. One such group of oligosaccharides are human milk oligosaccharides (HMOs), which make the third largest macronutrient group in the breast milk, but only couple of them are produced in mass synthetically. Cell factories and enzymatic means have been used to produce HMOs in larger scale, but these methods come with their own drawbacks e.g. hard to purify mixture of oligosaccharide products. On the contrary, using chemical synthesis, a single HMO with a high purity can be made. This would require multi-step synthesis while selectively protecting and deprotecting the polyhydroxy saccharides to form glycosidic bonds in the correct positions with the correct configurations.

Differences in chemical reactivity, changes in the reaction conditions, specific reagents, and sterically varied groups have been used to selectively protect different positions in saccharides. While these methods work, they often require multiple steps, metal catalysts or toxic reagents. A less toxic solution would be to use enzymes, more specifically lipases and esterases, which have been used to deprotect ester functionalities mostly in buffer solutions. Lipases, which have a hydrophobic lid protecting their catalytic site, have been used also in organic media for transesterification reactions. Commercially available immobilised lipases from *Candida antarctica* have been studied for years revealing many uses from synthesis of optically pure compounds to production of biofuel.

This doctoral thesis will focus on the use of immobilised *Candida antarctica* lipase-B (CAL-B) in transesterification reactions with mono- and disaccharides. The aims of the thesis are to find enzymatic methods using CAL-B for the synthesis of partially protected saccharides, which can be further used in the synthesis of HMOs. The thesis starts with a brief introduction of carbohydrate chemistry, followed by the introduction of lipases, and discussion of HMOs with some examples how to make them through different methods. The results of CAL-B transesterification reactions are described one saccharide at a time. As a result, an extensive overview of CAL-B reactions outcomes is given, while its interactions with different saccharides is discussed.

The results of the CAL-B transesterification reactions, both selective acetylation and deacetylation, have broadened the choice of methods to synthesise partially protected saccharides (**Publication I–III**). The use of the products has been demonstrated in the synthesis of a deviant HMO – 6'-galactosyllactose (**Publication I**). Recycling of the immobilised CAL-B has been studied both in deacetylation and acetylation reactions (**Publication II** and **III**). Additionally, the results of this thesis have been presented at international conferences in Belgium, Estonia, Germany, Latvia and in online conferences.

Abbreviations

2’-FL	2'-fucosyllactose
3-FL	3-fucosyllactose
6’-GL	6’-galactosyllactose
6’-SL	6’-sialyllactose
Ac	acetyl
AcCl	acetyl chloride
Ac_2O	acetic anhydride
AGA	automated glycan assembly
Asp	aspartic acid
Bn	benzyl
Bz	benzoyl
CAL-B	*Candida antarctica* lipase-B
CBr-Sep	cyanogen bromide activated agarose
CMC	critical micellar concentration
CMP	cytidine monophosphate
CPME	cyclopentyl methyl ether
CSA	camphorsulfonic acid
DCM	dichloromethane
DFL	2’,3-difucosyllactose
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethyl formamide
E. coli	*Escherichia coli*
EtOAc	ethyl acetate
eq.	equivalent
Fmoc	fluorenylmethyloxycarbonyl
Fuc	fucose
Fur	furanose
Gal	galactose
GC	gas chromatography
GDP	guanosine diphosphate
GH	glycoside hydrolases
Glc	glucose
GlcNAc	*N*-acetyl-glucosamine
GlcNPhth	*N*-phthalimido-glucosamine
GlcNTroc	*N*-2,2,2-trichloroethoxycarbonyl-glucosamine
GT	glycosyltransferases
GOS	galactooligosaccharide
His	histidine
HMO	human milk oligosaccharide
HPLC	high performance liquid chromatography

IUPAC	International Union of Pure and Applied Chemistry
Lac	lactose
LNB	lacto-*N*-biose
LNDFH I	lacto-*N*-difucohexaose I
LNFP I	lacto-*N*-fucopentaose I
LNFP II	lacto-*N*-fucopentaose II
LNFP III	lacto-*N*-fucopentaose III
LNnT	lacto-*N*-neotetraose
LNT	lacto-*N*-tetraose
MALDI	matrix-assisted laser desorption/ionisation
Man	mannose
MTBE	methyl *tert*-butyl ether
MS	mass spectrometry
M.S.	molecular sieves
n-BuOH	*n*-butanol
Neu5Ac	*N*-acetyl neuraminic acid
NIS	*N*-iodosuccinimide
NMR	nuclear magnetic resonance
PE	petroleum ether
Phth	phthalate ester
Rha	rhamnose
Ser	serine
TBAB	tetrabutylammonium bromide
TBAI	tetrabutylammonium iodide
TBDMS	*tert*-butyldimethylsilyl
TBDPS	*tert*-butyldiphenylsilyl
TCA	trichloroacetimidate
TFA	trifluoroacetic acid
TfOH	triflic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TLL	*Thermomyces langinosa* lipase
TMSOTf	trimethylsilyl trifluoromethanesulfonate
Troc	2,2,2-trichloroethoxycarbonyl
UDP	uridine diphosphate
Vin.Ac.	vinyl acetate

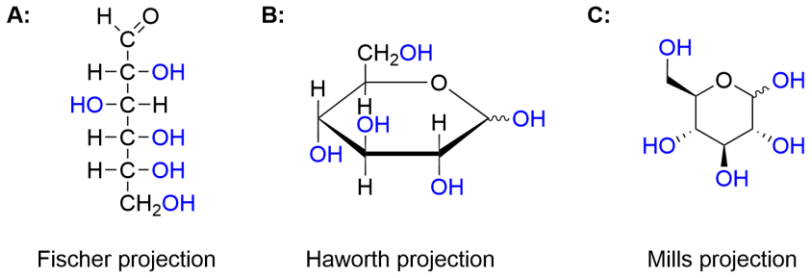
1 Literature overview

1.1 Carbohydrate chemistry

First sources of carbohydrates are from sugar canes which originated from Asia.[1] The sweet white crystals first widely known as "Indian salt" by Greeks and Romans were later named in 1857 by English chemist Miller as we know it today – sucrose.[2] Earliest advances in carbohydrate chemistry were due to the need to find an alternative source to sugarcane for extraction of sucrose. In 1747 German chemist Marggraf discovered that sucrose can be extracted from beetroot using alcohol.[3] However, it was not until 1801 – when Prussia was under embargo from Napoleon's France – that another German chemist, Achard, further developed the extraction process and opened the first sugar extraction factory using beetroot.[4] Marggraf also extracted some other saccharides he deemed were different from the sucrose he obtained from beetroot. One such saccharide was from raisins, which was later confirmed to be glucose. It took close to hundred years before glucose was confirmed to be a distinct saccharide different from sucrose and named in 1838 by French chemists Dumas *et al.* However, it was not until 1891 when Fischer provided conclusive proof by confirming the structure of several saccharides including glucose.[5,6] Today, we know that saccharides play an import role in life, starting from being energy sources for all organisms to being classified as essential medicines according to World Health Organisation.[7]

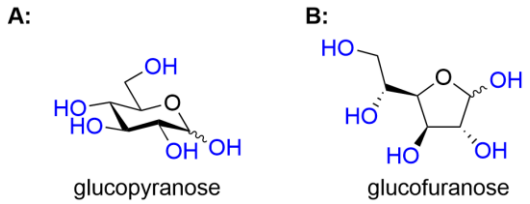
1.1.1 Structures and nomenclature of saccharides

The first projection of saccharides was named after its inventor Fischer.[5] The Fischer projection shows an open carbohydrate chain with either an aldehyde or a ketone at one end (Scheme 1A). Those saccharides can be then named as aldoses or ketoses, with "ald" and "ket" corresponding to aldehyde and ketone functionalities, while "ose" represents saccharides.[8] In the cyclic form of the saccharide, the Haworth projection allows to show more functional groups and longer chains connected to the saccharide core (Scheme 1B).[9] The wavy bond used in the Haworth projection is explained in next section (See Section 1.1.2.). The Mills projection provides a further simplified representation of saccharides closer to their true shape (Scheme 1C).[10] Mills also introduced dashed bonds, which indicate that the attached atom is going away from you into the page, and wedged bonds, where the attached atom is coming towards you, out of the page.



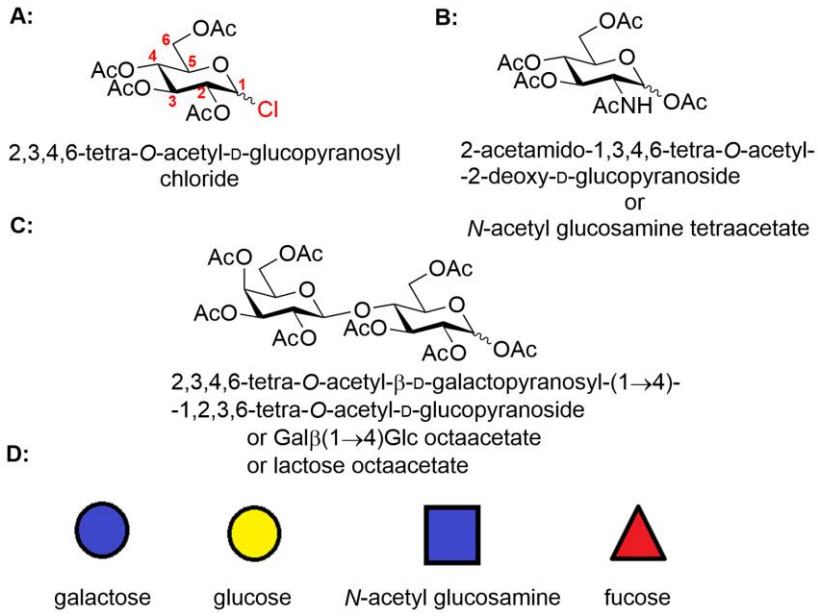
Scheme 1. *Three different projections showing a molecule of D-glucopyranose.* ***A:*** *Open-chain Fischer projection;* ***B:*** *Cyclic Haworth projection;* ***C:*** *Cyclic Mills projection.*

The most commonly used projection for saccharides is also the oldest one - the "chair" projection (Scheme 2A). The chair projection was originally developed by Sachse in 1890 to show that cyclohexane is not a strained molecule.[11] Today, saccharides with six-membered rings, called pyranoses, are most commonly represented using the chair conformation (Scheme 2A). The chair conformation is the closest how the saccharide molecule would look like on paper. Saccharides containing five-membered rings, called furanoses, are more depicted using the Mills projection (Scheme 2B). In both cases, the chirality of the saccharide is clearly seen.



Scheme 2. *Two most commonly used projections for pyranoses and furanoses.* ***A:*** *Chair conformation showing glucopyranose;* ***B:*** *Mills projection showing glucofuranose.*

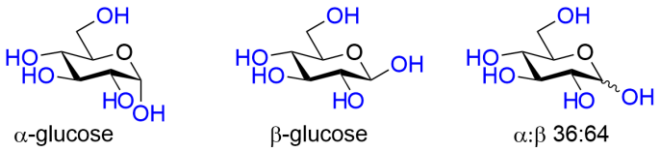
The International Union of Pure and Applied Chemistry (IUPAC) decided to give carbohydrates their own naming convention as well as numbering.[8] As a consequence, trivial names dominate in carbohydrate chemistry, simplifying the overall naming process. The protecting groups and any other modifications in a saccharide molecule are explicitly named. This can be seen when acetyl (Ac) protecting groups and chloride leaving group are used with glucose (Scheme 3A). Leaving groups depart from a saccharide during glycosylation reactions, those saccharides with leaving groups are named as donors. Saccharides that contain nucleophilic groups (e.g. one or more unprotected hydroxyl groups), which will make the bond connecting two saccharides are called acceptors. Unlike in most organic compounds, the naming of saccharides is not based on the oldest functional group but rather based on the core of a common saccharide. As such, *N*-acetyl glucosamine (GlcNAc) is used only when appropriate, otherwise it is known as 2-deoxy-glucose derivative (Scheme 3B). Abbreviations are widely used to further simplify the naming process. Describing oligosaccharides using abbreviations takes up much less space, a good example is with lactose (Lac) octaacetate, which is a disaccharide consisting of galactose (Gal) and glucose (Glc). Its full name is 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-D-glucopyranoside compared to the abbreviated version: Galβ(1→4)Glc octaacetate (Scheme 3C). Finally, symbols can also be used to completely replace names (Scheme 3D). These are mainly used when talking about multiple oligosaccharides.



Scheme 3. *Some examples of the unique way IUPAC and carbohydrate community have decided to name different saccharides;* ***A:*** *Example of acetyl protected glucose with a chloride leaving group;* ***B:*** *The naming rules for glucosamine;* ***C:*** *Different ways to name lactose octaacetate;* ***D:*** *Some symbols shown, which are used to describe saccharides.*

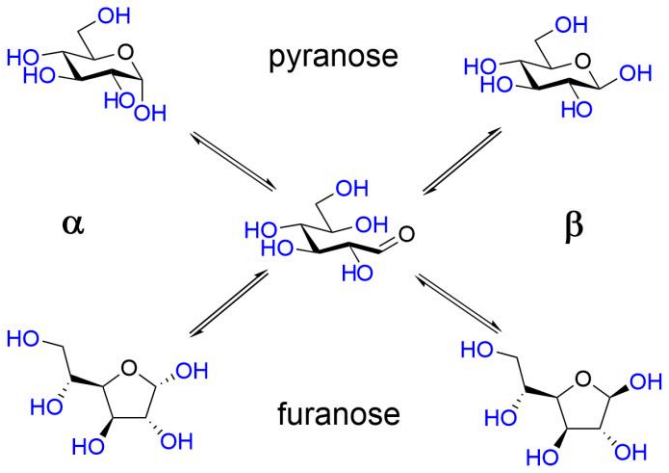
1.1.2 Anomeric configuration, mutarotation and anomeric effect

During the formation of a cycle by the open-chain saccharide, aldehyde in the first position is attacked by the fifth position's hydroxyl group, resulting in the formation of the pyranose ring. As such a chiral centre is formed, where the first position's hydroxyl group can be either in *trans* or *cis* configuration to the fifth position. The two different isomers, known as anomers, are designated as called alpha (*trans* configuration) in axial and beta (*cis* configuration) in equatorial orientation in chair conformation (Scheme 4). The wavy bond indicates the presence of both anomers in a mixture, where generally the anomeric ratio is specified.



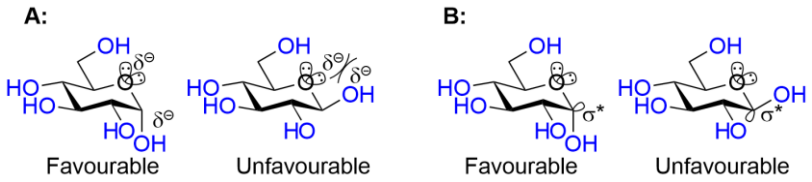
Scheme 4. *Different anomers of glucose and a mixture.*

In solution, there is an equilibrium between open-chain aldehyde and various closed cyclic forms (Scheme 5). There are four possible cycles formed: α- or β-pyranose, and α- or β-furanose. This reversible interconversion between the cyclic and acyclic forms is called mutarotation.[12–15] The amount of specific cyclic forms can be manipulated by changing the pH or solvent.[16,17] In general, the acyclic open-chain form is present only in trace amounts.



Scheme 5. *The mutarotation of glucose.*

The rules for the cyclohexane conformations state that bulky substituents must be in the equatorial position to minimise the steric hindrance.[18] As such the equatorial anomer, β-anomer, should be the major cyclic form present in solution. Indeed, for glucose the α:β ratio of 36:64 is favoured towards the β-anomer, but with considerably high α-anomer presence. In pentaacetates, the α:β ratio has flipped and now is 86:14, which means that other interactions need to be present to stabilise the steric effects. The main stabilising effect is called the anomeric effect.[19,20] Initially the anomeric effect was explained by unfavourable electrostatic effects (dipole-dipole and Coulombic interactions) between the ring oxygen's lone pair and the β-anomer's first position polar carbon-oxygen bond (Scheme 6A).[21] Currently, the anomeric effect is mainly contributed to favourable orbital-orbital interaction with the α-anomer's first position's exocyclic antibonding C-O σ* orbital and the ring oxygen's lone pair (Scheme 6B).[22]

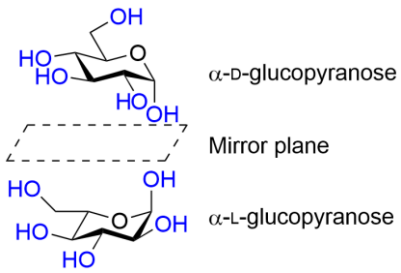


Scheme 6*. The anomeric effects stabilising forces;* ***A:*** *Coulombic unfavourable interaction with glucose* β*-anomer;* ***B:*** *Stabilising antibonding σ* orbital and lone pair orbital-orbital interaction with glucose α-anomer.*

1.1.3 Enantiomeric configuration

It was Louis Pasteur who discovered that a single chemical can exist in two different crystalline forms, which differ in optical properties.[23] What he discovered were two enantiomers of the same compound. Enantiomers are two compounds with the same physical and chemical properties, except optical, in an achiral environment. Enantiomers are mirror images – one enantiomer cannot be superimposed on the other (Scheme 7). Emil Fischer was the first one to classify optically active saccharides into enantiomeric

families with the D- and L-system, derived from Latin words *dextro* and *levo* (right and left).[5,6] The D- and L-system is based on the theoretical agreement. While the (+)- and (-)-system, which is also used, is based on the physical value of the optical rotation determined with the polarimeter. Most saccharides found in nature are D-enantiomers, with exceptions like L-fucose (Fuc) and L-rhamnose (Rha).



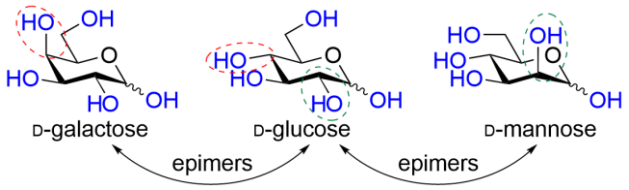
Scheme 7. *Two enantiomers of glucose.*

1.1.4 Epimers of saccharides

Epimers in general are two diastereoisomers which have an opposite configuration on one of the stereogenic centres. According to IUPAC[24]:

"Diastereoisomers are stereoisomers not related as mirror images. Diastereoisomers are characterised by differences in physical properties, and by some differences in chemical behaviour towards achiral as well as chiral reagents."

In the context of saccharides, epimers are two saccharides, that differ in configuration at one stereogenic centre. Galactose is an epimer of glucose due to a difference in the fourth position. Mannose (Man) is an epimer of glucose because of the second position's configuration, but mannose and galactose are not epimers of one another due to having two differences in configuration (Scheme 8).



Scheme 8. *Epimers of D-glucopyranose. With the red circle, the difference in D-galactopyranose and D-glucopyranose structure is shown. With the green circle, the difference between D-mannopyranose and D-glucopyranose structures is shown.*

1.1.5 Chemical methods of protection and deprotection of saccharides

Saccharides are polyhydroxy compounds, where alcohols differ in reactivity due to chemical differences and steric effects. Primary alcohols are the most reactive, while anomeric hemi-acetals can be modified using acetal chemistry. Secondary alcohols are harder to discern, but generally all axial positions are less reactive compared to the equatorial positions (Figure 1).[25] Because of these differences, some selective methods

have been developed to manipulate one position over the other. There still is great interest in developing selective protecting methods. Current methods, some of which are discussed in the following chapters, take either multiple steps, involve many different protecting groups, lack selectivity or result in low yields.

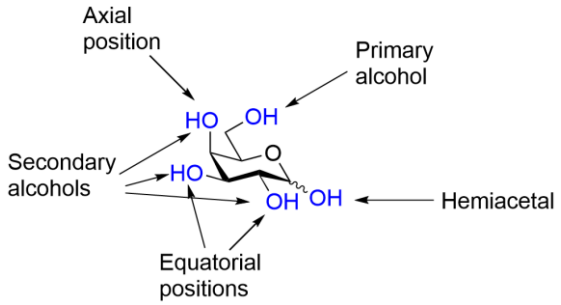


Figure 1. *Different positions of* D*-galactopyranose.*

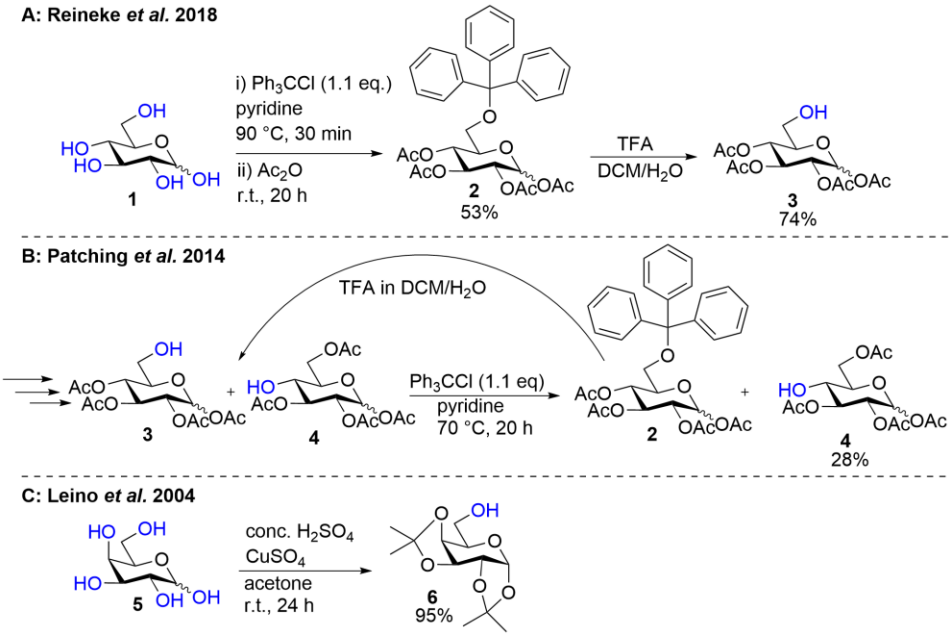
1.1.5.1 Protection and deprotection of primary alcohols

Primary positions in saccharide molecules are further away from the cyclic part of the compound, allowing the use of more bulkier protecting groups. Using this advantage in their strategy, in 2018 Reineke *et al.* selectively protected the sixth position of the fully unprotected D-glucopyranose **1** with a trityl group (Scheme 9A).[26] Next, they protected the remaining positions with acetyl protecting groups resulting in a product **2** in 53% yield. The sixth position was then deprotected using trifluoroacetic acid (TFA) in dichloromethane (DCM)/H_2O mixture. The tetraacetate **3** was isolated in 74% yield.

The downside of the trityl deprotecting reaction is using TFA in the last step as it induces acyl migration towards the sixth position. As such, hard to purify mixture of regioisomers is produced in the final step. In 2014, Patching *et al.* intentionally used this downside to produce the fourth position deprotected D-glucopyranoside tetraacetate **4** (Figure 9B).[27] After removing the trityl protecting group, they reintroduced it to the sixth position of compound **3**, where the migration had not taken place. This way the fourth position is left unprotected, and product **4** can be purified out of the reaction mixture. After four steps and three cycles of trityl group protection and deprotection, the overall yield of 28% was achieved for tetraacetate **4**.

The method described in the Scheme 9A illustrates a general approach how to temporarily protect the primary hydroxyl groups and selectively deprotect them when needed. Different bulky protecting groups like silyl ethers – *tert*-butyldiphenylsilyl (TBDPS)[28], or sulfonyls – *p*-toluenesulfonyl[29] are used in the same fashion.

Some protection strategies exist where the protecting group dictates which positions are protected. These methods work only for some specific saccharides, and the outcome depends on their configuration. For isopropylidene acetal protecting group, the 1,2-*cis*-diols pattern is favoured over 1,2-*trans*-diols and 1,3-diols. In 2003 Leino *et al.* protected D-galactopyranose **5** with two isopropylidene acetals leaving selectively only the sixth position unprotected (Scheme 9C).[30] Both 1α,2- and 3,4-isopropylidene formed were 1,2-*cis*-diols. Product **6** was isolated in 95% yield.



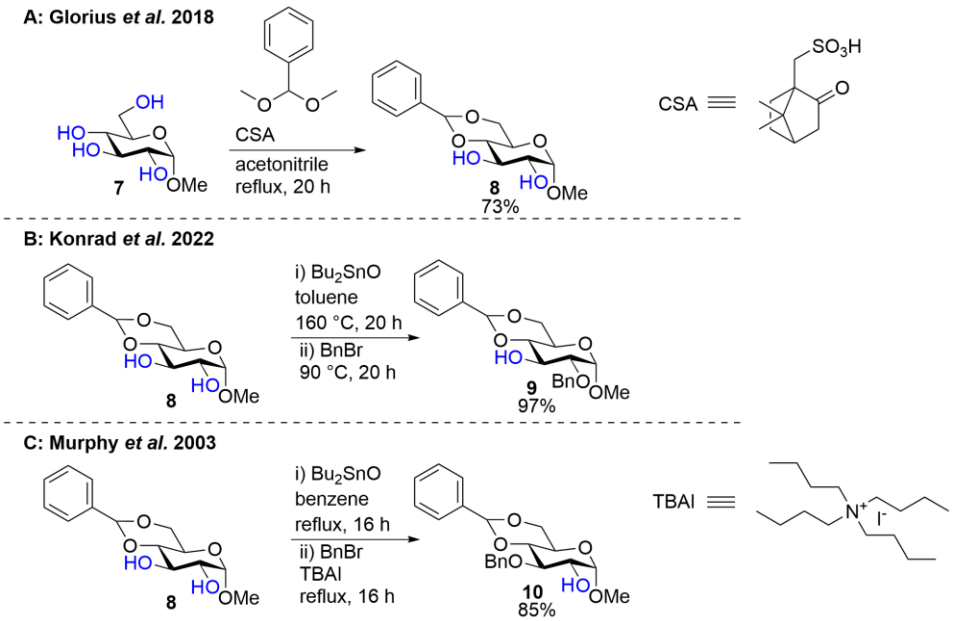
Scheme 9. *Selective protection and deprotection involving the primary hydroxyl group;* ***A:*** *A general method for the selective protection of the sixth position, example given with trityl protecting group;* ***B:*** *Using the TFA induced acyl migration and trityl protecting group to selectively synthesise the fourth position deprotected D-glucopyranoside tetraacetate* ***4****;* ***C:*** *Using acetals to selectively leave the sixth position unprotected.*

1.1.5.2 Selective protection and deprotection of secondary hydroxyl groups

Acetals are commonly used protecting groups in carbohydrate chemistry as they protect two positions simultaneously and can be selectively introduced. Benzylidene acetal is often used to protect the sixth and the fourth positions, as it is too bulky to be introduced elsewhere. The benzylidene acetal is introduced to methyl[31]- thio[32]- or other anomerically protected glycosides[33]. In 2018, Glorius *et al*. used methyl α-D-glucopyranoside **7** with benzaldehyde dimethyl acetal and camphorsulfonic acid (CSA) dissolved in acetonitrile under reflux in an overnight reaction (Scheme 10A).[34] After purification, benzylidene-protected product **8** was isolated in 73% yield.

Once the first, the fourth and the sixth positions are protected, selective protection of the second or the third positions can be carried out. The second position can be then protected using various protecting groups. A common protecting group – benzyl (Bn) can be introduced to glycoside **8** with sodium hydride[35], iron catalysts[36] and dibutyltin oxide as reported by Konrad *et al.* in 2022 (Scheme 10B).[37] The reaction was carried out in two steps. First, the dibutyltin oxide and benzylidene protected glycoside **8** were refluxed in toluene using Dean-Stark apparatus for overnight. The reaction mixture was concentrated resulting in the solid crude mixture, which was reacted with benzyl bromide. After purification, the second position protected product **9** was obtained in 97% yield. The preference to have the second position benzylated is attributed to its higher kinetic activity.[38] It is also thought that the stannylene acetal formed in the first step is a dimer, which due to stereoelectronic effects directs the attack towards the second position.[39]

In 2003, Murphy *et al.* showed that the same reagent (dibutyltin oxide) can be used to selectively protect the third position of the same benzylidene protected glycoside **8** via stannylene acetal (Scheme 10C).[40] The first step was almost identical to what Konrad *et al.* reported, also using dibutyltin oxide but dissolved in benzene, which was heated to reflux and during the reaction water was removed azeotropically. The reaction mixture was then concentrated by half of its volume and benzyl bromide with tetrabutylammonium iodide (TBAI) were added, resulting in selective protection of the third position. Product **10** was isolated in 85% yield. It is theorised that halide anions form complexes with the stannylene acetal, which directs the benzylation towards the formation of one regioisomer.[41,42]



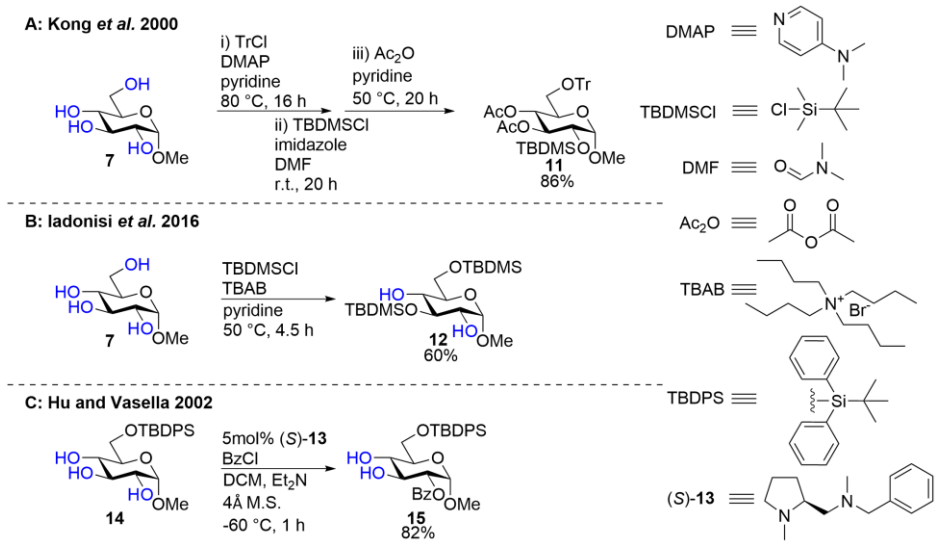
Scheme 10. *Selective protection of secondary hydroxyl groups using acetals;* ***A****: Introduction of 4,6-O-benzylidene acetal to methyl α-D-glucopyranoside* ***7****;* ***B****: Organotin mediated selective benzylation of the second position with glycoside* ***8****;* ***C****: Organotin and TBAI mediated selective benzylation of the third position with glycoside* ***8****.*

Regioselective protection of secondary hydroxyl groups of carbohydrates, without the use of complexes or acetals, requires protection of the anomeric position and any primary alcohols. In 2000, Kong *et al* developed one-pot full protection strategy for alkyl- and thioglycosides (Scheme 11A).[43] The sixth position was protected with the trityl group catalysed by 4-dimethylaminopyridine (DMAP), followed by the regioselective silylation of the second position using *tert*-butyldimethylsilyl chloride (TBDMSCl) and imidazole in *N*,*N*-dimethyl formamide (DMF). The rest of the positions were acetylated using pyridine and acetic anhydride (Ac_2O). The yield of product **11** after three steps was 86%. This reaction was an outlier, as other substrates gave the third position silylated products. The authors did not explain the change in regioselectivity for glycoside **7**.

The same bulky silyl protecting group TBDMS was used in 2016 by Iadonisi *et al*. to protect the sixth and the third positions of glycoside **7** (Scheme 11B).[44] To glycoside **7**, TBDMSCl, pyridine and tetrabutylammonium bromide (TBAB) were added. The resulting product **12** was obtained in 60% yield. The authors did not comment on the regioselectivity.

Comparing the conditions used for silylation, the main difference lies in the TBAB additive, which might have influenced the outcome.

Carbohydrates are chiral molecules, using chiral catalysts might have a regioselective outcome. This was tested by Hu and Vasella in 2002 with a chiral proline-based diamine **13** (Scheme 11C).[45] The methyl α-D-glucopyranoside **14** with the sixth position protected by TBDPS group, was used as the substrate. Using the (*S*)-enantiomer of **13**, led to selective benzoylation of the second position. Product **15** was isolated in 82% yield. The authors did not discuss the reason or mechanism behind the regioselective outcome of the reaction.



Scheme 11. *Regioselective protection of partially protected saccharides;* ***A****: Selective protection of the second position;* ***B****: Selective protection of the third position;* ***C****: Selective protection of the second position using organocatalysis.*

Secondary hydroxyl groups can be selectively deprotected, if there are multiple protection groups involved. Hydrazine hydrate or acetate can selectively deprotect ester groups, when D-glucopyranoside is protected with non-ester groups in the first, the fourth and the sixth positions.[46] The yields varied from 23% to 80%. Peracetylated D-glucopyranoside have been reported to be selectively deprotected by TFA in water[47], though evidently this method remains unreliable.

In conclusion for the chemical protection and deprotection of saccharides, the primary hydroxyl groups of saccharides can be selectively protected by bulky protecting groups. The introduction of these groups needs high temperature, while their deprotection can lead to acyl migration and thus unselective outcomes. Protection of secondary hydroxyl groups of saccharides requires to have anomeric hemiacetal group, and all the primary alcohols protected, unless acetal protecting groups or catalysts/reagents that form complexes are used. Acetal formation is carried out with acid catalysis at high temperatures, while complex-based methods often involve toxic metal reagents at even higher temperatures. There are many chemical methods not mentioned here, that can protect selectively either primary or secondary alcohols. Most of them require either harsh conditions, toxic reagents, multiple steps (e.g. the first and the sixth positions protection) or are low yielding.

1.2 Introduction to lipases

Lipases are enzymes belonging to hydrolases family. Their main function is to hydrolyse triglycerides into fatty acids and glycerol.[48] Lipases are only active in the presence of a biphasic layer – an aqueous layer, where the enzyme is dissolved, and a hydrophobic layer. That is because lipases have lids that open upon contact with hydrophobic phase allowing the substrate to access the catalytic site (Figure 2).[49] These conformational changes can also be induced by organic solvents.[50] This makes lipases resilient enzymes that do not denature and remain active even in the presence of organic media. Even though lipases function in organic solvents, water is still essential to dissolve the enzyme, form the correct conformation and to act as a reagent for the hydrolysis reaction. Lipases are enantioselective catalysts, with a broad range of substrates, and work both in aqueous and organic environments. [51–53]

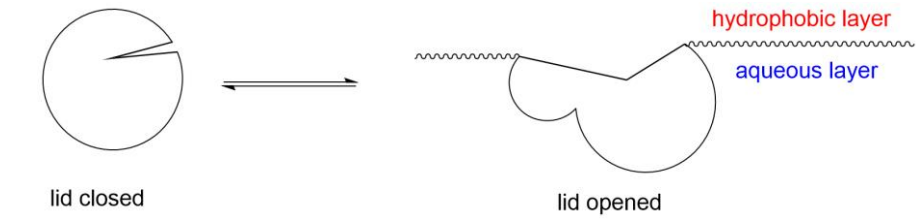
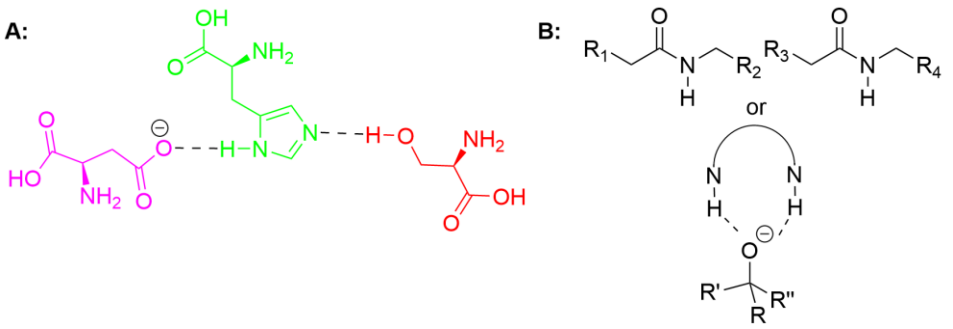


Figure 2. *Lipase activation when in contact with a bilayer interface.*

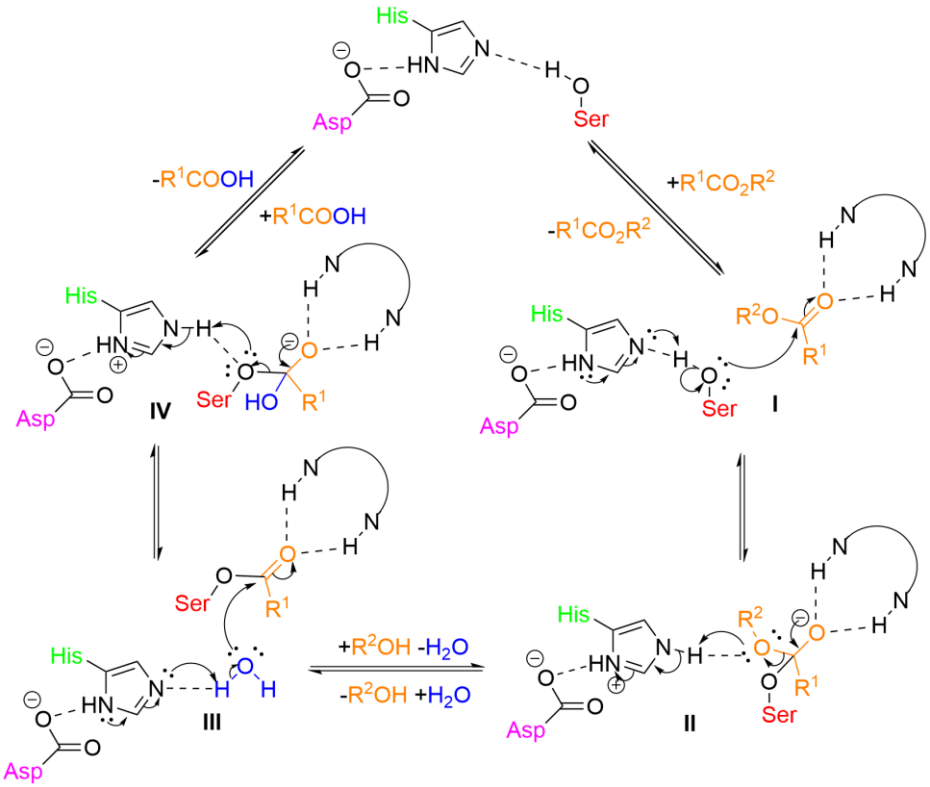
The catalytic site in lipases consists of a catalytic triad, which is generally made of serine (Ser), histidine (His) and aspartic acid (Asp).[54] The catalytic triad is stabilised by intermolecular hydrogen bonding and ionic interactions. The carboxylate anion of Asp is stabilised by His imidazole secondary amine's proton, while Ser hydroxyl group is hydrogen bonded to imidazole's tertiary amine (Scheme 12A). Further stabilisation during the catalytic cycle is provided by the oxyanion hole – a structural feature composed of hydrogen bond donors e.g. backbone amides, that stabilise the formed tetrahedral intermediate in the transition state via hydrogen bonding (Scheme 12B).[55]



Scheme 12. A: *The catalytic triad of a lipase consisting of aspartic acid, histidine and serine;* ***B:*** *Hydrogen bond stabilised tetrahedral oxyanion intermediate.*

The primary function of lipases is to hydrolyse esters. However, since the catalytic cycle is reversible, they are also capable of forming ester functional groups (Scheme 13). Furthermore, if water as a substrate is replaced with an alcohol, reversible

transesterification reactions can be carried out.[56] The catalytic cycle of the hydrolysis starts when the nucleophilicity of Ser hydroxyl group is increased by deprotonation, allowing the formed alcoholate to attack the substrate (Scheme 13**I**). The resulting tetrahedral intermediate's anion is stabilised by charge delocalisation by the oxyanion hole (Scheme 13**II**). Elimination of the alcohol occurs (Scheme 13**II**), and the formed acyl-enzyme complex is then attacked by water, which is activated by the Asp-His complex (Scheme 13**III**). Finally, through the second tetrahedral intermediate both Ser and the formed carboxylic acid product are released reforming the catalytic triad, therefore completing the catalytic cycle (Scheme 13**IV**).[49,57]



Scheme 13. *Catalytic cycle of a lipase hydrolysis reaction.*

Lipases differ from other hydrolases like esterases, which have the same catalytic cycle as lipases, by the presence of a unique lid feature.[58] The lid also dictates the enzyme activity and kinetics in nature, where enzymes are in aqueous solutions. At low substrate concentrations, the lid remains closed, and no catalytic activity is observed. Once the substrate solubility limit is reached and surpassed, micelles are formed, which after reaching critical micellar concentration (CMC) form the lipophilic phase. Then the catalytic activity of the lipase starts. This mode of activation is called interfacial activation.[59] The kinetics of lipases are in sharp contrast to other hydrolases, which follow the Michaelis-Menten activity, where the enzyme activity is correlating to the substrate concentration (Figure 3).[48,60]

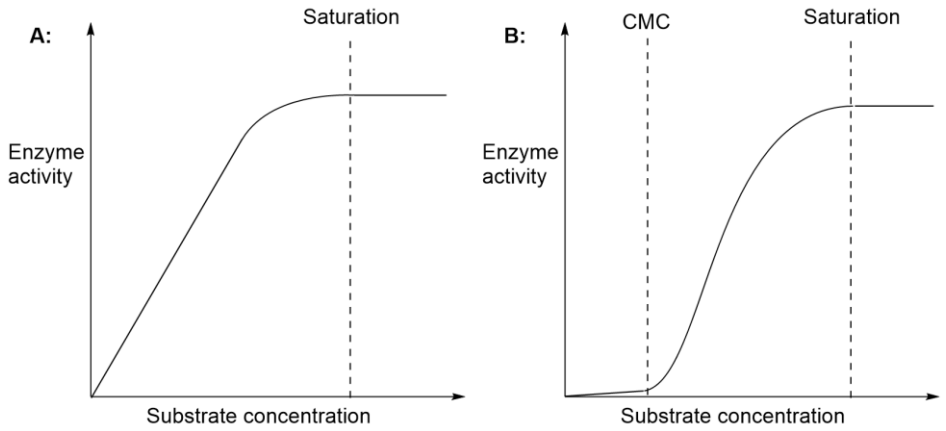


Figure 3. A: *Normal Michaelis-Menten kinetics graph;* ***B:*** *Graph corresponding to lipase kinetics.*

1.2.1 *Candida antarctica* lipase-B

The yeast named *Candida antarctica* was first collected from the bottom of the lake Vanda in Antarctica during an expedition in 1964/65.[61] It was not until 1988 when Novo Nordisk screened a large number of microbes to find industrial use lipases, that it was discovered that *Candida antarctica* produces lipases.[62] Two lipases, named lipase-A and lipase-B, were identified and expressed. Lipase-A turned out to be especially attractive due to its high thermal stability (>90 °C), the ability to hydrolyse tertiary and bulky substrates, and its chemoselectivity towards amine groups.[63,64] *Candida antarctica* lipase-B (CAL-B) turned out to be similarly very stable enzyme that can work in high temperatures (80–90 °C)[65], and has since become one of the most researched lipases.[66] CAL-B has many uses both in industry and in academia: resolution of chiral compounds[67–69], hydrolysis of esters[70,71], esterification of hydroxyl groups[72–75] and transesterification reactions.[76–78] These reactions with CAL-B have been applied in the synthesis of active pharmaceutical ingredients[79–81] and natural compounds.[82–85] Just like other lipases, CAL-B is an interfacial enzyme due to having a lid, which activates upon contact with hydrophobic surfaces. As such, CAL-B has shown high catalytic activity in organic solvents, especially in ethers like methyl *tert*-butyl ether (MTBE), tetrahydrofuran (THF) and cyclopentyl methyl ether (CPME).[86,87] This makes CAL-B highly valuable for organic synthesis, where compounds typically are insoluble in water.

Interfacial activation can be used to immobilise lipases on the hydrophobic support, stabilising the active form, thus increasing the catalytic activity and enabling the use of even higher temperatures.[88] The process of immobilisation[89], along with the chemical[90] and physical properties[91] of the resin, influences both the catalytic activity and stability of the immobilised enzyme. One of the most popular commercially available immobilised CAL-B, Novozyme 435, is immobilised on Lewatit VP OC 1600 a macroporous poly(methyl methacrylate) support. Novozyme 435 is used broadly from synthesis of optically pure compounds to biodiesel production.[92] Immobilised CAL-B has a simpler work-up procedure of just filtration, making it suitable for continuous-flow reactors and enabling enzyme recycling. Immobilised CAL-B has been successfully recycled from the reactions in organic media for at least 5 reaction cycles.[93,94]

1.2.2 Enzymatic methods of protection and deprotection of saccharides

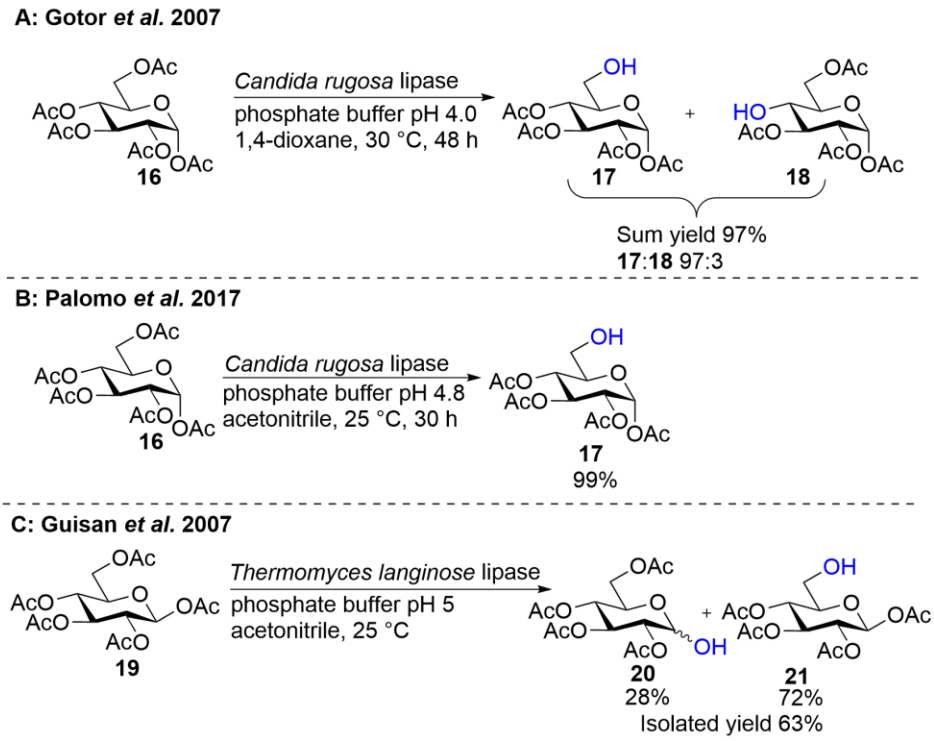
The most common outcome of enzymatic deprotection of peracetylated saccharides is the deprotection at either the first or the sixth positions. While some enzymes can be non-selective and deprotect the saccharide fully. Enzymes can be used both as free forms or immobilised, where immobilised enzymes in theory could be reused. Enzymatic transesterifications, both for introducing protecting groups and deprotecting, are carried out in variety of media: buffer solutions, buffer-organic co-solvent mixtures, ionic liquids or even pure organic solvents. The reaction conditions vary significantly, with temperatures ranging from room temperature up to 60 °C, and the reaction time ranging from 0.5 h to seven days. Overall, the outcome of the enzymatic manipulation depends on which enzyme, substrate and conditions are used.[95]

1.2.2.1 Enzymatic hydrolysis reactions

A selective enzymatic hydrolysis was demonstrated by Gotor *et al.* in 2007 with α-D-glucopyranoside pentaacetate **16** (Scheme 14A).[96] The reaction was performed in a phosphate buffer at pH 4 together with an organic co-solvent. For initial optimisation and lipase selection, the co-solvent was acetonitrile. During the lipase selection process, it was concluded that lipase from *Candida rugosa* had the highest activity. Surprisingly, CAL-B was completely inactive under these conditions. Two regioisomers, the sixth position deprotected compound **17** and the fourth position deprotected compound **18**, were isolated together. Initial results with phosphate buffer at pH 4, acetonitrile as co-solvent at 40 °C after 48 h led to 55% conversion and 2:1 ratio of regioisomers **17**:**18**. After optimisation, including replacing acetonitrile with 1,4-dioxane as the co-solvent, the yield was increased to 97% and the regioisomeric ratio to 97:3 in favour of product **17**.

In 2017, Palomo *et al.* also used lipase from *Candida rugosa*, but they immobilised the enzyme onto a commercial octyl-agarose carrier (Scheme 14B).[97] Using phosphate buffer at pH 4.8, acetonitrile as co-solvent at 25 °C in 30 h they managed to get 99% isolated yield towards the sixth position deprotected product **17**. Compared to Gotor *et al.* in 2007, the small change in pH, temperature and immobilising the enzyme led to a highly selective and high yielding reaction with only single regioisomer as the product. Depending on how they immobilised *Candida rugosa*, the reusability of the immobilised enzyme dropped either by 61% or 25% after the fourth cycle.

In 2007, Guisan *et al*. employed a lipase from *Thermomyces langinose* (TLL), which they immobilised on three different carriers: octyl-agarose, cyanogen bromide activated agarose (CBr-Sep) and polyethyleneimine-agarose (Scheme 14C).[98] They used β-anomer of D-glucopyranose pentaacetate **19** as the substrate. All three TLL immobilised forms yielded two regioisomers: the first position deprotected product **20** and the sixth position deprotected product **21**, with the latter being predominant in all cases. Only for the CBr-Sep immobilised TLL the monodeacetylated products had >50% yield determined by high performance liquid chromatography (HPLC). For CBr-Sep-TLL, the HPLC yield were 28% for product **20** and 72% for product **21**. Product **21** was isolated in 63% yield from the mixture. The authors also tested CAL-B and lipase from *Aspergillus niger* but did not mention how these performed with substrate **19**.



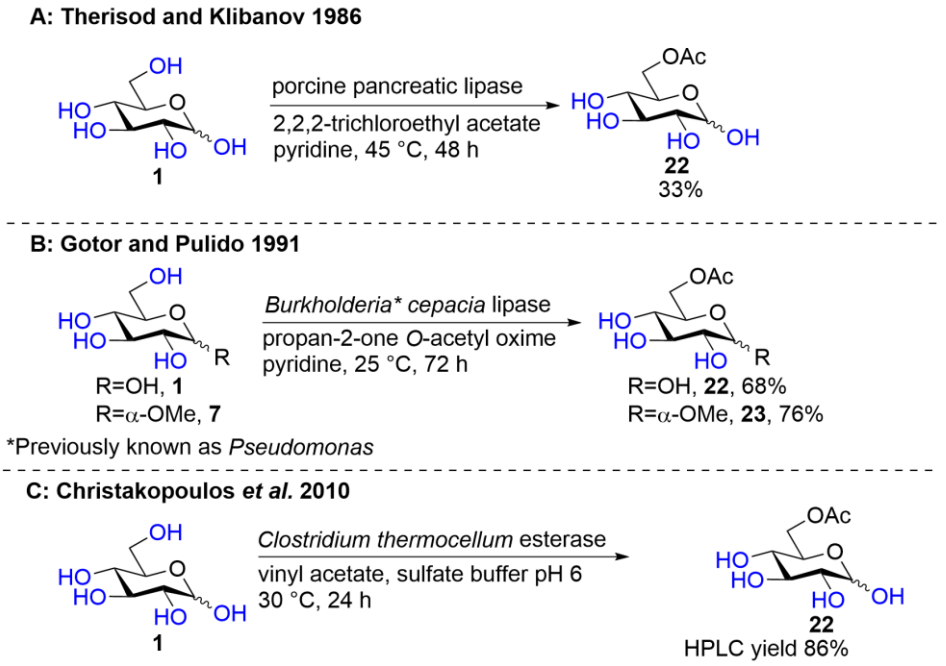
Scheme 14*. Enzymatic deacetylation reactions;* ***A****: Using Candida rugosa lipase in buffer/co-solvent system with α-D-glucopyranoside pentaacetate* ***16*** *resulted in a mixture of products;* ***B****: The reaction with α-D-glucopyranoside pentaacetate* ***16*** *and immobilised Candida rugosa lipase resulted in selective deacetylation;* ***C****: Immobilised Thermomyces langinosa lipase reaction with β-D-glucopyranoside pentaacetate* ***19*** *resulted in a mixture of products.*

1.2.2.2 Enzymatic acetylation and transesterification reactions

Acetylation reactions were performed by Therisod and Klibanov in 1986 using porcine pancreatic lipase (Scheme 15A).[99] Pyridine was chosed as the solvent due to its ability to dissolve saccharides. Both pyridine and the enzyme were dried prior to the use. They used 2,2,2-trichloroethyl acetate as the acetylation reagent. The reaction with unprotected D-glucopyranose **1** was run for two days at 45 °C with 300% w/w enzyme:saccharide, even though after two days only 50% conversion was achieved. The reaction progress was followed by gas chromatography (GC), where they noticed the presence of a minor side product, which was not identified. The authors did not comment, why they chose to end the reactions after two days. The major product **22** was the sixth position mono-acetylated product isolated in 33% yield.

In 1991, Gotor and Pulido used a commercial *Burkholderia* (previously known as *Pseudomonas*) *cepacia* lipase together with oxime esters – irreversible acyl transfer agents (Scheme 15B).[100] Pyridine was used as a solvent, while the reaction was run at 25 °C for three days with 444% w/w enzyme:substrate. The authors did not discuss why they chose the said temperature, how they followed the reaction or why they stopped after three days. Using fully unprotected D-glucose **1** in the acetylation reaction, they managed to get 68% yield of the sixth position acylated product **22**. When they used methyl α-D-glucose **7**, the yield of the sixth position protected compound **23** increased to 76%.

In 2010, Christakopoulos *et al.* used an esterase from *Clostridium thermocellum*, which functions both as an acetyl esterase and a carbohydrate esterase – is active with saccharides and synthetic esters and uses them as substrates (Scheme 15C).[101] They used vinyl acetate as the acetylating reagent in a buffer solution. Using D-glucopyranose **1** as substrate, the reaction was run for 24 h and followed by thin layer chromatography (TLC) or HPLC. 0.4% w/w pure enzyme:substrate was used. The reaction yielded the sixth position acetylated product **22** in 86% HPLC yield. While the authors isolated the product for nuclear magnetic resonance (NMR) and HPLC-mass spectrometry (MS) analysis, they did not mention the isolated yield. The authors, also, did not specify why the exact temperature and pH were used in the reactions.



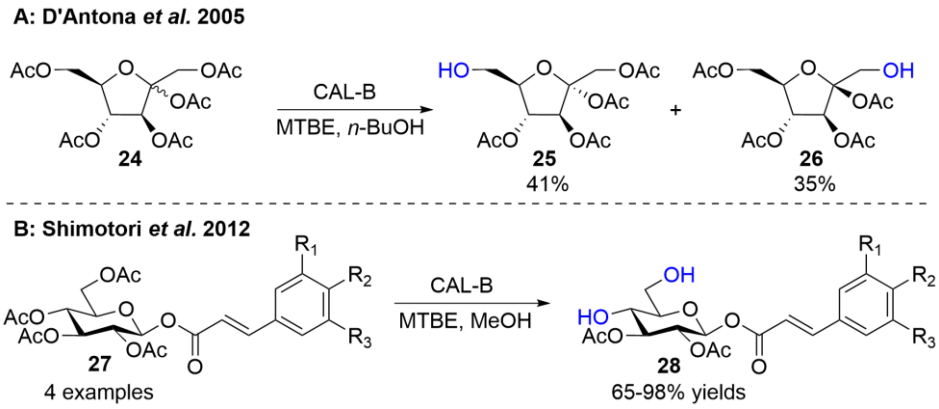
Scheme 15. *Enzymatic acetylation and transesterification reactions;* ***A****: Selective transesterification using porcine pancreatic lipase;* ***B****: Selective acetylation using Burkholderia cepacia lipase;* ***C****: Selective transesterification using Clostridium thermocellum esterase.*

1.2.2.3 *Candida antarctica* lipase-B catalysed transesterification reactions

CAL-B has been used in transesterification reactions for the selective acetylation and deacetylation of saccharides. In 2005, D'Antona *et al.* selectively deacetylated fructofuranoside pentaacetate **24** by using Novozyme 435, immobilised CAL-B (Scheme 16A).[102] For the deacetylation reactions, they used MTBE as solvent, *n*-butanol (*n*-BuOH) as the nucleophilic reagent, 100% w/w CAL-B:substrate at 45 °C. The outcome was that the primary hydroxyl groups were deacetylated in 7 h either in the sixth position for α-anomer (product **25**, 41% yield) or in the first position for β-anomer (product **26**, 35% yield).

In 2012 Shimotori *et al.* expanded the scope of the CAL-B-mediated deacetylation reactions with different glucoside derivatives **27**, targeting the selective deacetylation of the fourth and the sixth positions of D-glucose fragment (Scheme 16B).[103] They used

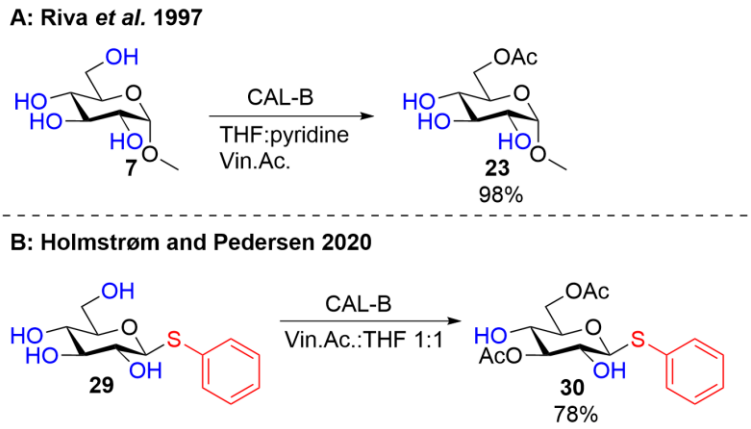
similar conditions, except the nucleophilic reagent was changed to methanol and the temperature was increased to 50 °C. The yields varied, depending on the substrate, from 65% to 98% after 24 h reaction time.



***Scheme 16**. **A**: CAL-B deacetylation reaction with D-fructofuranose pentaacetate **24**; **B**: CAL-B deacetylation reactions with D-glucopyranoside based glycosides **27**.*

In 1997, Riva *et al.* carried out CAL-B-catalysed acetylation reactions using different anomerically protected monosaccharides. The reactions mainly resulted in the sixth position acetylation products. For methyl α-D-glucopyranoside **7** they used THF:pyridine 4:1 solvent solution, vinyl acetate (Vin.Ac.) as the acetylation reagent together with 100% w/w CAL-B:substrate (Scheme 17A).[75] The outcome of the reaction was the sixth position protected product **23** isolated in 98% yield.

In 2020, Holmstrøm and Pedersen used saccharides with thio- and ether protecting groups in the anomeric position as substrates.[74] The major products were the third and the sixth positions protected saccharides. Initially, they screened various solvents using D-glucopyranoside equipped with thiophenol leaving group **29**, vinyl acetate as the nucleophilic reagent and 20% w/w CAL-B:substrate. They found that the neat vinyl acetate and acetonitrile increased the amount of the second and the sixth positions protected regioisomer. In contrast, solvents such as MTBE, THF and 2-MeTHF favoured the formation of the desired the third and the sixth positions protected product **30**. Increasing the amount of vinyl acetate and using it as a co-solvent together with THF were found to be optimal. These conditions led to selectively protected in the third and the sixth positions product **30** in 78% yield in 140 h with 20% w/w CAL-B (Scheme 17B). They, however, did not use any other substrates where anomeric position was left unprotected.



***Scheme 17. A**: CAL-B acetylation reaction with unprotected methyl α-D-glucopyranoside **7**; **B**: CAL-B acetylation reaction with thio-β-D-glucopyranoside **29**.*

In summary, the enzymatic reactions with saccharides depend heavily on which substrate and enzyme were used as well as which conditions were applied. While there has been a lot of research conducted with lipases and esterases, the main outcome for the ester deprotection has been esters from primary hydroxyl groups or anomeric position deprotection. For acetylation reactions, the sixth position protection remains most common and with only few examples of using fully unprotected saccharides. The selective protection or deprotection of secondary hydroxyl groups has been barely reported. Mostly because generally mixtures of regioisomers are the main products, which are hard to purify and analyse.

1.3 Human milk composition

Human breast milk has evolved to nourish infants and fulfil almost every need they have for growth – nutrients, antibodies and growth factors among others. Breast milk is not only vital for babies' development, but also for protection against illnesses like diarrhoea, respiratory and gastrointestinal infections, which are the leading causes of death in children.[104,105] As such, the use of the phrase "breast is best" is well-founded and encouraged by medical professional and scientists alike.[106] It has to be mentioned that not all women are able to breastfeed or produce enough milk, while other complications may inhibit the ability to breastfeed like baby rejecting the breast.[107,108] For those who are able, breastfeeding is encouraged, if possible, for at least for the first six months of the infant's life.[109] While human milk composition varies from mother to mother and is influenced by many factors such as diet, environment, health and the stage of lactation, the macronutrient profile remains relatively among all lactating mothers (Figure 4).[110,111] Water is the main component in human milk with ~88% of the total mass, followed by macronutrients with ~12% and <1% of other components (ash etc.).[112,113] Macronutrients compose of mainly lactose (~53%), fats (~30%), human milk oligosaccharides (HMOs, ~11%) and lipids (~6%).[114]

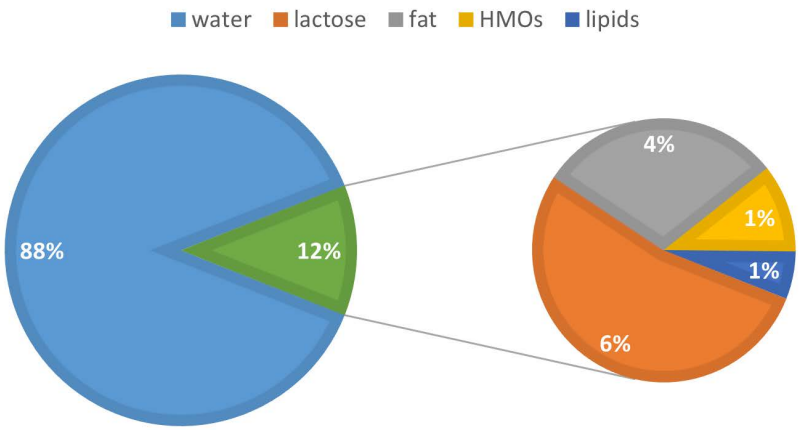


Figure 4. *Composition of human milk. First pie chart shows the distribution of water and macronutrients. Second pie chart shows a more detailed macronutrient composition.*

1.3.1 Structure and role of HMOs

Lactose is the main energy source in human milk, while HMOs, which are also carbohydrates, are not digested by the infant and used as a nutritional source.[115] Instead, the main function of HMOs is to be prebiotics – the energy source for beneficial gut bacteria.[116] Besides being prebiotics, HMOs also act as decoys for pathogenic bacteria and toxins by binding with them, thus inhibiting the growth and decreasing the effect of pathogens on the infants body.[117,118] Furthermore, HMOs modulate T-cells to produce more balanced lymphocytes improving the immune responses, help to develop the brain and cognition by providing essential nutrients, decrease the risk of mastitis for the lactating mother and may prevent allergies in adult patients.[119] The core of HMO structure is generally based around lactose, with additional saccharides bonded to it. HMOs are made of five monosaccharide units: D-Glc, D-Gal, D-GlcNAc, L-Fuc and *N*-acetyl-L-neuraminic acid (L-Neu5Ac) (Figure 5).[120] Both L-Fuc and L-Neu5Ac are always bonded to the rest of the HMO structure with α-glycosidic bonds and they are terminal saccharides, which means no saccharide is bonded to them. Similarly, D-Gal and D-GlcNAc are always bonded with β-glycosidic bonds, and they can be either terminal or have other saccharides bonded to them. The glycosidic bonds are formed in HMOs so that generally there is a reducing saccharide, which is almost always D-Glc. The glycosidic bond formed with L-Fuc is generally in the second, the third or the fourth positions, D-Gal and D-GlcNAc in the third, the fourth or the sixth positions and L-Neu5Ac in all but the first position. The length of HMOs can be more than 50 monosaccharide units, as identified by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS).[121] HMOs with up to 14 monosaccharide units have been elucidated and characterised.[122] More than 150 HMOs have been characterised, while estimates suggest there may be more than 1000 different HMOs in human breast milk.[123]

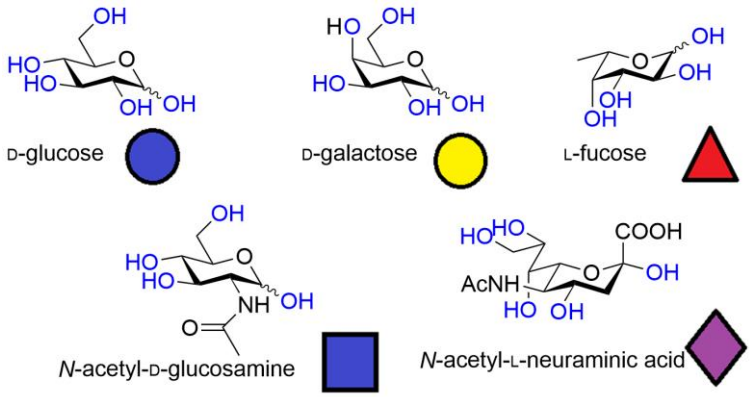


Figure 5. *Five building blocks of HMOs with their respective symbols.*

In human milk, fucosylated HMOs, where at least one of the monosaccharide units is L-Fuc, are the most common with ~63% relative abundance. These are followed by sialylated HMOs containing one or more L-Neu5Ac monosaccharide with ~12%. Sialylated HMOs are called after sialic acid saccharides, which are α-keto acid saccharides with a nine-carbon backbone, they are also called acidic HMOs. Next are Gal-GlcNAc moiety containing oligosaccharides, belonging to the neutral HMO group (fucosylated HMOs are also part of neutral HMOs), with ~12%. The remaining ~13% consists of rare and deviant structures like galactooligosaccharide (GOS) trisaccharides[124,125] Ten HMOs constitute ~80% of all HMOs present in breast milk. Among these, three are trisaccharides (~45% out of total), three are tetrasaccharides (~15%) and four are penta- and hexasaccharides (~20%) (Figure 6).[124]

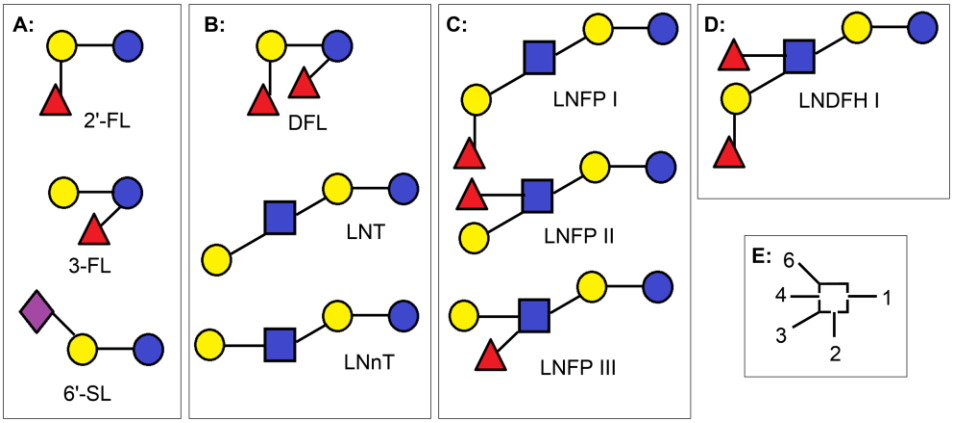


Figure 6. *Ten most abundant HMOs in human milk.* ***A****: Trisaccharides, fucosylated: 2'-fucosyllactose (2'-FL) and 3-fucosyllactose (3-FL) and sialylated: 6'-sialyllactose (6'-SL);* ***B****: Tetrasaccharides, fucosylated: 2',3-difucosyllactose (DFL) and Gal-GlcNAc moiety oligosaccharides: lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT);* ***C****: Pentasaccharides fucosylated: lacto-N-fucopentaose I, II and III (LNFP I, II and III);* ***D****: Hexasaccharide fycosylated: lacto-N-difucohexaose I (LNDFH I);* ***E****: Glycosidic linkage information scheme.*

1.3.2 Enzymatic synthesis of HMOs

The glycosidic bond in HMOs can be formed by glycosyltransferases (GT) and glycoside hydrolases (GH). GTs are enzymes that perform glycosidic reactions in the nature, while GHs are hydrolases and normally perform hydrolysis, but they can transglycosylate as well. GTs have limited stability, they are hard to purify and as such are expensive enzymes that also use expensive nucleotide donors (Figure 7A). GHs, in contrast, are more stable, robust and affordable enzymes, that do not use as expensive starting materials (Figure 7B). However, GHs also have a high hydrolysis activity especially with wild-type enzymes, thus lowering the yields. Increasing the acceptor amount (up to 25 eq.) can shift the equilibrium towards transglycosylation.[126,127]

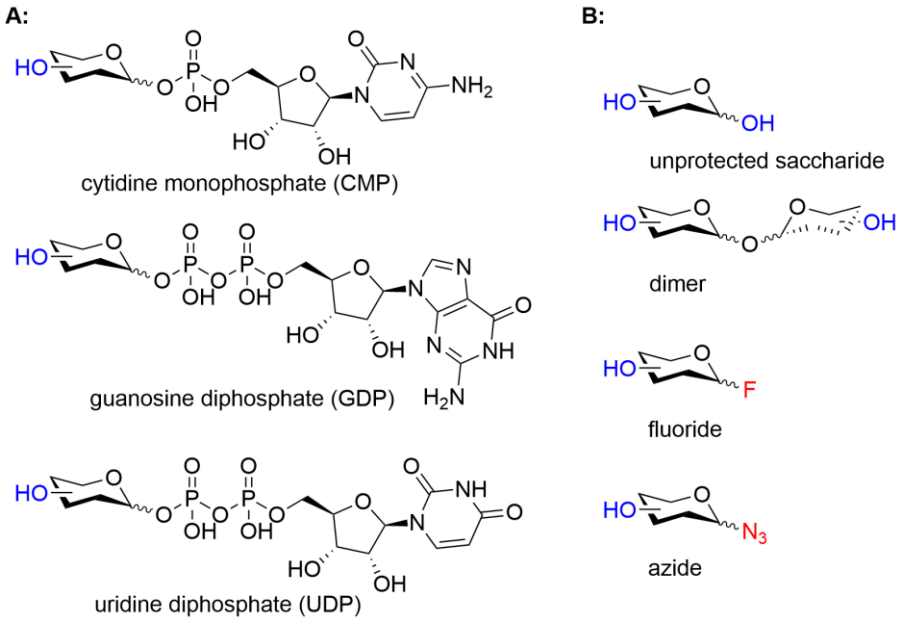


***Figure 7. A**: Substrates for glycosyltransferases (GTs) – saccharides equipped with different nucleotides; **B**: Substrates for glucoside hydrolases (GHs).*

1.3.3 Production of HMOs with cell factories

Cell factories have been used in industry to produce amino acids, biofuel, antibiotics etc.[128] Over the past couple of decades, industrial HMO synthesis has been moving towards cell factories. Although HMOs are not naturally synthesised in microorganisms (e.g. *Escherichia coli* (*E. coli*)), the microbial host organism already has the necessary building blocks (saccharide nucleotides) for HMO synthesis.[129] The host's DNA needs to be modified to produce not only GTs necessary for the HMO synthesis, but also native enzymes that consume intermediates or starting compounds in competing processes have to be knocked out (Figure 8). Purification of HMOs from the host cell would be impractical and intracellular accumulation of oligosaccharides might lead to osmotic stress, as such transporters should be used to deliver HMOs out of cell.[130] During production of HMOs different overreaction side products (like DFL in 2'-FL synthesis) and side products will appear, which could be toxic for the host cell or difficult to purify.

Therefore, it is crucial that produced HMO is free of impurities not naturally found in breast milk to ensure safety for infant consumption.[131]

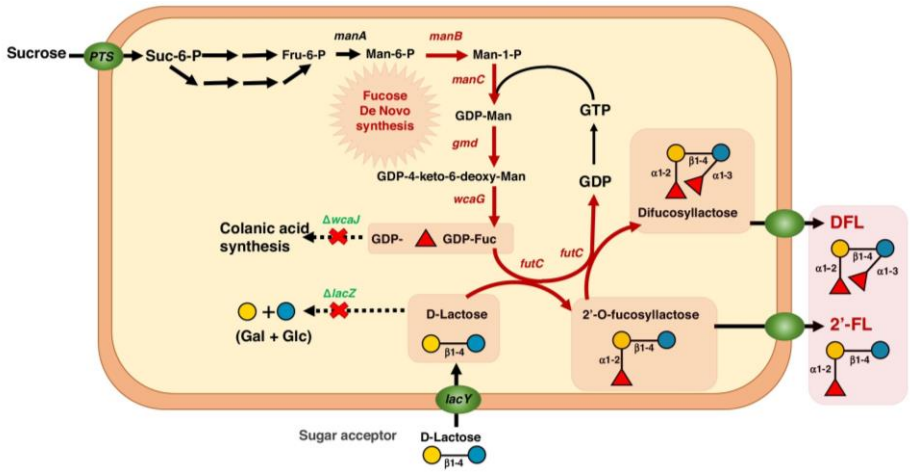


***Figure 8**. Example of a cell factory for 2'-FL production.[1]*

1.3.4 Chemical synthesis of HMOs

Glycosylation reactions involve two substrates: a donor - electrophilic compound, and an acceptor – nucleophilic compound. For glycosylation reactions to work a catalyst or a promotor is required. In chemical synthesis, various types of catalysts have been used to form glycosidic bonds: Brønsted-Lowry acids[132], Lewis acids[133], transition metals[134], organocatalysts[135] etc. Compounds formed in glycosylation reaction via covalent bonds (i.e. glycosidic bonds) are called glycoconjugates.[136] While compounds that have formed a mixed acetal (or a ketal) after hydroxyl group had attacked a saccharide are called glycosides.[8] Glycoside name can also be applied to sulphur and selenium connected compounds – thioglycosides and selenoglycosides, respectively. Unlike proteins, oligosaccharides and glycoconjugates do not have a template-driven synthetic pathways. As such, chemical synthesis of oligosaccharides (e.g. HMOs) is investigated by one target compound at a time in a multi-step synthesis.[137] As saccharides are polyhydroxy compounds the use of protecting groups is necessary.[138] The acceptor must be selectively partially protected to ensure that the glycosidic bond forms in the desired position. The donor must be fully protected and functionalised with a leaving group to prevent self-polymerisation.

Although there is no defined template for glycosylation, solid-supported automated synthesis using automated glycan assembly (AGA) has been developed in hopes to streamline and automate oligosaccharide synthesis.[139] One of the most advanced systems, Glyconeer 3.1, developed by Seeberger *et al.*, is capable of automated glycosylation, protection, deprotection and functionalisation (Figure 9).[140] The reactions can be conducted across a wide temperature range from -40 °C to 90 °C in anhydrous

1 [1] Reprinted from "Current Opinion in Biotechnology", 56, K. Bych, M. H. Miks, T. Johanson, M. J. Hederos, L. K. Vigsnæs and P. Becker "Production of HMOs using microbial hosts — from cell engineering to large scale production", 130-137, 0958-1669/© (2018), with permission from Elsevier

conditions. Using solid support, the initial acceptor is bound until the targeted oligosaccharide is ready to be cleaved using selective photocleavage. The AGA system should allow selective glycosylation, while any unreacted acceptors are capped to ensure they do not participate in next cycle. Both starting materials, donor and solid-support bound acceptor, need to be selectively protected by orthogonal protecting groups. That way, selective deprotection can occur only at the correct position(s), where glycosidic bond(s) are meant to be created.

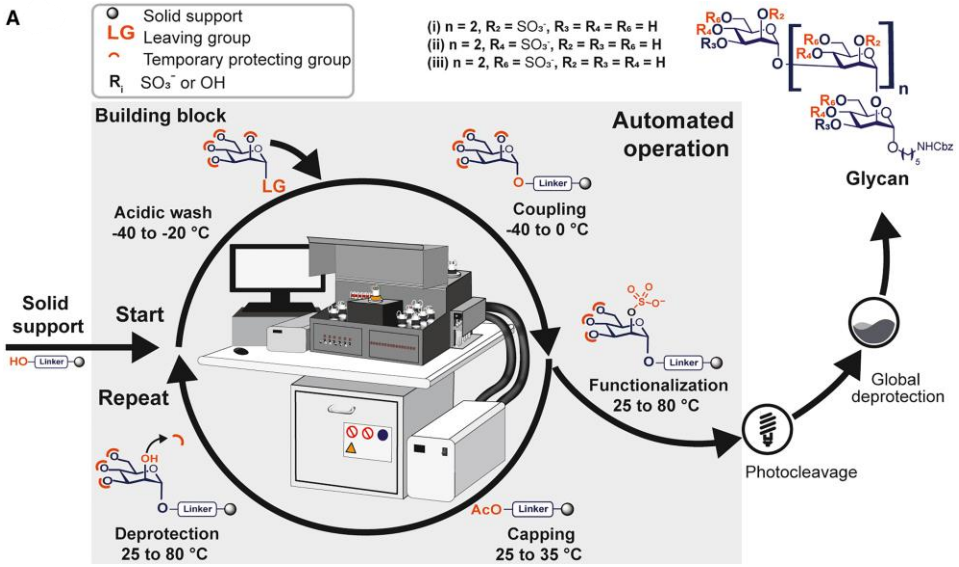
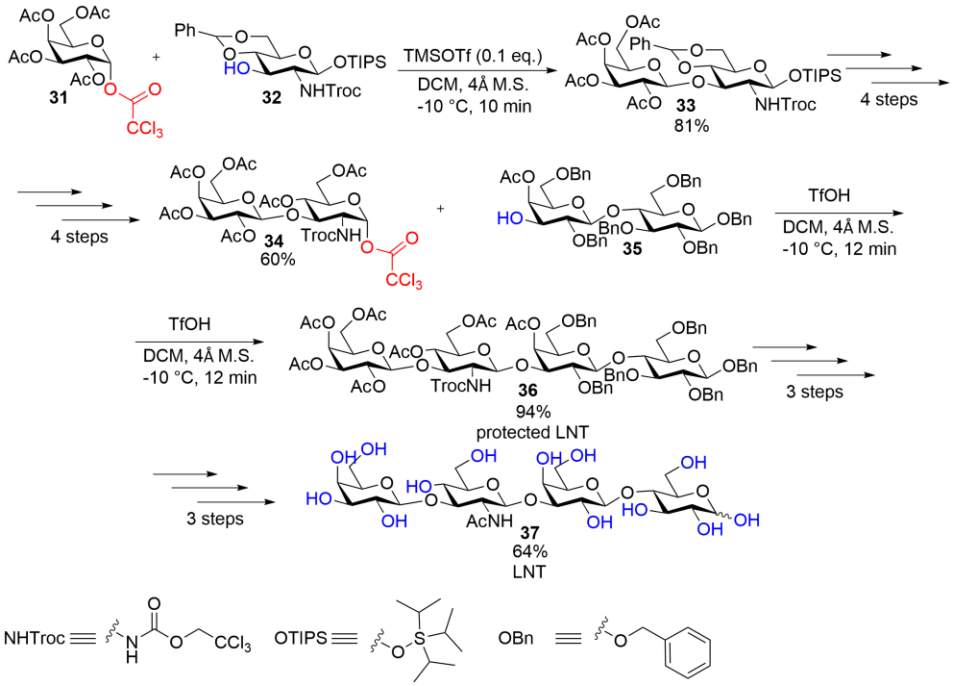


Figure 9. *Illustration of the Glyconeer 3.1 operations. Figure from Seeberger et al. 2024[140] reused under creative common license with permission from Elsevier.*[2]

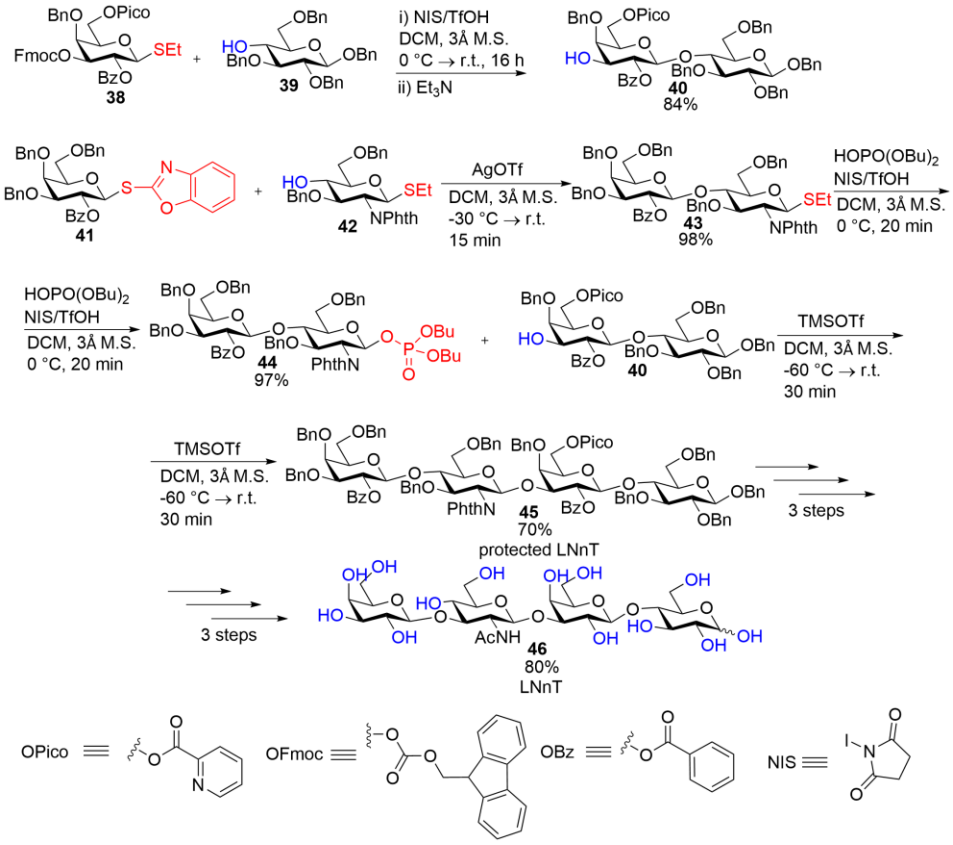
Chemical synthesis of oligosaccharides can be carried out by adding one saccharide at a time in a linear fashion [1+X] or forming and then adding oligomers (e.g. disaccharides [2+X]) together in a convergent way. For the synthesis of tetrasacccharide HMO called LNT, Craft and Townsend in 2017 used [2+X] method (Scheme 18).[141] The synthesis was carried out in total of 25 steps. Starting materials D-galactopyranoside donor **31** and D-glucopyranosamine acceptor **32** were synthesised in 3 steps (yield not reported) and 6 steps (38% yield), respectively. The first glycosylation was carried out by using trimethylsilyl trifluoromethanesulfonate (TMSOTf) as a catalyst in DCM with 4Å molecular sieves (M.S.) under argon at -10 °C with only 10 min reaction time. The protected disaccharide **33** lacto-*N*-biose (LNB) was isolated in 81% yield. Preparation of the LNB donor **34** required an additional 4 steps, while lactose acceptor **35** took 7 steps resulting in 27% yield. The second glycosylation reaction was carried out using triflic acid (TfOH) as the catalyst under similar conditions as previously in 12 minutes. The resulting protected LNT **36** was isolated in 94% yield. The final deprotection of LNT **37** took 3 steps resulting in 64% yield (27 mg), with a total yield of 11%. LNT was purified, isolated and characterised by NMR. The authors have commented in a follow-up review article that this route has been further optimised to produce 5 g of LNT over ten days.[142]

[2]Figure reused under Attribution-NonCommercial-NoDerivatives 4.0 International licence https://creativecommons.org/licenses/by-nc-nd/4.0/.



Scheme 18. *Chemical convergent synthesis of lacto-N-tetraose HMO.*

The synthesis of lacto-*N*-neotetraose (LNnT) was carried out by Demchenko *et al*. in 2019 using both the convergent [2+X] and the linear [1+X] approaches (Scheme 19, only convergent is shown).[143] The synthesis was carried out in total of 35 steps. The lactose acceptor **40** was derived from D-galactopyranoside donor **38** (synthesised in 7 steps with 67% yield) and D-glucopyranoside acceptor **39** (synthesised in 6 steps with 13% yield). The glycosylation reaction was catalysed by TfOH with a *N*-iodosuccinimide (NIS) promoter in DCM using 3Å M.S. and in 16 h. After completion, the fluorenylmethoxycarbonyl protecting group (Fmoc) was removed with triethylamine. The resulting lactose acceptor **40** was obtained in 84% yield. The disaccharide donor **43** was synthesised from D-galactopyranoside donor **41** (synthesised in 8 steps with 22% yield) and D-glucopyranosamine acceptor **42** (synthesised in 7 steps with 13% yield). The glycosylation reaction catalysed by silver triflate was conducted in DCM with 3Å M.S. starting at -30 °C and warmed up to room temperature. The total reaction time was 15 minutes and resulted in 98% isolated yield of the disaccharide donor **43**. The thioethyl leaving group was then exchanged to phosphate ester with dibutyl phosphor acid, NIS and TfOH in 97% yield. The disaccharide donor **44** with phosphate ester leaving group and lactose acceptor **40** were used in the glycosylation reaction catalysed by TMSOTf. The reaction was run in DCM with 3Å M.S. at -60 °C and warmed up to room temperature. The total reaction time was 30 minutes and resulted in protected LNnT **45** in 70% isolated yield. The full deprotection took three steps and resulted in 80% (21 mg) isolated yield for LNnT **46**. The yield of the convergent [2+2] LNnT synthesis was 66% over three glycosylation reactions, compared with the linear approach with 57% yield. Over 35 steps, the total yield for the synthesis of LNnT was 0.1% mostly due to the low yields for selective protection and deprotection of starting materials.



Scheme 19. *Chemical convergent synthesis of lacto-N-neotetraose HMO.*

In summary, the chemical synthesis of HMOs is performed in a multistep way using different protecting groups to selectively protect the starting materials. Chemical synthesis allows to get a single HMO in high purity, but the complex strategies and many steps required make it time consuming. As such every extra step reduces the overall yield and increases the time needed to get to the target HMO. Ultimately, complexity of the process currently limits the scale of production. For these reasons, the industrial manufacture of HMOs and other complex oligosaccharides is not currently feasible through chemical synthesis.

2 Aims of the present work

Oligosaccharides and natural carbohydrate-containing compounds have glycosidic bonds formed at specific positions with defined anomeric and enantiomeric configurations. Enzymes, pure ones or in cell factories, can be utilised to produce such oligosaccharides, these often result in complex mixtures that are difficult to purify. In contrast, chemical synthesis enables to produce a single oligosaccharide in high purity. However, this usually requires multiple steps and extensive use of protecting groups to get the correct partially protected starting material for the glycosylation reactions. Lipase-catalysed reactions offer an efficient alternative for selective deprotection of ester-protected saccharides. This strategy can significantly decrease the steps needed for the synthesis of the starting materials. The main focus of this work was the enzymatic synthesis of selectively partially protected saccharides, which are valuable building blocks for the synthesis of natural oligosaccharides.

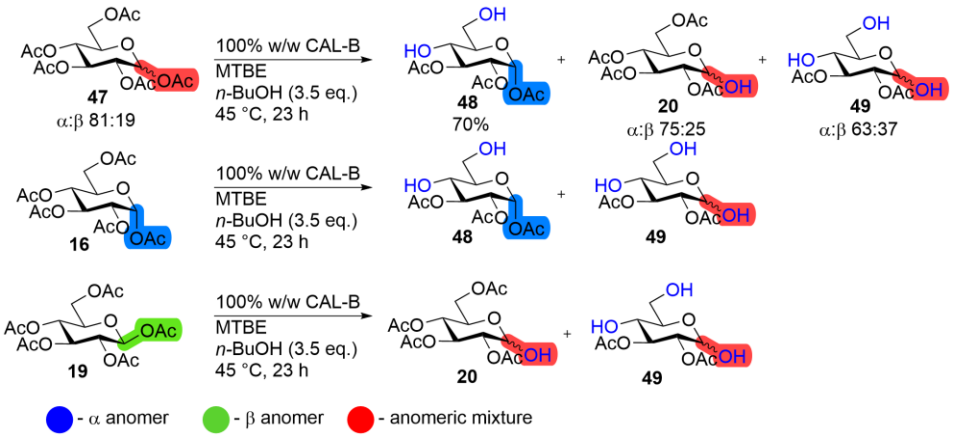
The specific aims of the thesis were:

- Synthesis of selectively deacetylated saccharides or thiosaccharides from peracetylated saccharides via CAL-B mediated transesterification reactions;
- Carry out transesterification reactions with CAL-B to selectively acetylate fully unprotected saccharides or thiosaccharides;
- To develop new methods for the synthesis of natural oligosaccharides, more specifically 6'-galactosyllactose.

3 Results and discussion

3.1 Optimisation and insight to deacetylation reactions with CAL-B

The substrate chosen to evaluate whether the deacetylation reactions with CAL-B works selectively was D-glucopyranoside pentaacetate **47**. Product **48** was isolated in 70% yield when the transesterification reaction was performed using 100% w/w CAL-B with *n*-BuOH as nucleophilic reagent in MTBE at 45 °C for 23 h. It was noted that the main product the fourth and the sixth positions deprotected **48** was anomerically pure. Several side products were also isolated, most notably the first position deprotected D-glucopyranose tetraacetate **20** and the first, the fourth and the sixth positions deacetylated D-glucopyranose diacetate **49**. Evidently, the anomers of D-glucopyranoside pentaacetate **47**, reacted differently. The dominant α-D-glucopyranoside pentaacetate **16**, gave the major product **48** and smaller amounts of diacetate **49**, while the β-D-glucopyranoside pentaacetate **19** yielded either anomeric deprotection product **20** or diacetate **49** (Scheme 20).



Scheme 20. *Different products of deacetylation reactions with D-glucopyranoside pentaacetates based on anomeric configuration. Below the scheme is the colour code used from now on: blue ● shows α-anomers, green ● shows β-anomers and red ● shows an anomeric mixture.*

Nevertheless, the optimisation was carried out with the anomeric mixture of pentaacetate **47** (Table 1). Solvent screening revealed that toluene halved the reactivity, while most other solvents gave trace amounts of the fourth and the sixth positions deacetylated product **48** (Table 1, entries 2-6). CPME showed comparable performance as MTBE (Table 1, entries 1, 7). As such, CPME was used interchangeably with MTBE throughout the CAL-B deacetylation reactions studied both in **Publication I** and **Publication II**. Lowering the concentration of the reaction mixture did increase yield for product **48** slightly, increasing the concentration did not influence the outcome (Table 1, entries 8, 9). Increasing the amount of CAL-B accelerated the reaction, which increased the amount of overreaction product diacetate **49**, thereby lowering the isolated yield of main product **48** to 63% (Table 1, entry 10). Decreasing the amount of CAL-B had the opposite effect with increasing the yield of product **48** to 82% (Table 1, entry 11). Temperature had significant effect on the outcome of the reaction. Decreasing the

temperature to 30 °C slightly increased yield, though full conversion was not achieved. Both room temperature and 55 °C decreased significantly the isolated yield of product **48** (Table 1, entries 12–14). Using both 55 °C and 200% CAL-B led to full conversion in 4 h, but the yield of product **48** dropped to 63% due to overreaction (Table 1, entry 15). In the absence of the nucleophilic reagent, mostly anomeric position was deacetylated with only 20% of product **48** was isolated (Table 1, entry 16).

Table 1. *Optimisation of the CAL-B deacetylation reaction with D-glucopyranoside pentaacetate* ***47****.*[a]

Entry	*Deviation from initial conditions*	*Yield of 48 (%)*[b]
1	-	70
2	Toluene as solvent and 55 °C	Traces[c]
3	Acetone as solvent and 55 °C	32[c]
4	MeCN as solvent and 55 °C	8[c]
5	Ethanol as solvent and 55 °C	Traces[c]
6	Chloroform as solvent and 55 °C	Traces[c]
7	CPME as solvent and 55 °C	69
8	0.05 M of pentaacetate **47**	69
9	0.013 M of pentaacetate **47**	74
10	200% w/w CAL-B	63
11	50% w/w CAL-B	82
12	Room temperature	54
13	30 °C	77
14	55 °C	54
15	55 °C, 200% w/w CAL-B and 4 h reaction time	63
16	No *n*-BuOH added	20

[a] – Reaction conditions: D-glucopyranoside pentaacetate **47** (100 mg), 100% CAL-B (w/w), *n*-BuOH (3.5 eq.), MTBE (10 mL), 45 °C, 23 h; [b] – isolated yield; [c] – Relative ratio of the product against reaction mixture, determined by ^{1}H NMR.

When the deacetylation reaction of α-D-glucopyranoside pentaacetate **16** with CAL-B was followed over time, it was found that the reaction peaked at 8 h with 89% conversion to product **48**. After 8 h, further deacetylation started to occur, leading to the formation of diacetate **49**, which decreased the yield of product **48** to 82% after 23 h. It was also noted that an intermediate, with the sixth position deacetylated, appeared and disappeared during the reaction, indicating that deacetylation is a stepwise process. The first step being the sixth position's deacetylation followed by the fourth position's deacetylation. The opposite stepwise deacetylation was ruled out, as the amount of the fourth position deacetylated side product remained constant once the starting material was consumed. [77]

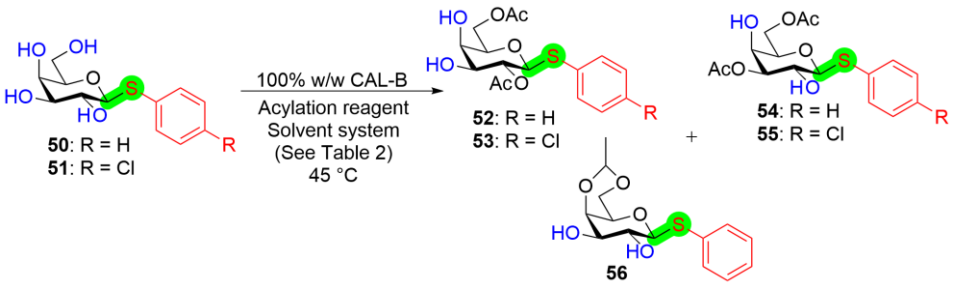
Computational studies, carried out by Alfonso T. Garcia- Sosa and Uko Maran, showed that α-D-glucopyranoside pentaacetate **16** is stabilised by additional hydrogen bonding and has a better fit in the active cite of CAL-B compared to D-glucopyranoside pentaacetate β-isomer **19**.[77] This could partially explain the observed difference in outcome between those two anomers.

To explore the versatility of the method, different alcohols were screened as nucleophilic reagents in the CAL-B-mediated deacetylation reaction of α-glucopyranoside pentaacetate **16**. It was found that *n*-BuOH can be replaced by lower boiling point

alcohols like methanol and ethanol without any significant loss of yield (~2–5%). It was further confirmed by deacetylation reactions with β-glucopyranoside pentaacetate **19** and EtOH in 100 mg scale, which resulted in only 2% lower NMR yield, and with α-galactopyranoside pentaacetate **76** and EtOH in a 10-gram (100 times scaled up) reaction, where only a 4% drop in isolated yield was observed (the latter experiment was conducted by Nora Deil).

3.2 Optimisation of acetylation reactions with CAL-B

The goal set for the acetylation reactions with CAL-B was to selectively protect D-galactopyranose, leaving the third position unprotected. The substrate chosen for this investigation was thio-β-D-galactopyranoside **50** (Scheme 21). As both thiophenol and *p*-chlorothiophenol leaving groups attached to monosaccharides showed no difference in reactivity with CAL-B, after initial reactions with thiophenol, it was replaced with *p*-chlorothiophenol as less foul-smelling alternative.



Scheme 21. *Acetylation reaction with thio-β-D-galactopyranosides **50** and **51** with CAL-B.*

Starting with Riva *et al.*[75] conditions, the acetylation reaction gave only traces of desired product resulting in mainly mixture of monoacetylated products (Table 2, entry 1).[144] Replacing THF with MTBE afforded acceptable yield of the second and the sixth positions acetylated product **52** with 49%, but with 20% of the undesired the third and the sixth positions acetylated product **54** (Table 2, entry 2). When using only pure solvent, both MTBE and chloroform gave surprisingly an 4,6-*O*-ethylidene acetal **56** in ~50% yield (Table 2, entries 3, 4). While acetals are common protecting groups, their enzymatic formation is rare, except glycosidic acetal bonds. Acetone gave a mixture of products with both vinyl acetate and acetic anhydride as acetylation reagents (Table 2, entries 5, 6). Acetonitrile had an interesting effect on the reaction. Depending on the acetylation reagent, one isomer dominated over the other. Using acetic anhydride in acetonitrile favoured the formation of side product **54** as the major product, while vinyl acetate in acetonitrile selectively produced the desired isomer **52** in high yields (Table 2, entries 7, 8). Following the procedure reported by Holmstrøm and Pedersen in 2020[74], increasing the amount of vinyl acetate and the reaction concentration with 20% w/w CAL-B led to full conversion. Although, in THF the reaction required four days, and the amount of the side product **55** had increased compared to the reaction in acetonitrile (Table 2, entries 9, 10). Increasing CAL-B loading to 60% w/w decreased the reaction time to 24 h without affecting the isomeric ratio of the crude mixture (Table 2, entries 11, 12). As the last step in the synthesis of starting material **51** is Zemplén deacetylation[145], where acetic acid or dry ice can be used to neutralise sodium methoxide, both sodium acetate

and sodium carbonate were tested as additives (Table 2, entries 13, 14). Sodium acetate increased the amount of the side product **55** seen in the crude mixture, while sodium carbonate had no effect on the reaction. Using 100% w/w CAL-B did not decrease the reaction time to a single-day reaction, while optimising the product purification gave 80% isolated yield (Table 2, entry 15). Both increasing or lowering the reaction concentrations led to increased amount of side product **55** seen in the crude mixture (Table 2, entries 16, 17). When there was no CAL-B used, the reaction did not proceed (Table 2, entry 18). The best conditions were achieved using 60% or 100% w/w CAL-B in acetonitrile:vinyl acetate mixture as 40% w/w CAL-B had small amounts of starting material left unreacted (Table 2, entries 12, 15).

***Table 2**. Optimisation of CAL-B acetylation reaction with thio-β-D-galactopyranosides **50** and **51**.*[a]

Entry	Sub. Used	Deviation from initial conditions	Time (h)	Product yield (%)[b]		
				52/53	54/55	56
1	**50**	-	168	Traces	Traces	-
2	**50**	MTBE:pyridine as solvent	48	49	20	-
3	**50**	Chloroform as solvent	72	-	-	51
4	**50**	MTBE as solvent	72	-	-	49
5	**50**	Acetone as solvent	96	Traces	Traces	-
6	**50**	Acetone as solvent, Ac_2O as acyl reagent	96	Traces	Traces	-
7	**50**	MeCN as solvent, Ac_2O as acyl reagent	48	18*	50*	-
8	**50**	MeCN as solvent	48	72	-	-
9	**51**	THF:Vin.Ac. 1:1 (2.4 mL), 20% CAL-B w/w	96	83* (56)	17*	-
10	**51**	MeCN:Vin.Ac. 1:1 (2.4 mL), 20% CAL-B w/w	48	92* (57)	8*	-
11	**51**	MeCN:Vin.Ac. 1:1 (2.4 mL), 40% CAL-B w/w	24	94*	6*	-
12	**51**	MeCN:Vin.Ac. 1:1 (2.4 mL), 60% CAL-B w/w	24	93 (72[c])*	7*	-
13	**51**	MeCN:Vin.Ac. 1:1 (2.4 mL), 60% CAL-B w/w, NaOAc (2 eq.)	24	72*	28*	-
14	**51**	MeCN:Vin.Ac. 1:1 (2.4 mL), 60% CAL-B w/w, Na_2CO_3 (2 eq.)	24	93*	7*	-
15	**51**	MeCN:Vin.Ac. 1:1 (2.4 mL)	24	93* (80)	7*	-
16	**51**	MeCN:Vin.Ac. 1:1 (4.8 mL)	24	74*	26*	
17	**51**	MeCN:Vin.Ac. 1:1 (1.2 mL)	24	83*	17*	
18	**51**	No CAL-B	96	-	-	-

[a] – reaction conditions: thioglycoside **50** or **51** (60 mg), vinyl acetate (20 eq.), THF:pyridine 4:1 (5 mL), 100% w/w CAL-B (60 mg), 45 °C; [b] – Isolated yield shown or in (); [c] – purified by crystallisation with petroleum ether:ethyl acetate (PE:EtOAc) * – determined from ^{1}H NMR from the crude mixture; Sub. – substrate.

3.3 Transesterification reactions with CAL-B summaries

All the results from transesterification reactions (both deacetylation and acetylation reactions) with CAL-B from **Publications I–III** are summarised based on the saccharide used. This approach provides a comprehensive overview of how a single saccharide reacts with CAL-B and collectively presents all the different saccharides used in **Publications I–III**. For clarity, deacetylation reactions will not be distinguished whether they were performed in **Publication I** or **Publication II**. Acetylation reactions were exclusively carried out in **Publication III**.

3.3.1 Glucopyranose

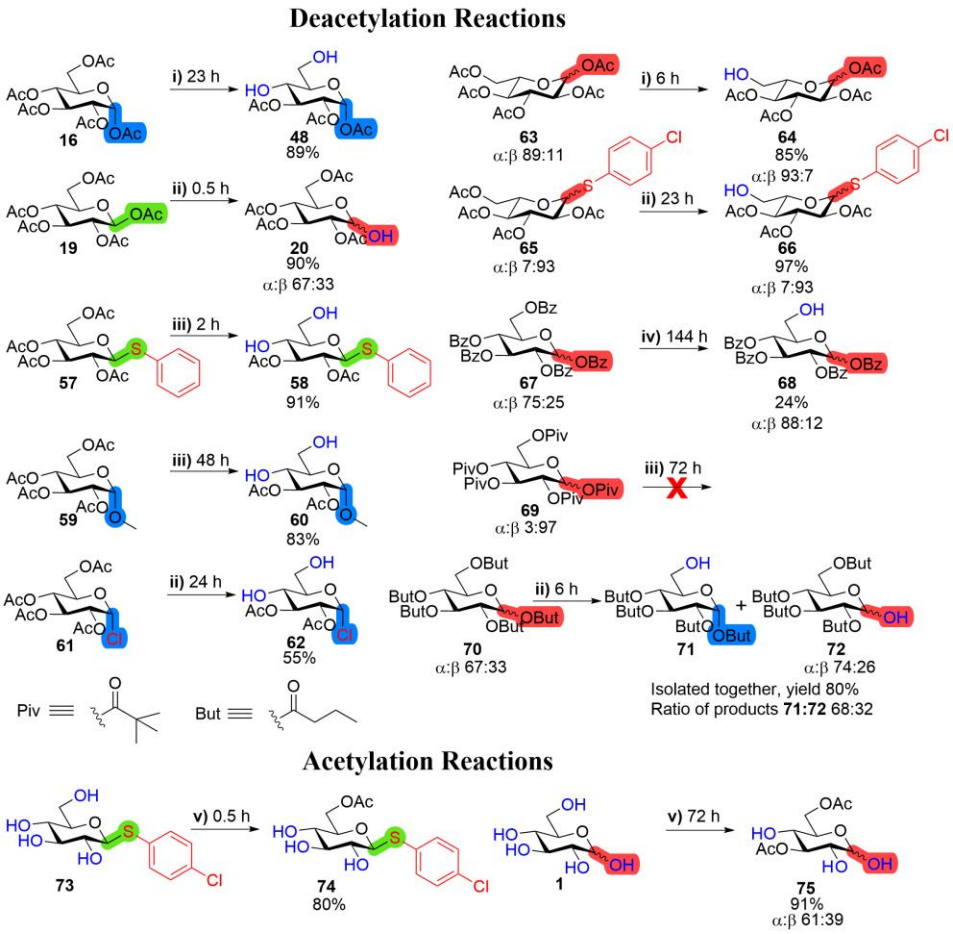
As mentioned in Section 2.2, depending on the anomeric configuration, the CAL-B deacetylation reactions outcome changes. Briefly summarising the differences for pentaacetates – the α-anomer has the fourth and the sixth positions deacetylated, forming product **48** selectively in 23 h with 89% yield (Scheme 22). While the β-anomer has the first position deacetylated forming tetraacetate **20** in 90% in only 30 minutes. Traditionally, the first position is deacetylated by either using hydrazine acetate (1:1)[146] or primary amines like benzyl amine[147] or ethylene diamine[148]. The advantages of using CAL-B for the anomeric deacetylation are that the crude mixture is sufficiently pure for subsequent reaction, easy work-up consisting of filtering and concentrating the reaction mixture, and no neutralisation or washing steps are required. α-Pentaacetate D-glucopyranose **16** deacetylation reaction was conducted with 50% w/w CAL-B at 45 °C in MTBE with *n*-BuOH (3.5 eq.) as nucleophilic reagents – condition **i)**, for β-anomer, the same conditions were used except with 100% w/w CAL-B – condition **ii)**. Changing the anomeric protecting group to thiophenol leaving group, the β-tetraacetate **57** reacted slowly with 100% w/w CAL-B. Increasing the amount of CAL-B to 200% w/w and the temperature to 60 °C, condition **iii)**, significantly accelerated the reaction, affording the fourth and the sixth positions deacetylated product thioglycoside **58** in 91% yield after 2 h. Having methoxy group in the anomeric position, the α-tetraacetate **59** had a slow rate of reaction even at harsher conditions of 200% w/w CAL-B and 60 °C. The reaction took 48 h while also producing the fourth and the sixth positions deacetylated product **60** in 83% yield. Using chloride as the leaving group had a three-step reaction profile.[76] The first step consisted of selective transesterification, where most of the starting material had reacted (~68% conversion) to selectively give the fourth and the sixth positions deacetylated product **62**. The first step took ~7 h, which was followed by the second step, lag phase. The lag phase took place between the 7th and 24th hours of the reaction time, during which minor amounts <5% of overreaction products appeared. In the next 24 h (between 24th and 48th hour) the third phase, non-selective deacetylation, happened. Interestingly, after 48 h there was no product **62** leftover, yet 32% of the unreacted starting material **61** remained in the solution. Two hypothesis were drawn: the reaction follows product activation reaction characteristics[149], and both product **62** and overreaction products inhibit the access of the starting material **61** to the CAL-B's catalytic site. Harsher conditions did not lead to any improvements on the reaction outcome. The substrates with L-configuration **63** and **65** changed the configurational preference of CAL-B, and the positions targeted. In this case, anomeric configuration no longer influenced the outcome, as both α- and β-anomers of pentaacetate **63** reacted the same way resulting in the sixth position deacetylated product **64** in 85% yield. The reaction was relatively fast, with only 6 h reaction time with 50% CAL-B. *p*-Chlorothiophenol leaving group in the anomeric position did not influence the

deacetylation target position as the isolated yield was 97% for the sixth position deacetylation product **66.** Changing the protecting groups to more sterically hindered ones as seen in D-glucopyranoside **67** clearly inhibited the transesterification reaction. Even under harsh conditions (90 °C, 200% w/w CAL-B – condition **iv**), only 24% yield of the sixth position deprotected product **68** was obtained after six days. The branched aliphatic protecting group, pivaloyl ester substrate **69**, was completely inactive under these conditions even after three days. In contrast, the linear aliphatic protecting group, *n*-butyl ester substrate **70**, on the other hand acted similarly to acetyl protected D-glucopyranosides **16** and **19**. Meaning that the anomers reacted differently – α-anomer gave selectively the sixth position deprotected product **71**. While β-anomer had its first position deprotected resulting in product **72**. Both products were isolated together with the total 80% yield. The ratio of the products corresponded to the starting materials anomeric ratio, solidifying the idea that the anomers react differently.

The sixth position deprotection traditionally is carried out by first introducing a bulky protecting group like pivaloyl or trityl into the sixth position, followed by full acetylation of the monosaccharide and then selective deprotection of the bulky protecting group.[150] Direct selective deprotection of the sixth position with CAL-B reduces the number of steps needed, and avoids the formation of the fourth position deacetylated side-product associated with acyl migration.

Acetylation reactions with D-glucopyranoses were carried out with thio-β-D-glucopyranoside **73** with *p*-chlorothiophenol leaving group and with D-glucopyranose **1**. Most likely due to better solubility in a vinyl acetate:acetonitrile 1:1 system, the thioglycoside **73** reacted in just half an hour resulting in the sixth position protected product **74** in 80% isolated yield. Fully unprotected D-glucopyranose **1** required three days to reach full conversion, resulting with high selectivity in the third and the sixth positions protected product **75** in 91% yield. This result was unexpected, as the solvent system showed preference towards the second and the sixth positions protected product during the optimisation. Increasing the amount of enzyme or temperature did not increase the rate of the reaction, but instead decreased the isolated yield.

In summary for glucopyranoses, in the case of deacetylation reactions, anomers of acetylated D-glucopyranosides react differently with CAL-B and the outcome depends on the anomer. Based on the results with different functional groups in anomeric position, it can be concluded that in the absence of ester functionality in the first position, the deacetylation typically occurs at the fourth and the sixth positions. It seems, that the L-configuration allows only the sixth position's deacetylation with CAL-B transesterification reactions and there are no anomeric differences on the reaction outcome. Bulkier protecting groups inhibit the reaction and show no clear anomeric preference, except in the case of linear aliphatic ester protecting groups. Acetylation reactions led to the protection of the sixth position for thio-β-D-glucopyranoside and the third and the sixth positions for unprotected D-glucopyranose.



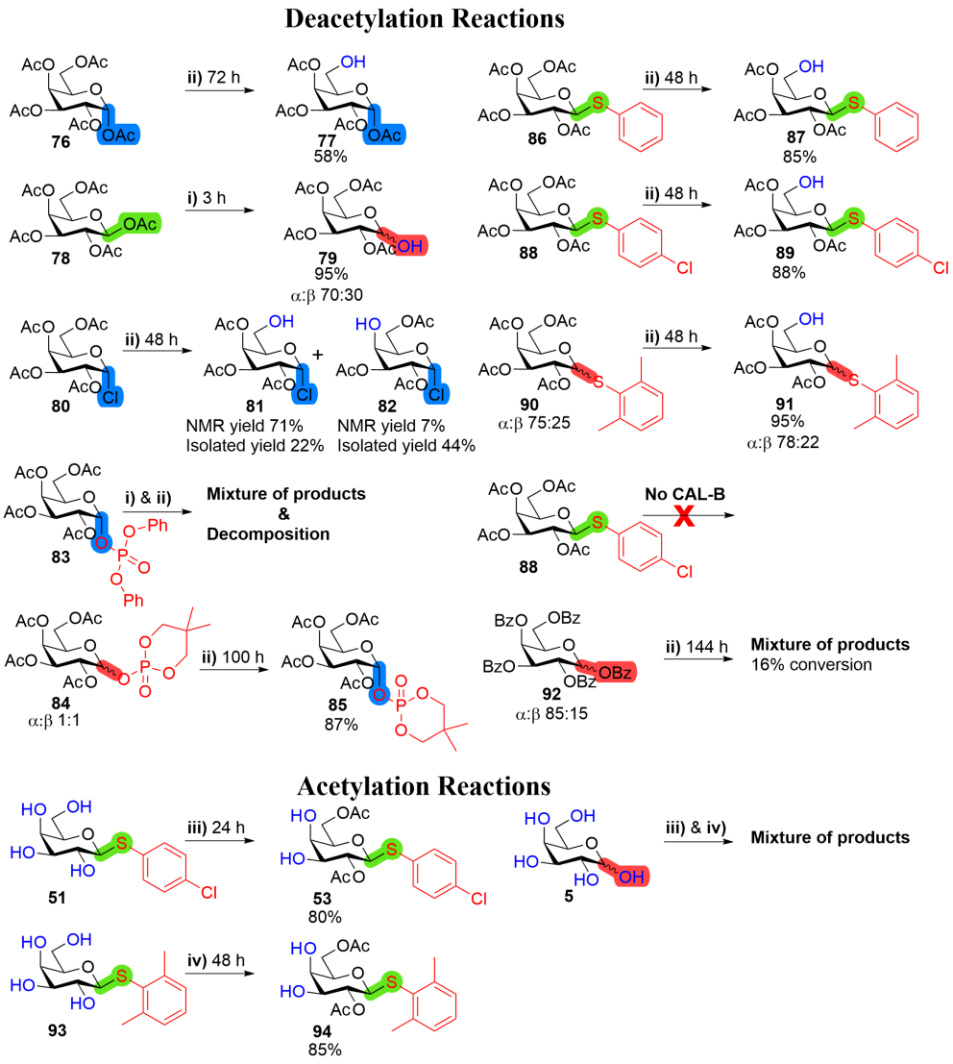
Scheme 22. *Transesterification reactions with glucopyranose substrates. Reaction conditions:* ***i)*** *saccharide (50 mg), 50 % CAL-B (w/w), MTBE or CPME (10 mL), n-BuOH (3.5 eq.), 45 °C;* ***ii)*** *like* ***i)*** *except 100 % CAL-B (w/w);* ***iii)*** *like* ***i)*** *except 200 % CAL-B (w/w), 60 °C;* ***iv)*** *like* ***i)*** *except 200 % CAL-B (w/w), 90 °C;* ***v)*** *saccharide (25–100 mg), MeCN:Vin.Ac. 1:1 (0.08 M of substrate), 100% CAL-B (w/w), 45 °C; yields shown are isolated yields.*

3.3.2 Galactopyranose

The anomeric differentiation for CAL-B carried over from the glucopyranose series to the galactopyranoses. The α-anomer of D-galactopyranoside pentaacetate **76** reacted sluggishly, with increased temperature to 60 °C and 200% w/w CAL-B -condition **ii)**, the sixth position deacetylated product **77** was isolated after three days in 58% yield (Scheme 23). Full conversion was not achieved and there were overreaction products appearing over time. Comparingly, the β-anomer of D-galactopyranoside pentaacetate **78** reacted in just 3 h with 95% isolated yield of the anomeric deprotection product **79**. The crude reaction mixture's ^{1}H NMR spectrum was sufficiently pure, that 100 times scale up reaction (with 10 grams of pentaacetate **78**) was used from a crude state. Both peracetylated β-anomers of D-glucopyranoside and D-galactopyranoside reacted the same way, but the reaction was six times faster for D-glucopyranoside. With chloride in the anomeric position, the transesterification reaction stopped after 24 h with ~75%

conversion of the substrate **80**. After 24 h, the main product of the sixth position deacetylated **81** started to undergo acyl migration forming the fourth position deacetylated product **82**. After 48 h, the crude mixture showed only 7% of the fourth position deacetylated product **82** and 71% of the main product **81**. After column chromatography, the products swapped and 22% of the sixth position deacetylated product **81** was isolated, while the fourth position deacetylated product **82** was isolated in 44% yield. Slightly acidic silica gel promoted acyl migration between the fourth and sixth positions. An acyclic aromatic phosphate ester leaving group bonded to D-galactopyranoside tetraacetate **83** turned out to be unstable, while also producing multitude of unstable products with CAL-B. Using cyclic aliphatic phosphate ester as leaving group with D-galactopyranoside tetraacetate **84** showed initially no reaction, but increasing the temperature to 60 °C and the amount of CAL-B to 200% w/w led to mainly anomeric enrichment with the initial α:β ratio of 50:50 changing to 100:0. It is surmised that the phosphate ester interacts with the enzyme's catalytic site, temporarily breaking and subsequently reforming the glycosidic bond , resulting in the more stable α-anomer. Next, in the thio-D-galactopyranoside series, all the thiogalactopyranoside compounds needed 200% w/w CAL-B at 60 °C to reach full conversion in 48 h. Starting from D-galactopyranoside tetraacetate **86** equipped with a thiophenol leaving group, it was selectively deacetylated in the sixth position resulting in product **87** with 85% yield. Adding an electron-withdrawing chloride to thiophenol gave a similar outcome, with the sixth position deacetylated product **89** isolated in 88% yield. If thiophenol had two electron-donating methyl groups, the transesterification reaction's product **91** yield was increased to 95%. Furthermore, there was no anomeric preference for D-galactopyranoside tetraacetate **90** with 2,6-dimethylthiophenol leaving group in the CAL-B transesterification reaction. It is not clear, whether it is the steric or electronic effect, that increases the reaction yield in the thio-D-galactopyranoside series. When CAL-B was omitted from the reaction, no transesterification occurred with *p*-chlorothiophenol equipped tetraacetate **88**. While if acetyl groups were changed to benzoyl protecting groups, with substrate **92**, there was barely any conversion, and the deprotection was non-selective.

Turning to acetylation reactions, thio-β-D-galactopyranoside **51** was used to optimise the conditions in Section 2.3 with the final result of 80% isolated yield towards the second and the sixth positions acetylated product **53**. Changing the leaving group to 2,6-dimethylthiophenol with substrate **93** did not influence the acetylation reaction outcome, besides increasing the yield 5% to 85% for the second and the sixth positions acetylated product **94**. Surprisingly, fully unprotected D-galactopyranose **5** gave a non-selective mixture of products with both milder and harsher conditions.



Scheme 23. *Transesterification reactions with galactopyranose substrates. Reaction conditions:* ***i)*** *saccharide (100 mg), 100 % CAL-B (w/w), MTBE or CPME (10 mL), n-BuOH (3.5 eq.), 45 °C;* ***ii)*** *like* ***i)*** *except 200 % CAL-B (w/w), 60 °C;* ***iii)*** *saccharide (60–100 mg), MeCN:Vin.Ac. 1:1 (0.08 M of substrate), 100% CAL-B (w/w), 45 °C;* ***iv)*** *like* ***iii)*** *except 200 % CAL-B (w/w), 60 °C; yields shown are isolated yields.*

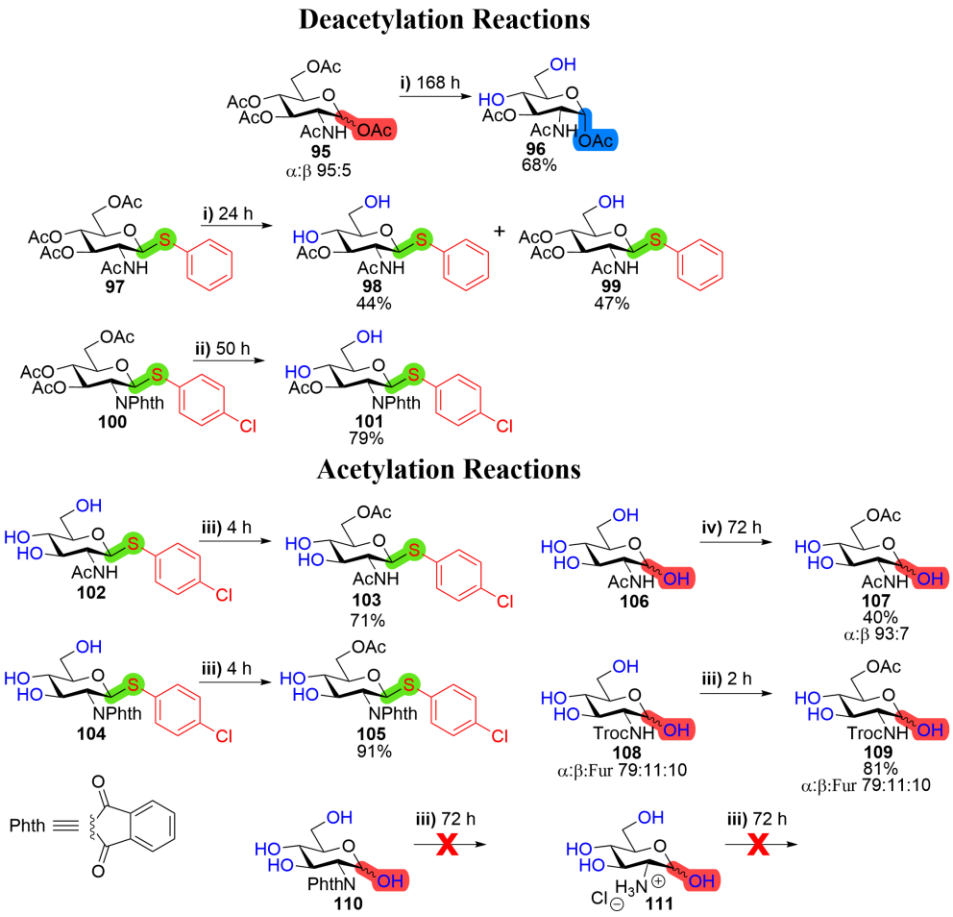
In conclusion of the galactopyranose series, the deacetylation reactions had a similar anomeric discrimination, as seen in the glucopyranose series. Compering the two epimers shows that the fourth position influences whether the outcome of the deacetylation reactions is monodeacetylated (Gal) or a diol (Glc). Overall, galactopyranose series had a decreased rate of reaction when compared to glucopyranose counterparts. Variations in the thiophenol leaving groups did not affect the deacetylation's target position, and yields remained consistent. Chloride and phosphate leaving groups induced acyl migration. Bulkier benzoyl protecting group cannot be used in selective deacetylation reactions. In acetylation reactions, similarly to deacetylation reactions, different thiophenol leaving groups had a minimal effect on the reaction outcome, while unprotected galactose, unfortunately, yielded a non-selective product mixture.

3.3.3 Glucopyranosamine

The anomers of D-glucopyranosamine pentaacetate **95** reacted similarly to previous two monosaccharides, where CAL-B's deacetylation reaction outcome was different according to anomeric configuration (Scheme 24). Only pentaacetate **95** α-anomer's product was discovered as the fourth and the sixth positions deacetylated diol **96** after seven days of reaction time. There was too little β-anomer present, to determine its product. Increased amount of MTBE had to be used in the deacetylation reactions due to poor solubility of both peracetylated D-glucopyranosamine **95** and thioglucopyranosamine **97**. The thiophenol equipped D-glucopyranosamine tetraacetate **97** reacted in 24 h but gave two separate products. The fourth and the sixth positions deacetylated diol **98** and the sixth position deacetylated product **99** were isolated. It seems that the reaction partially stopped at the sixth position deacetylation. Exchanging the *N*-protecting group to phthalate ester showed more selective reaction with substrate **100**. The fourth and the sixth positions deacetylated product **101** was isolated in 79% yield, but the reaction took longer, and full conversion was reached in 50 h.

In the acetylation reactions with CAL-B, both the *N*-acetyl **102** and the *N*-phthalate ester **104** protected thio-β-D-glucopyranosamines reacted within 4 h, producing the sixth position protected products **103** and **105**. Due to the formation of the overreaction products with *N*-acetyl thio-β-D-glucosamine **102**, the reaction was stopped with some unreacted starting material left. Interestingly almost no side products were seen for *N*-phthalate ester thio-β-D-glucosamine **104** in the acetylation reaction with CAL-B. As such, purification by crystallisation was carried out and *N*-phthalate ester product **105** was purified in three batches with the total yield of 91%. Carrying on with fully unprotected D-glucosamines. *N*-Acetyl D-glucopyranosamine **106** needed increased amount of CAL-B at higher temperatures and even then, only 40% isolated yield of the sixth position protected **107** was reached. While *N*-Troc D-glucosamine **108**, mainly α/β mixture containing 10% furanose (Fur), reacted in just 2 h, retaining its original α:β:Fur ratio. The sixth position acetylated product **109** was isolated in 81% yield. Both phthalate ester protected D-glucopyranosamine **110** and hydrochloric acid salt of D-glucopyranosamine **111** showed no reaction over three days. These two substrates did not dissolve at all in the reaction mixture, which seems to be a limitation in the CAL-B catalysed acetylation reactions.

D-Glucopyranosamine reacts similarly to D-glucopyranose in both acetylation and deacetylation reactions with CAL-B. The transesterification reaction's outcome depends on the second position amine's protecting group. Bulkier phthalate ester protecting group allows both the fourth and the sixth positions deacetylation selectively. Smaller acetyl protecting group cuts the reaction short with half of the products being just the sixth position deacetylated product. In acetylation reactions with CAL-B, both for the thioglycosides and for the fully unprotected D-glucopyranosamines, the acetylation occurs in the sixth position. The rate of the reaction and the yield is heavily connected with the solubility. The solubility trend in the increasing order for D-glucosamine is: **110** = **111** < **106** < **108**.



Scheme 24. *Transesterification reactions with glucopyranosamine substrates. Reaction conditions:* ***i)*** *saccharide (50–100 mg), 100 % CAL-B (w/w), MTBE or CPME (10–20 mL), n-BuOH (3.5 eq.), 45 °C;* ***ii)*** *like* ***i)*** *except saccharide (100 mg), MTBE (10 ml);* ***iii)*** *saccharide (60–100 mg), MeCN:Vin.Ac. 1:1 (0.08 M of substrate), 100% CAL-B (w/w), 45 °C;* ***iv)*** *like* ***iii)*** *except 200 % CAL-B (w/w), 60 °C; yields shown are isolated yields; Fur – furanose.*

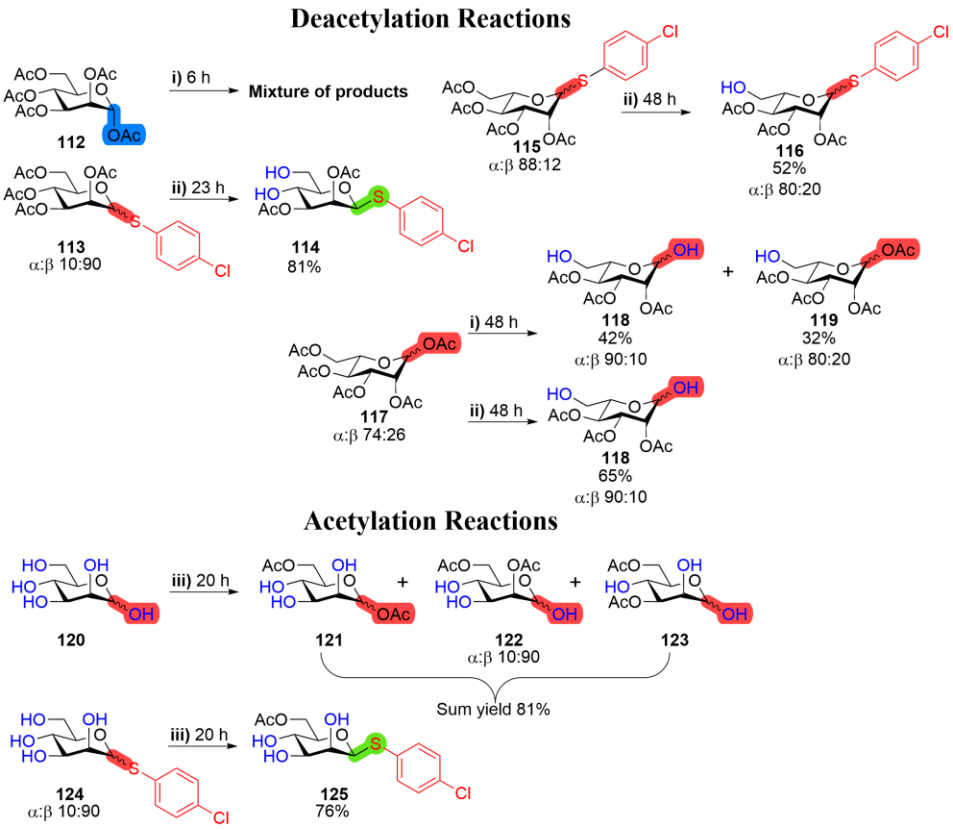
3.3.4 Mannopyranose

The deacetylation reaction with α-D-mannopyranoside pentaacetate **112** and CAL-B resulted in a non-selective mixture of products (Scheme 25). The products ranged from monodeacetylated to monoacetylated and no single compound was could be isolated separately. Compared to its epimer α-D-glucopyranose pentaacetate **16**, it is clear, that the second position's configuration influences whether the reaction is selective or not. For thio-D-mannopyranoside **113** only the β-anomer reacted selectively giving the fourth and the sixth positions deacetylated product **114** in 81% yield. The α-anomer of substrate **113** did not react at all. Similarly to thio-L-glucopyranoside epimer **65**, the thiomannopyranoside L-enantiomer **115** was deacetylated in the sixth position giving the product **116** in 52% yield. The L-mannopyranoside pentaacetate **117** reacted selectively resulting in two isolated products: the first and the sixth positions deacetylated triacetate **118** in 42% yield, and the sixth position deacetylated tetraacetate **119** in 32% yield. Increasing the amount of CAL-B and temperature improved the yield of triacetate **118** to

65% yield. As the tetraacetate **119** anomeric distribution was similar to the starting ratio, there was no anomeric discrimination. Furthermore, increasing the temperature led to decrease in the amount of tetraacetate **119** present. This could be an indicator of a stepwise reaction, first step would be the sixth position's deacetylation, followed by the first position's deacetylation. Compared to its L-glucopyranose epimer **63**, the second position influences whether the anomeric position is deacetylated or not.

Acetylation reactions with D-mannopyranose **120** and CAL-B resulted in inseparable mixture of diacetates **121**-**123** in 81% yield. The reaction was significantly slower and less selective compared to its epimer D-glucopyranose **1**. Both compounds did selectively produce diacetates, but D-glucopyranose **1** had only one major product. Thio-D-mannopyranoside **124** reacted similarly to its glucose epimer resulting in the sixth position acetylated product **125** in 76% yield. Similarly to thio-D-mannose tetraacetate **113**, the acetylation reaction with substrate **124** only worked with β-anomer.

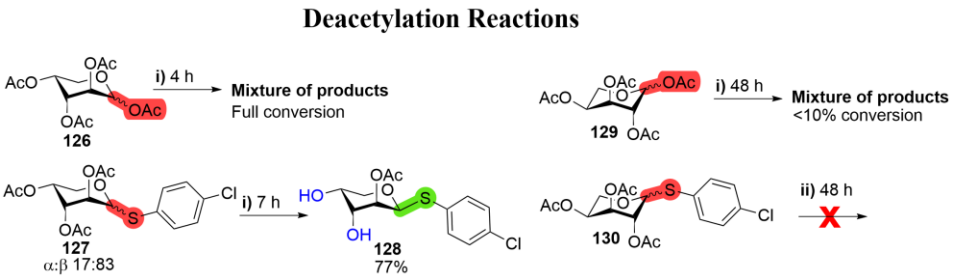
Thio-D-mannopyranosides **113** and **124** and thio-L-mannopyranoside **115** behaved similarly to their glucose epimer counterparts in both deacetylation and acetylation reactions. On the other hand, D-mannopyranoses **112** and **120** and L-mannopyranoside **117** outcomes differed significantly compared to their glucose epimers. With D-enantiomers (Glc and Man), the second position's configuration influences whether the reaction is selective. As for L-enantiomers (Glc and Man), the second positions configuration influences whether there is only one position deacetylated or two positions.



Scheme 25. *Transesterification reactions with mannopyranose substrates. Reaction conditions:* ***i)*** *saccharide (100 mg), 100 % CAL-B (w/w), MTBE or CPME (10 mL), n-BuOH (3.5 eq.), 45 °C;* ***ii)*** *like* ***i)*** *except 200 % CAL-B (w/w), 60 °C;* ***iii)*** *saccharide (100 mg), MeCN:Vin.Ac. 1:1 (0.08 M of substrate), 100% CAL-B (w/w), 45 °C; yields shown are isolated yields.*

3.3.5 Arabinopyranose

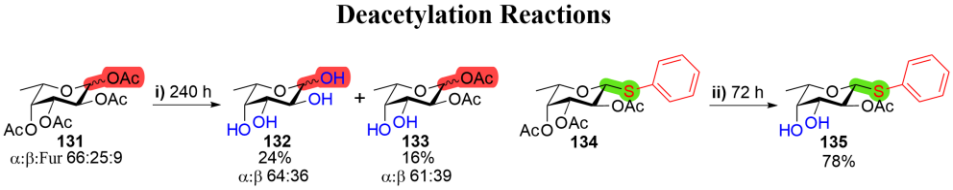
In the arabinopyranose series, CAL-B showed a clear preference for the D-enantiomer over the L-enantiomer in deacetylation reactions (Scheme 26). While both tetraacetates did not react selectively, D-enantiomer **126**, reacted in 4 h with full conversion while L-enantiomer **229** took over 48 h to reach <10% conversion. For thioarabinopyranoside triacetates, the D-enantiomer **127** showed anomeric preference towards the β-anomer. The deacetylation reaction resulted in the third and the fourth positions deprotected product **128** in 77% yield in 7 h. While L-enantiomer **130** did not react at all in 48 h at elevated temperature with 200% CAL-B.



Scheme 26. *Transesterification reactions with arabinopyranose substrates. Reaction conditions:* ***i)*** *saccharide (100 mg), 100 % CAL-B (w/w), MTBE or CPME (10 mL), n-BuOH (3.5 eq.), 45 °C;* ***ii)*** *like* ***i)*** *except 200 % CAL-B (w/w), 60 °C; yields shown are isolated yields.*

3.3.6 6-deoxy-L-galactopyranose (L-fucose)

Tetraacetate L-fucose **131** reacted differently than all the other substrates tested on the deacetylation reaction with CAL-B (Scheme 27). After ten days, full conversion was not reached, while resulting in fully unprotected L-fucose **132** in 24% yield. Partially deprotected in the third and fourth positions L-fucose **133** was also isolated in 16% yield. Thio-L-fucose **134** required elevated temperature and additional amounts of CAL-B to reach 78% yield of the third and the fourth positions deprotected product **135** in 72 h.

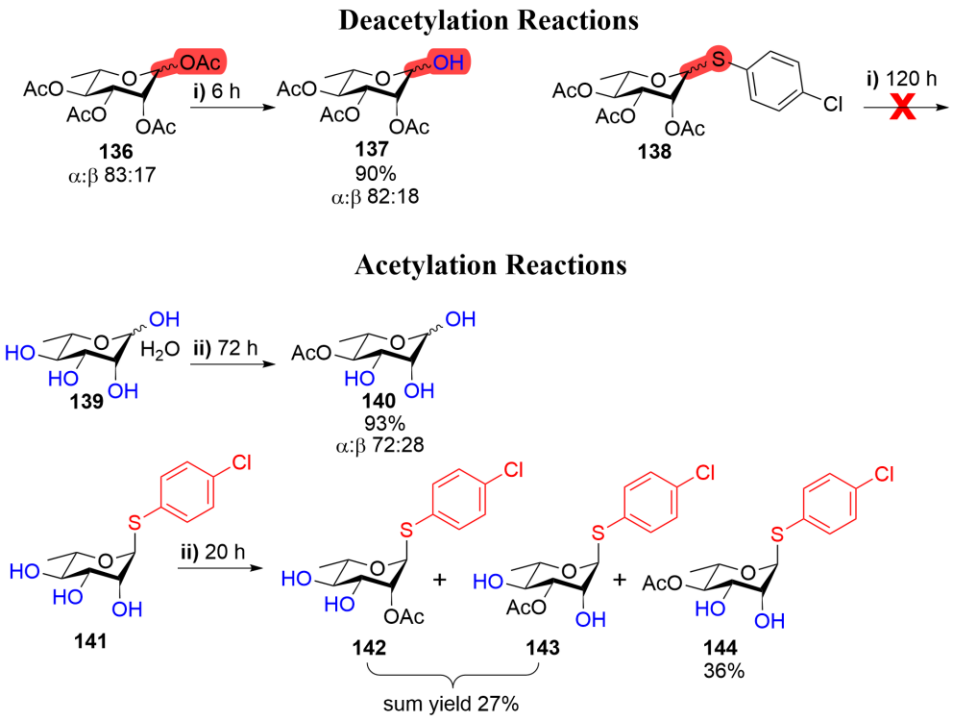


Scheme 27. *Transesterification reactions with 6-deoxy-L-galactopyranose substrates. Reaction conditions:* ***i)*** *saccharide (100 mg), 100 % CAL-B (w/w), MTBE or CPME (10 mL), n-BuOH (3.5 eq.), 45 °C;* ***ii)*** *like* ***i)*** *except 200 % CAL-B (w/w), 60 °C; yields shown are isolated yields.*

3.3.7 6-deoxy-L-mannopyranose (L-rhamnose)

L-Rhamnopyranoside tetraacetate **136** reacted in high selectivity in the CAL-B deacetylation reaction, resulting in the first position deacetylated product **137** in 90% yield (Scheme 28). Comparing substrate **136** to L-mannopyranoside pentaacetate **117**, the difference lies in the sixth position's acetoxy group. Clearly, the absence of the acetoxy group enhances the reactivity, while also influences the target position of the deacetylation reaction. The absence of the acetoxy group in the sixth position also did not allow any reaction to occur with thio-L-rhamnopyranoside triacetate **138** in five days.

In acetylation reactions with CAL-B, L-rhamnopyranose monohydrate **139** reacted very selectively giving the fourth position protected triol **140** in 93% yield over three days. The triol **140** has been previously synthesised, but from benzyl L-rhamnopyranoside and in four steps.[151] Thio-L-rhamnopyranoside **141** did not share the same success, as three monoacetylated products **142**–**144** were obtained. Only the third position acetylated **144** was managed to isolate separately in 36% yield, the second and first positions acetylated **142** and **143** were isolated together in 27% yield, giving the total yield of 63%.

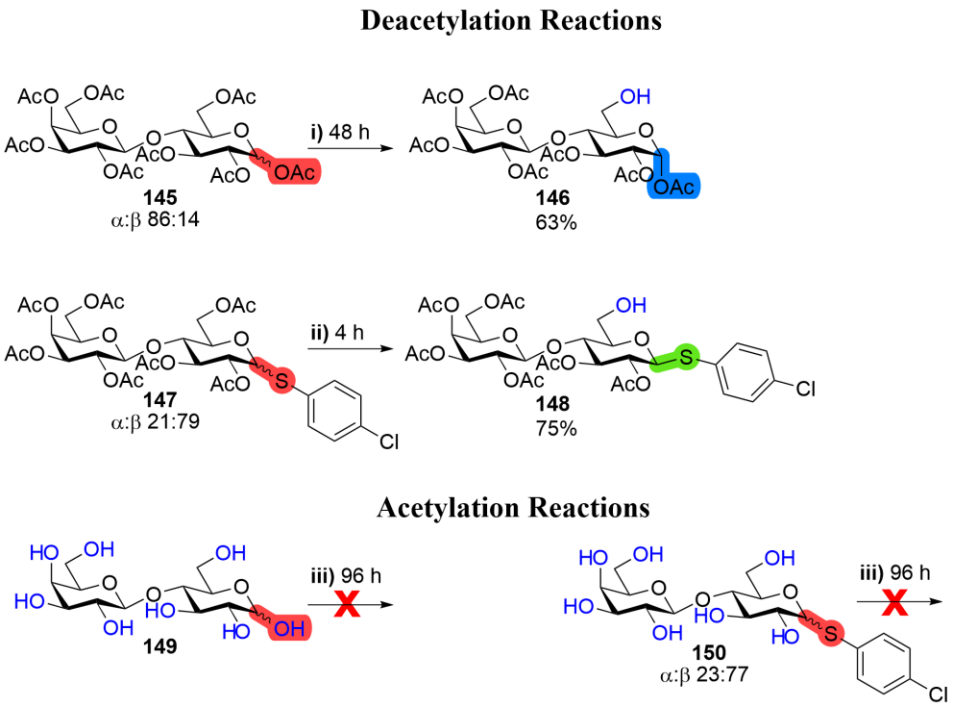


Scheme 28. *Transesterification reactions with 6-deoxy-L-mannopyranose substrates. Reaction conditions:* ***i)*** *saccharide (100 mg), 200 % CAL-B (w/w), MTBE or CPME (10 mL), n-BuOH (3.5 eq.), 60 °C;* ***ii)*** *saccharide (100 mg), MeCN:Vin.Ac. 1:1 (0.08 M of substrate), 100% CAL-B (w/w), 45 °C; yields shown are isolated yields.*

3.3.8 Galactopyranosylβ(1→4)glucopyranose (lactose)

The only disaccharide tested with CAL-B was D-lactose (Scheme 29). In the deacetylation reactions, both D-lactose octaacetate **145** and thio-D-lactose heptaacetate **147** had their sixth position deacetylated in 63% and 75% yields respectively. Furthermore, both reactions were anomerically discriminative as D-lactose octaacetate **145** deacetylation reaction's product **146** was α-anomer. While the thio-D-lactose heptaacetate **147** deacetylation reaction's product **148** was β-anomer. These two reactions suggest, that the non-reducing terminal saccharide (Gal) does not participate in the CAL-B reaction and the reducing saccharide (Glc) influences the outcome. Further testing is needed with various saccharides to prove this assumption.

There was no reaction in the acetylation with D-lactose **149** and thio-D-lactose **150** with CAL-B because of insolubility of substrates in the reaction medium.



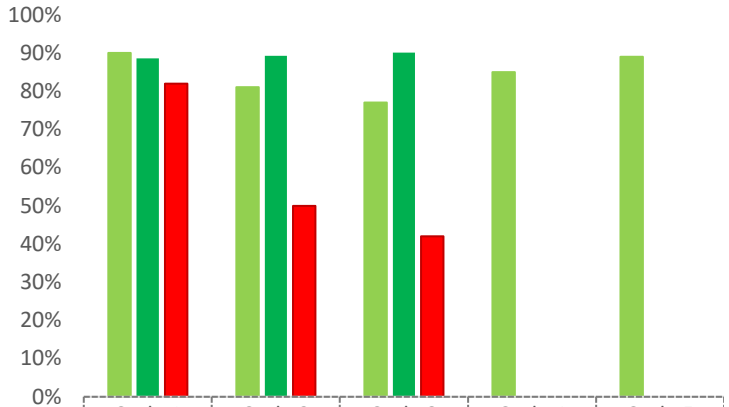
***Scheme 29**. Transesterification reactions with galactopyranosylβ(1→4)glucopyranose substrates. Reaction conditions: **i)** saccharide (100 mg), 100 % CAL-B (w/w), MTBE or CPME (10 mL), n-BuOH (3.5 eq.), 45 °C; **ii)** like **i)** except 200% CAL-B, 60 °C; **iii)** saccharide (100 mg), MeCN:Vin.Ac. 1:1 (0.08 M of substrate), 100% CAL-B (w/w), 45 °C; yields shown are isolated yields.*

3.4 CAL-B recycling from transesterification reactions

The recyclability of CAL-B was tested for the deacetylation reactions in two different reactions (Figure 10). First the recycling tests were conducted with β-D-glucopyranoside pentaacetate **19**, with 0.5 h reaction time, for five consecutive cycles to test the impact of repeated washing and filtration on CAL-B activity. The second reaction chosen was with α-D-glucopyranoside pentaacetate **16**, which took 23 h and was run for three cycles. It was mainly chosen to test the effect of prolonged mechanical stirring. After each reaction cycle, the immobilised CAL-B was washed with DCM (~50 mL) and air-dried for 1 h before reuse. The same recycling procedure was applied to acetylation reactions with thio-β-D-galactopyranoside **51**, with reaction time of 24 h. The recycling was run for three cycles.[76,144]

The outcome of the recycling tests was positive for the deacetylation reactions, shown as green bars in Figure 10. Initially with β-D-glucopyranoside pentaacetate **19** there was a downward trend, with the isolated yield of product **20** decreased from 90% to 77%. After the fourth cycle, the isolated yield shot up again and rebounded to 89% after the fifth cycle. This was probably due to experimental error i.e. possibly due to inconsistent substrate dissolution or stirring efficiency. More consistent results were obtained with α-D-glucopyranoside pentaacetate **16** with a longer reaction time, where isolated yield of product **48** fluctuated by only 1%. For the acetylation reactions, it was concluded that the recycling was not feasible (Figure 10, red bars). The yield of the isolated product **53**

dropped after the second cycle to 50% from 80% and further declined to 42%, after third cycle. It is hypothesised that CAL-B might be acetylated during the reaction, which either changes the configuration, inhibits the access of the starting material to the catalytic site or acetylates the catalytic site directly. Further research is needed to prove this theory.

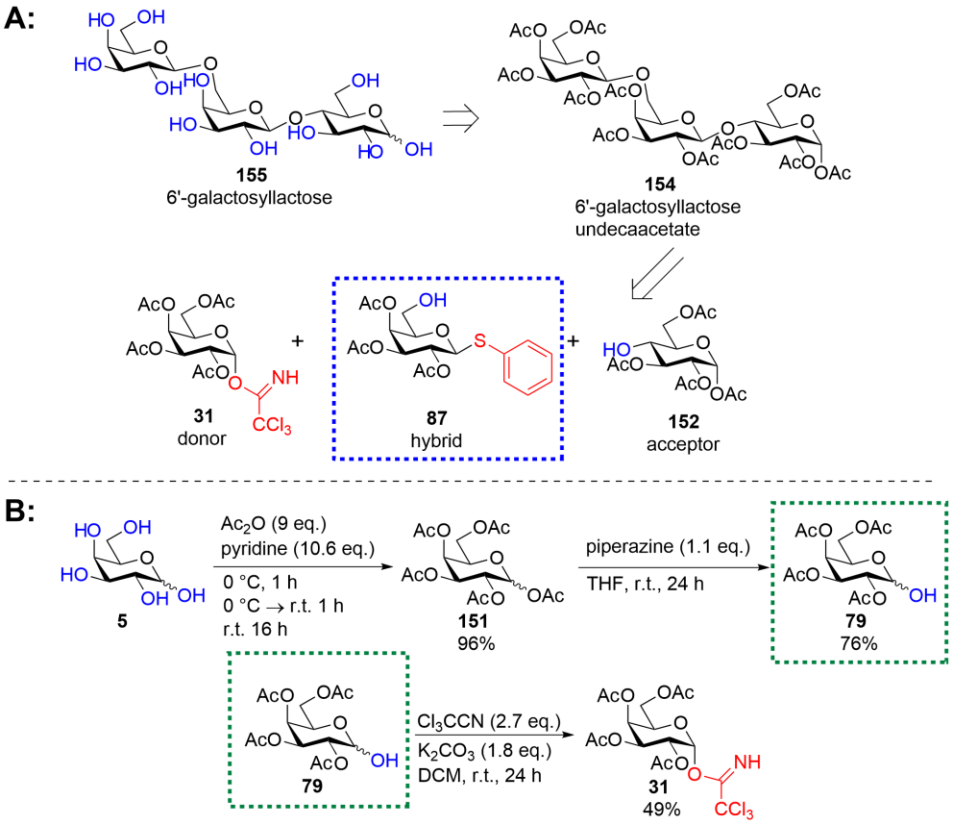


	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
Isolated Yield of 20	90%	81%	77%	85%	89%
Isolated Yield of 48	89%	89%	90%		
Isolated Yield of 53	82%	50%	42%		

Figure 10. *Recycling tests conducted for transesterification reactions with CAL-B, green bars are for deacetylation reactions and red is for the acetylation reaction; Reaction conditions: 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranoside* ***16*** *(100 mg), 100 % CAL-B (w/w), MTBE (10 mL), n-BuOH (3.5 eq.), 45 °C; 1,2,3,4,6-penta-O-acetyl-α-D-glucopyranoside* ***19*** *(100 mg), 100 % CAL-B (w/w), MTBE (10 mL), n-BuOH (3.5 eq.), 45 °C; 4-chlorophenyl 1-thio-β-D-galactopyranoside* ***51*** *(100 mg), MeCN:Vin.Ac. 1:1 (0.08 M of substrate), 100% CAL-B (w/w), 45 °C.*

3.5 Synthesis of 6'-galactosyllactose

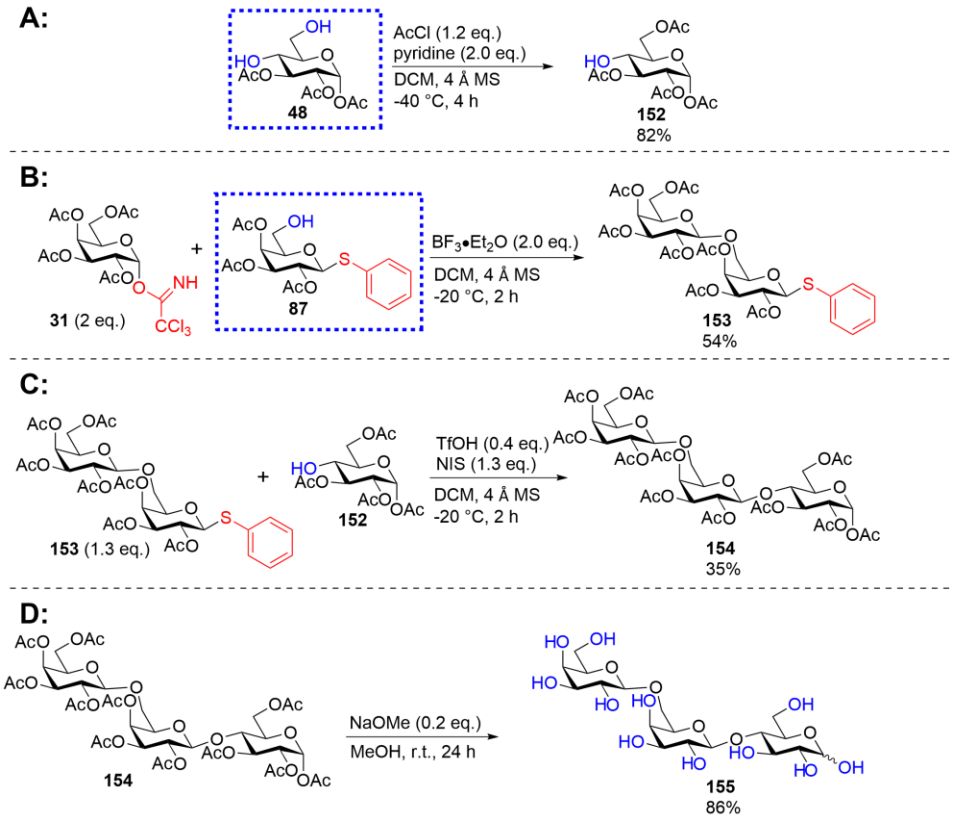
To demonstrate the synthetic utility of the partially protected saccharides obtained from CAL-B transesterification reactions, CAL-B deacetylation products were used in the synthesis of a deviant HMO and GOS, 6'-galactosyllactose (6'-GL). The 6'-GL synthesis needed three monosaccharide units: terminal saccharide D-galactopyranose – donor, middle saccharide – D-galactopyranose – hybrid, and reducing saccharide D-glucopyranose – acceptor (Scheme 30A). First, the donor was synthesised in three steps from unprotected D-galactopyranose **5** (Scheme 30B). D-Galactopyranose **5** was fully acetylated using Ac_2O and pyridine overnight resulting in 96% yield (10.4 g) for D-galactose pentaacetate **151**.[152] Next, using piperazine in THF in an overnight reaction, the anomeric position was selectively deacetylated yielding product **79** in 76% yield (6.5 g).[153] Initially, chemical synthesis was used to prepare D-galactose tetraacetate **79**. After optimising and finding the CAL-B reaction for anomeric deprotection of β-D-galactopyranoside pentaacetate, D-galactose tetraacetate **79** was synthesised enzymatically via transesterification (see Section 2.4.2). Final step for donor synthesis was introduction of the trichloroacetimidate (TCA) leaving group. This was carried out by using trichloroacetonitrile as the reagent in anhydrous DCM under argon with potassium carbonate. The reaction yields varied from 30–49% for the synthesis of α-D-galactopyranoside-TCA tetraacetate **31**. As the donor **31** decomposes over time, this step was conducted on a smaller scale (~350 mg).



***Scheme 30. A**: Retrosynthetic pathway to 6'-GL from three monosaccharide building blocks. **B**: D-Galactopyranoside-TCA donor **31** synthesis; blue box – product of CAL-B transesterification reactions; green box – can now be made by CAL-B.*

The thio-β-D-galactopyranoside hybrid **87** (0.9 g, 90% yield) was synthesised via CAL-B deacetylation from tetraacetate **86** (see Section 2.4.2). While the α-D-glucopyranoside acceptor **152** (Scheme 31A) was made in one step from CAL-B deacetylation reaction's product D-glucose triacetate **48** (for synthesis of **48** see Section 2.2). D-Glucopyranoside triacetate **48** was dissolved in DCM with pyridine (2 eq.) and acetyl chloride (AcCl, 1.2 eq.) was added dropwise at -40 °C.[154] The reaction was over after 4 h and yielded the acceptor **152** in 82% yield. Next, glycosylation reaction was carried out between D-galactopyranoside donor **31** (2 eq.) and D-galactopyranoside hybrid **87** with $BF_3{\cdot}Et_2O$ (2 eq.) as the catalyst in anhydrous DCM at -20 °C (Scheme 31B). The formed Galβ(1→6)Gal-SPh disaccharide **153** was isolated in 54% yield (390 mg). This glycosylation reaction was possible because the TCA and SPh leaving groups are activated under different conditions. Thus, the resulting disaccharide **153** became the next donor with SPh leaving group. The glycosylation reaction between acceptor **152** and the disaccharide donor **153** (1.3 eq.) was catalysed by TfOH (0.4 eq.) and NIS (1.3 eq.) at -20° C in anhydrous DCM (Scheme 31C). The resulting 6'-galactosyllactose undecaacetate **154** was isolated in 35% yield (101 mg). For the final full deacetylation, several methods were tested, but Zemplén deacetylation was chosen as the best method. 6'-galactosyllactose undecaacetate

154 was stirred in dry MeOH and fresh NaOMe in MeOH (total 0.2 eq. NaOMe) was added (Scheme 31D). After work-up with acidic AmberLite™ 120 H^+ pinkish solids were got, as such purification by column chromatography was necessary. The yield for unprotected 6'-galactosyllactose was 86% (37 mg). The overall yield after nine steps was 4%. However, the yields for D-galactopyranoside donor **31** and D-glucopyranoside acceptor **152** synthesis have since been improved. Further optimisation of the glycosylation steps is ongoing with alternative catalysts under investigation.



***Scheme 31. A**: D-Glucopyranoside acceptor **152** synthesis; **B**: Synthesis of Galβ(1→6)Gal-SPh disaccharide donor **153**; **C**: Synthesis of 6'-galactosyllactose undecaacetate **154**; **D**: Deprotection as the final step in 6'-galactosyllactose **155** synthesis; blue box – product of CAL-B transesterification reactions.*

4 Conclusions

- A method for the selective deacetylation of pyranose saccharides in organic media with CAL-B was developed. During optimisation and substrate scope exploration, several factors influencing the reaction were discovered together with different characteristics:
    - The configuration of the starting pyranose saccharide plays an important role on the outcome and rate of the reaction.
        - Anomers react differently – β-anomers tend to have first position deprotected, while α-anomers direct the deacetylation to the sixth and/or fourth position.
        - D-and L-enantiomers reactions with CAL-B depends on the starting saccharide, while no unversal trends were established, enantiomers react differently in all cases.
        - Reactions with different epimers show that configuration of every position influence CAL-B's rate of reaction and which positions are deacetylated.
    - Glucose-based saccharides react the same way resulting in the fourth and sixth positions being deacetylated.
    - Different alcohols can be used as nucleophilic reagents with minimal impact on the outcome.
    - CAL-B can be recycled and used repeatedly in deacetylation reactions.
    - Bulkier protecting groups inhibit or do not react with CAL-B with exception of linear aliphatic ester protecting groups.
- A method for selective acetylation of partially deprotected thiopyranosides and fully unprotected pyranoses in organic media was developed. The main features of the acetylation reaction with CAL-B include:
    - With thiogalactopyranoside:
        - Depending on the solvent, the rate of the reaction and isomeric ratio of the products can be increased significantly.
        - Regioselectivity of the acetylation depends on the acetylation reagent.
        - Using pure chloroform or MTBE as solvent resulted in a fourth and sixth position protected acetal.
    - With other thiopyranosides, the sixth position was typically acetylated, or the reactions lacked regioselectivity altogether.
    - With fully unprotected saccharides, the reaction outcome depended on the starting saccharide and no general pattern emerged.
    - Both with unprotected thiopyranosides and fully unprotected saccharides, the solubility of the starting material was the main limitation.
- The synthetic utility of partially protected saccharides obtained from CAL-B transesterification reaction was shown in the synthesis of 6'-galactosyllactose – a deviant HMO and a GOS.
- Using CAL-B-mediated synthesis of GOS donors and acceptors substantially increases the total yield of the 6'-galactosyllactose synthesis (from 4% to 9%).

5 Experimental section

5.1 General experimental information

Full assignment of ^{1}H and ^{13}C chemical shifts were based on the ^{1}D and ^{2}D (COSY, HSQC, HMBC) FT NMR spectra measured with a Bruker Avance III 400 MHz instrument. Residual solvent signals were used ($CDCl_3$: δ = 7.26 ^{1}H NMR, 77.2 $^{13}C\{^{1}H\}$ NMR; CD_3OD: δ = 3.31 ^{1}H NMR, 49.0 $^{13}C\{^{1}H\}$ NMR; $(CD_3)_2SO$: δ = 2.50 ^{1}H NMR, 39.5 $^{13}C\{^{1}H\}$ NMR; D_2O: δ = 4.79 ^{1}H NMR) as internal standards. High-resolution mass spectra were recorded with an Agilent Technologies 6540 UHD Accurate-Mass QTOF LC/MS spectrometer by using AJ-ESI ionisation. Prior to analysis the instrument was calibrated in the mass range of m/z 50–3200. Optical rotations were obtained with an Anton Paar GWB Polarimeter MCP 500. Melting points were determined using polarising optical microscope Nagema-K8. Precoated Merck silica gel 60 F_{254} plates were used for TLC (thin layer chromatography) and column chromatography was performed with Merck 60 (0.040–0.063 mm) mesh silica gel. Commercial reagents, and solvents were generally used as received. DCM was distilled over CaH or phosphorous pentoxide, ethyl acetate (EtOAc) and acetone over phosphorus pentoxide, MeOH and toluene over sodium. Petroleum ether (PE) had a boiling point of 40–60 °C. Silicon oil bath on magnetic stirrer with heating was used as a heat source for reactions requiring heating. Immobilised *Candida antarctica* Lipase-B on hydrophobic acrylic resin, Novozyme 435, with 10000 (propyl laurate unit/g) activity was a kind gift from Novozymes A/S.

5.2 General procedures

5.2.1 General procedure for deacetylation with CAL-B

Ester protected saccharide or thiosaccharide (100 mg) was dissolved in MTBE or CPME (10 mL) where *n*-BuOH (3.5 eq.) was added and stirred at 45 °C/60 °C/90 °C. Stirring was set to 100 rpm and CAL-B (50 or 100 or 200 mg) was added. The reaction was followed by TLC, and upon completion the reaction mixture was filtered, immobilised enzymes were washed with DCM (~50 mL), and the filtrate was concentrated in vacuo. The crude mixture was purified by silica gel column chromatography to yield the purified target product(s).

5.2.2 General procedure for acetylation with CAL-B

Unprotected saccharide or thiosaccharide (60 or 100 mg) was dissolved in acetonitrile:vinyl acetate 1:1 (0.08 M to substrate), heated up to 45 or 60 °C and stirred for 10 min. Stirring was set to 100 rpm and CAL-B (20–200% w/w) was added. The reaction vessel was equipped with air-cooler or water-cooler with $CaCl_2$ tube. The reaction was followed by TLC and upon completion, the reaction mixture was filtered. Immobilised enzymes were washed with DCM or MeOH or MeOH/H_2O (~50 mL) and the filtrate was concentrated in vacuo. The crude mixture was purified by silica gel column chromatography to yield the purified target product(s).

5.3 Experimental procedures and characterisation of products

5.3.1 4-chlorophenyl 3-*O*-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**101**)

According to General Procedure for Deacetylation with 4-chlorophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside **100** (100 mg, 0.178 mmol), MTBE (10 mL), *n*-BuOH (3.5 eq., 57 µL) and CAL-B (100 mg) at 45 °C. The reaction was run for 50 h resulting in white crystalline solid 67 mg (79%), TLC – DCM:EtOAc 1:1, R_f = 0.39; column chromatography eluent system DCM:EtOAc 9:1 → 2:3; m.p. – 191–195 °C (from MeOH); $[\alpha]D20$ -54.2 (acetone, c 0.08)

^{1}H NMR (400 MHz, MeOD) δ 7.80-7.91 (m, 4H), 7.40 (d, *J* = 8.5 Hz, 2H, SPh-ArH), 7.26 (d, *J* = 8.5 Hz, 2H, SPh-ArH), 5.74 (d, *J* = 10.5 Hz, 1H, H-1), 5.57 (dd, *J* = 8.7, 10.1 Hz, 1H, H-3), 4.16 (t, *J* = 10.3 Hz, 1H, H-2), 3.95 (dd, *J* = 1.8, 12.1 Hz, 1H, H-6a/6b), 3.78 (dd, *J* = 5.2, 12.2 Hz, 1H, H-6a/6b), 3.63 (dd, *J* = 10.0, 18.7 Hz, 1H, H-4), 3.58 (ddd, *J* = 1.9, 5.2, 9.9 Hz, 1H, H-5), 1.86 (s, 3H); ^{13}C{^{1}H} NMR (101 MHz, MeOD) δ 172.2 (Ac-C=O), 169.3 (Phth-C=O), 168.6 (Phth-C=O), 136.0 (Phth-C), 135.8 (Phth-C), 135.3 (SPh-C), 135.0 (2xSPh-C), 132.9 (Phth-C), 132.4 (Phth-C), 132.1 (SPh-C), 130.1 (2xSPh-C), 124.6 (Phth-C), 124.5 (Phth-C), 84.3 (C-1), 82.5 (C-5), 75.9 (C-3), 69.7 (C-4), 62.4 (C-6), 55.4 (C-2), 20.5; HRMS (AJS-ESI): $[M+Na]^+$ for $C_{22}H_{20}ClNO_7SNa^+$ calculated 500.0541 found 500.0551.

5.3.2 4-chlorophenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-L-mannopyranoside (**115**)

Using modified procedure from literature[152]: L-mannopyranoside pentaacetate **117** (0.5 g, 1.28 mmol) was dissolved under argon in dry DCM (2.5 mL) and cooled to 0 °C. 4-chlorothiophenol (226 mg, 1.2 eq.) was added. $BF_3{\cdot}Et_2O$ (0.386 mL, 2.4 eq.) was added dropwise to the reaction mixture. The reaction mixture was stirred for 2h and slowly reached room temperature during this time. The reaction mixture was diluted with DCM, washed with cold sat. aq. $NaHCO_3$ and water. Organic phase was dried and concentrated in vacuo. The crude product was purified by column chromatography resulting in honey-like gel 346 mg (57%) with α:β 88:12; TLC – PE:EtOAc 2:1; R_f = 0.48; column chromatography eluent system PE:EtOAc 1:0 → 2:1;

α-^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40-7.44 (m, 2H), 7.27-7.31 (m, 2H), 5.48 (dd, *J* = 1.6, 2.9 Hz, 1H, H-2), 5.46 (d, *J* = 1.3 Hz, 1H, H-1), 5.25-5.36 (m, 2H, H-3,4), 4.50 (ddd, *J* = 2.4, 6.2, 8.9 Hz, 1H, H-5), 4.29 (dd, *J* = 6.0, 12.2 Hz, 1H, H-6a/6b), 4.10 (dd, *J* = 2.3, 12.2 Hz, 1H, H-6a/6b), 2.16 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H); ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 170.5, 169.9, 169.9, 169.7, 134.5, 133.3 (2xC), 131.0, 129.4 (2xC), 85.6 (C-1), 70.7 (C-2), 69.6 (C-5), 69.3 (C-3), 66.3 (C-4), 62.4 (C-6), 20.9, 20.7 (2xC), 20.6.

β-^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45-7.48 (m, 2H), 7.35-7.39 (m, 2H), 5.65 (dd, *J* = 0.9, 3.5 Hz, 1H, H-2), 5.25-5.36 (m, 1H, H-4), 5.05 (dd, *J* = 3.5, 10.1 Hz, 1H, H-3), 4.87 (d, *J* = 1.0 Hz, 1H, H-1), 4.25-4.33 (m, 1H, H-6a/6b), 4.17 (dd, *J* = 2.5, 12.2 Hz, 1H, H-6a/6b), 3.69 (ddd, *J* = 2.5, 6.5, 9.9 Hz, 1H, H-5), 2.21 (s, 3H), 2.10 (s, 3H), 2.05 (s, 3H), 1.99 (s, 3H); ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 170.6, 170.2, 170.1, 169.6, 134.6, 133.5 (2xC), 131.5, 129.2 (2xC), 85.5 (C-1), 76.6 (C-5), 71.8 (C-3), 70.4 (C-2), 65.7 (C-4), 62.8 (C-6), 20.8 (2xC), 20.6, 20.6; HRMS (AJS-ESI): $[M+Na]^+$ for $C_{20}H_{23}ClO_9SNa^+$ calculated 497.0644 found 497.0645.

5.3.3 4-chlorophenyl 2,3,4-di-*O*-acetyl-1-thio-L-mannopyranoside (**116**)

According to General Procedure for Deacetylation with 4-chlorophenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-L-mannopyranoside **115** (100 mg, 0.21 mmol), MTBE (10 mL), *n*-BuOH (3.5 eq., 67 µL) and CAL-B (200 mg) at 60 °C. The reaction was run for 48 h resulting in honey-like gel 47 mg (57%) with α:β 80:20, 36% of starting material was also isolated from column chromatography; TLC – PE:EtOAc 1:2; R_f = 0.64; column chromatography eluent system DCM:EtOAc 4:1 → 1:4;

α-^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.46 (m, 2H), 7.27-7.33 (m, 2H), 5.50 (dd, *J* = 1.6, 2.9 Hz, 1H, H-2), 5.47 (d, *J* = 1.3 Hz, 1H, H-1), 5.23-5.39 (m, 2H, H-3,4), 4.26 (ddd, *J* = 3.2, 3.2, 8.9 Hz, 1H, H-5), 3.58-3.78 (m, 2H, H-6a,6b), 2.29 (dd, *J* = 6.3, 8.0 Hz, 1H, OH-6), 2.15 (s, 3H), 2.11 (s, 3H), 2.03 (s, 3H); ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 170.9, 170.1, 170.0, 134.6, 133.4 (2xC), 131.2, 129.6 (2xC), 85.9 (C-1), 72.1 (C-5), 71.0 (C-2), 69.2 (C-3), 66.6 (C-4), 61.3 (C-6), 21.0, 20.9, 20.8.

β-^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.46 (m, 2H), 7.27-7.33 (m, 2H), 5.66 (dd, *J* = 1.0, 3.5 Hz, 1H, H-2), 5.23-5.39 (m, *J* = 6.9 Hz, 1H, H-4), 5.09 (dd, *J* = 3.5, 10.2 Hz, 1H, H-3), 4.92 (d, *J* = 1.1 Hz, 1H, H-1), 3.58-3.78 (m, 2H, H-6a,6b), 3.52 (ddd, *J* = 2.7, 5.2, 9.8 Hz, 1H, H-5), 2.20 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H); ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 170.4, 170.3, 170.2, 134.7, 133.5 (2xC), 131.5, 129.6 (2xC), 85.6 (C-1), 79.1 (C-5), 71.8 (C-3), 70.7 (C-2), 65.9 (C-4), 61.8 (C-6), 20.9, 20.8, 20.7; HRMS (AJS-ESI): $[M+Na]^+$ for $C_{18}H_{21}ClNO_8SNa^+$ calculated 455.0538 found 455.0544.

5.3.4 2,3,4-tri-*O*-acetyl-L-mannopyranoside (**118**)

According to General Procedure for Deacetylation with L-mannopyranoside pentaacetate **117** (100 mg, 0.26 mmol), CPME (10 mL), *n*-BuOH (3.5 eq., 82 µL) and CAL-B (200 mg) at 60 °C. The reaction was run for 48 h resulting in honey-like gel 51 mg (65%) with α:β 90:10; TLC – PE:EtOAc 1:2; R_f = 0.18; column chromatography eluent system PE:EtOAc 1:0 → 0:1;

α-^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (dd, *J* = 3.3, 10.1 Hz, 1H, H-3), 5.26 (d, *J* = 2.9 Hz, 1H, H-2), 5.24 (s, 1H, H-1), 5.19 (t, *J* = 10.2 Hz, 1H, H-4), 4.35 (s, 1H, OH-1), 4.05 (ddd, *J* = 2.3, 4.9, 10.0 Hz, 1H, H-5), 3.69 (dd, *J* = 1.9, 12.5 Hz, 1H, H-6a/6b), 3.63 (dd, *J* = 5.0, 12.5 Hz, 1H, H-6a/6b), 2.88 (s, 1H, OH-6), 2.15 (s, 3H), 2.08 (s, 3H), 2.01 (s, 3H); ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 171.1, 170.5, 170.3, 92.3 (C-1), 70.6 (C-5), 70.3 (C-2), 68.8 (C-3), 66.8 (C-5), 61.6 (C-6), 21.0, 20.9, 20.9;

β-^{1}H NMR (400 MHz, $CDCl_3$) δ 5.42 (dd, *J* = 0.8, 3.1 Hz, 1H, H-3), 5.14 (d, *J* = 9.5 Hz, 1H, H-4), 5.11 (dd, *J* = 3.0, 10.0 Hz, 1H, H-2), 5.02 (s, 1H, H-1), 3.58-3.76 (m, 2H, H-6a,6b), 3.53 (ddd, *J* = 2.2, 5.1, 9.1 Hz, 1H, H-5), 2.21 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H); ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 170.9, 170.7, 170.3, 92.9 (C-1), 74.8 (C-5), 71.2 (C-2), 70.2 (C-3), 66.0 (C-4), 61.4 (C-6), 20.9, 20.9, 20.7; HRMS (AJS-ESI): $[M+Na]^+$ for $C_{12}H_{18}O_9Na^+$ calculated 329.0843 found 329.0848.

5.3.5 1,2,3,4-tetra-*O*-acetyl-L-mannopyranoside (**119**)

According to General Procedure for Deacetylation with L-mannopyranoside pentaacetate **117** (100 mg, 0.26 mmol), CPME (10 mL), *n*-BuOH (3.5 eq., 82 µL) and CAL-B (100 mg) at 45 °C. The reaction was run for 48 h resulting in honey-like gel 29 mg (32%) with α:β 80:20; TLC – PE:EtOAc 1:2; R_f = 0.43; column chromatography eluent system PE:EtOAc 1:0 → 0:1;

α-^{1}H NMR (400 MHz, $CDCl_3$) δ 6.09 (d, *J* = 1.6 Hz, 1H, H-1), 5.39 (dd, *J* = 3.4, 10.2 Hz, 1H, H-3), 5.31 (t, *J* = 10.1 Hz, 1H, H-4), 5.27 (dd, *J* = 2.0, 3.2 Hz, 1H, H-2), 3.85 (ddd, *J* = 2.3,

4.2, 9.8 Hz, 1H, H-5), 3.57-3.80 (m, 2H, H-6a,6b), 2.36 (s, 1H, OH-6), 2.17 (s, 6H), 2.08 (s, 3H), 2.02 (s, 3H);
β-^{1}H NMR (400 MHz, $CDCl_3$) δ 5.87 (d, *J* = 0.8 Hz, 1H, H-1), 5.49 (d, *J* = 3.0 Hz, 1H), 5.25 (d, *J* = 9.9 Hz, 1H), 5.17 (dd, *J* = 3.3, 10.1 Hz, 1H), 3.57-3.80 (m, 3H, H-5,6a,6b), 2.20 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H), 2.01 (s, 3H); NMR matches with literature data.[155]

Table 3. *Supporting information concerning compounds discussed in the thesis, but not presented in the Experimental section can be found in the corresponding publication. [a] – New compounds or compounds lacking analytical data before publishing.*

Entry	Compound no. in thesis	Compound no. in publication			New compound[a]
		I	**II**	**III**	
1	**1**			**23**	
2	**5**			**25**	
3	**16**	**1α**	**42**		
4	**19**	**1β**	**1**		
5	**20**	**2a**	**2**		
6	**31**	**27**			
1	**47**	**1**			
3	**48**	**2**	**43**		**Yes**
4	**49**	**2c**			**Yes**
7	**50**			**1**	
8	**51**			**2**	
9	**52**			**3**	**Yes**
10	**53**			**4**	**Yes**
11	**54**			**5**	**Yes**
12	**55**			**6**	**Yes**
13	**56**			**7**	**Yes**
14	**57**	**13**			
15	**58**	**14**			
16	**59**	**15**			
17	**60**	**16**			
18	**61**		**7**		
19	**62**		**8**		**Yes**
20	**63**		**23**		
21	**64**		**24**		**Yes**
22	**65**		**25**		**Yes**
23	**66**		**26**		**Yes**
24	**67**		**35**		
25	**68**		**36**		
26	**69**		**38**		
27	**70**		**39**		
28	**71**		**40**		**Yes**
29	**72**		**41**		
30	**73**			**10**	
31	**74**			**11**	**Yes**

33	**75**			**24**	
34	**76**		**5**		
35	**77**		**6**		
36	**78**	**5**	**3**		
37	**79**	**4**	**4**		
38	**80**		**9**		
39	**81**		**10**		
40	**82**		**11**		**Yes**
41	**83**		**18**		
42	**84**		**19**		**Yes**
43	**85**		**19**		
44	**86**	**18**			
45	**87**	**19**			
46	**88**		**12**		
47	**89**		**13**		**Yes**
48	**90**		**14**		
49	**91**		**15**		**Yes**
50	**92**		**37**		
51	**93**			**8**	
52	**94**			**9**	**Yes**
54	**95**	**6**			
55	**96**	**7**			
56	**97**	**22**			
57	**98**	**23**			**Yes**
58	**99**	**24**			
59	**102**			**12**	
60	**103**			**13**	**Yes**
61	**104**			**14**	
62	**105**			**15**	**Yes**
63	**106**			**26**	
64	**107**			**27**	
65	**108**			**28**	
66	**109**			**29**	**Yes**
67	**110**			**S1**	
68	**111**			**S2**	
69	**112**		**20**		
70	**113**		**21**		
71	**114**		**22**		**Yes**
72	**120**			**30**	
73	**121**			**31**	
74	**122**			**32**	
75	**123**			**33**	**Yes**
76	**124**			**16**	**Yes**
77	**125**			**17**	**Yes**
78	**126**		**27**		
79	**127**		**29**		**Yes**

80	**128**		**30**		
81	**129**		**28**		
82	**130**		**31**		
83	**131**	**8**			
84	**132**	**9**			
85	**133**	**10**			**Yes**
86	**134**	**20**			
87	**135**	**21**			**Yes**
88	**136**		**32**		
89	**137**		**33**		
90	**138**		**34**		
91	**139**			**34**	
92	**140**			**35**	
93	**141**			**18**	**Yes**
94	**142**			**19**	**Yes**
95	**143**			**20**	**Yes**
96	**144**			**21**	**Yes**
97	**145**	**11**			
98	**146**	**12**			
99	**147**		**16**		**Yes**
100	**148**		**17**		**Yes**
101	**149**			**36**	
102	**150**			**22**	**Yes**
103	**151**	**3**			
105	**152**	**26**			
106	**153**	**28**			
107	**154**	**29**			**Yes**
108	**155**	**30**			**Yes**
				Total:	38

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Abstract

Transesterification of Monosaccharides with *Candida Antarctica* Lipase-B and Their Use in The Synthesis of Human Milk Oligosaccharides

This doctoral thesis provides a brief general overview of the carbohydrate chemistry, highlighting the structural peculiarities and differences among various saccharides. Additionally, a short summary is given about lipases, more specifically, immobilised CAL-B. Followed by a short introduction to human milk oligosaccharides (HMOs) and together with their different synthesis methods.

The synthesis of natural oligosaccharide and glycoconjugates is a complex task involving multi-step reaction pathways. These compounds can be made by enzymatic, cell factory or chemical means. Cell factories utilise microbial hosts with specifically modified genomes to manufacture target oligosaccharides. Currently, cell factories are the only means to produce commercially viable amounts of human HMOs. However, cell factories do have several drawbacks like hard to purify products and reproducibility issues, due to complexity of working with living organisms. Enzymatic approach often requires expensive enzymes e.g. glycosyltransferases, and nucleotide activated glycosides. Understandably, due to expensive materials used, the enzymatic oligosaccharide synthesis is conducted at small scale, while it can produce a library of structurally diverse oligosaccharides. Chemical synthesis of natural oligosaccharides is time consuming due to the need to selectively protect and arm the saccharides taking part in the glycosylation reactions. While glycosylation reactions itself are not fully understood and need to be studied substrate by substrate basis.

In order to produce the partially protected saccharides needed in chemical synthesis, several methods including toxic transition metal can be used. In contrast, enzymatic methods are not toxic and often regioselective. In this doctoral thesis, immobilised *Candida antarctica* lipase-B (CAL-B) was tested in transesterification reactions in organic media to find the means to selectively protect and deprotect various saccharides. Using CAL-B, the synthesis routes of several known saccharides were significantly shortened, while 42 new (or analytical data improved) saccharides were synthesised. An extensive overview of CAL-B transesterification reactions is shown by saccharide basis. It also includes a discussion of how different saccharides, configurations and protecting groups influences the transesterification reactions outcome. The immobilised CAL-B was successfully recycled from the deacetylation reactions, maintaining activity over multiple cycles. However, it was discovered that CAL-B cannot be recycled from the acetylation reactions. To demonstrate the synthetic utility of the products made from CAL-B deacetylation reactions, the deviant HMO 6'-galactosyllactose, was synthesised using two CAL-B-derived products.

Lühikokkuvõte

Monosahhariidide ümberesterdamine *Candida antarctica* lipase-B abil ja nende kasutamine rinnapiimas olevate oligosahhariidide sünteesis

Käesolev doktoritöö sisaldab lühikest üldist ülevaadet sahhariidide keemiast, näidates nende struktuuri omapära ja erinevate suhkrute struktuurseid erinevusi. Lisaks antakse lühike kokkuvõte lipaaside, täpsemalt immobiliseeritud CAL-B-i kohta ja tuuakse näiteid rinnapiima oligosahhariidide erinevatest sünteesimeetoditest.

Looduslike oligosahhariidide ja teiste sahhariide sisaldavate ühendite süntees on keeruline. Tavaliselt on tarvis pikki, mitmeid etappe sisaldavaid sünteesiskeeme. Neid ühendeid saab sünteesida nii rakuvabrikute, ensüümide kui ka keemilise sünteesi abil. Rakuvabrikud kasutavad soovitud oligosahhariidide saamiseks spetsiifiliselt muudetud genoomiga mikroobe. Hetkel on rakuvabrikud ainus viis, kuidas saada suurtes kogustes inimese emapiimas olevaid oligosuhkruid (HMO-d). Rakuvabrikutel on ka omad miinused. Kuna töö käib elusorganismidega on tihti raskusi katsete reprodutseeritavusega ja saadud ainete puhastamisega. Looduslikke oligosahhariidide saamiseks ensümaatiliselt on vaja kalleid ensüüme ja lähteaineid, nt. glükosüültransferaase ja nukleotiididega aktiveeritud sahhariide. Sellest tulenevalt on tavaliselt ensümaatiliselt sünteesitud ainult väikeseid koguseid, samas saab ensüümide abil toota palju erinevaid oligosuhkruid. Keemilise sünteesi abil saab valmistada spetsiifilise struktuuriga kõrge puhtusega oligosahhariidi, kuid see on aeganõudev protsess kuna glükosüleerimise reaktsioonides osalevad sahhariidid peavad olema selektiivselt kaitstud ja omama kindlaid lahkuvaid rühmi. Kuna glükosüleerimise mehhanism ei ole täpselt teada peab iga suhkru sünteesi eraldi uurima.

Keemilise sünteesi jaoks vajalikke osaliselt kaitstud suhkrute saamiseks on erinevaid teid, teiste seas ka toksilisi metallkatalüüsi sisaldavaid. Samas ensümaatilised reaktsioonid ei ole toksilised ja on tihti regioselektiivsed. Käesolevas doktoritöös uuriti erinevate suhkrute näidete abil tahkele kandjale seotud *Candida antarctica* lipaas-B (CAL-B) võimet selektiivselt lisada ja eemaldada kaitsvaid rühmi orgaanilistes lahustites. CAL-B-i abil lühendati tuntud ühendite sünteesiteekondi mitmete etappide võrra ja sünteesiti 42 uut (või täiendati seni puudulike spektraalsete andmetega) sahhariidi. Doktoritöö sisaldab põhjalikku kokkuvõtet CAL-B-i ümberesterdamise reaktsioonide tulemustest, mis on esitatud konkreetsete sahhariidide kaupa. Lisaks on arutletud, kuidas erinevad suhkrud, nende geomeetriad ja kaitsmisrühmad mõjutavad CAL-B-i reaktsioonide tulemusi. Tõestati, et kandjale seotud CAL-B-i saab taaskasutada estrite hüdrolüüsil, kuid esterdamise reaktsioonis ei olnud korduvkasutus enam võimalik. Näitamaks uuritud lähenemise kasulikkust ja efektiivsust kasutati ensümaatilise meetodiga saadud vaheprodukte ühe HMO-6'-galaktosüüllaktoosi sünteesis.

Appendix 1

Publication I

Hunt, K. E.; García-Sosa, A. T.; Shalima, T.; Maran, U.; Vilu, R.; Kanger, T. Synthesis of 6'-Galactosyllactose, a Deviant Human Milk Oligosaccharide, with the Aid of *Candida Antarctica* Lipase-B. *Org. Biomol. Chem.* **2022**, *20* (23), 4724–4735. https://doi.org/10.1039/D2OB00550F.

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Synthesis of 6′-galactosyllactose, a deviant human milk oligosaccharide, with the aid of *Candida antarctica* lipase-B†

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Research on human milk oligosaccharides (HMOs) has increased over the past decade showing great interest in their beneficial effects. Here we describe a method for the selective deacetylation using immobilised *Candida antarctica* lipase-B, Novozyme N435 (N435), of pyranose saccharides in organic media with the aim of simplifying and improving the pathways for the synthesis of HMOs. By first studying in depth the deacetylation reaction of peracetylated D-glucose two reaction conditions were found, which were used on different HMO building blocks, peracetylated saccharides and thioglycosides. D-Glucose based saccharides showed selectivity towards the fourth and the sixth position deacetylation. While α-anomer of peracetylated D-galactose remained unreactive and β-anomer favoured the first position deacetylation. Peracetylated L-fucose, on the other hand, had no selectivity as the main product was fully unprotected L-fucose. Taking the peracetylated D-glucose deacetylation reaction product and selectively protecting the primary hydroxyl group in the sixth position left only the fourth position open for the glycosylation. Meanwhile, the deacetylation product of D-galactose thioglycoside, with the sixth position deacetylated, had both acceptor and donor capabilities. Using the two aforementioned products derived from the N435 deacetylation reactions a deviant HMO, 6′-galactosyllactose (6′-GL) was synthesised.

Introduction

Human milk oligosaccharides (HMOs) form a unique class of oligosaccharides that consists of five main subunits: D-glucose, D-galactose, *N*-acetyl-D-glucosamine, L-fucose and *N*-acetylneuraminic acid. These monosaccharides are connected by glycosidic bonds leading to a wide variety of linear or branched oligosaccharides with degrees of polymerisation from 3 to 30. Over 200 HMOs have been identified.[1] HMOs are essential for the development of newborn infants, especially for their gut microbiota, where they boost probiotic bacterial growth.[2] Furthermore, HMOs can act as decoys for such pathogens as respiratory and intestinal viruses, help against diarrhoea and alleviate allergic symptoms.[3] Because of the increasing importance of baby food and prebiotic supplements there is an increasing need for the production of HMOs. To obtain HMOs, a shift from chemical synthesis to biotechnology, such as enzymatic[4] and cell factory[5] methods, has taken place in recent years. Chemical synthesis is still important as it makes it possible to get a single compound instead of mixtures obtained by cell factories.[6]

In chemical synthesis, a selective manipulation of protection groups of the saccharides is crucial as general synthetic targets are molecules with specific glycosidic bonds.[7–9] The synthesis of glycosyl acceptors is usually a multi-step procedure using various temporary and permanent protection groups.[10] To minimise the number of steps needed to obtain the target molecule different tools have been utilised including enzymes. Enzymes have been used to form glycosidic bonds,[11,12] selectively protect and deprotect mono- and oligosaccharides.[13,14] *Candida antarctica* lipase-B (CAL-B) is one of the most widely used enzymes in chemical applications, it has high regio- and enantioselectivity towards substrates and a wide range of uses from the production of biodiesel to the resolution of racemic mixtures to optically active compounds.[15,16] In carbohydrate chemistry CAL-B has mainly been used in selective esterification[17,18] and transesterification reactions.[19,20] One of the major advantages of CAL-B is its ability to also function in organic solvents, increasing its range

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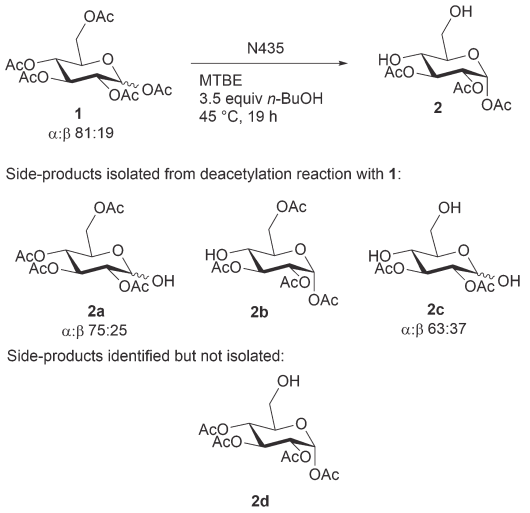
†Electronic supplementary information (ESI) available: NMR spectra of all synthesised compounds. See DOI: https://doi.org/10.1039/d2ob00550f

of uses considerably.[21] D'Antona *et al.*[22] showed that CAL-B can deacetylate peracetylated D-fructose and its derivatives, and Ryczek *et al.*,[23] Schramm *et al.*[24] and Shimotori *et al.*[25,26] have shown the deacetylation of glucose derivatives. There have been few examples of using CAL-B to deacetylate peracetylated pyranose containing monosaccharides with varying degrees of success.[14,20,27–30] Several other enzymes have shown high selectivity to deacetylating pyranoses, but usually in buffer solutions or in mixtures of buffer and organic solvents, which can lead to troublesome work-up procedures to isolate the partially deprotected products out of aqueous media.[14,20,31–33] Enzymes as natural compounds often exhibit a moderate stability even at room temperature. The immobilisation of the enzymes is used to improve their stability. The properties of the immobilised enzyme depend on the immobilisation methods and on solid support.[34] In this study, we used a commercially available immobilised *Candida antarctica* lipase-B Novozym 435[16] (N435) for deacetylation reactions in non-aqueous media with several peracetylated monosaccharides, D-lactose, and pyranose monosaccharide derivatives with a thiophenol leaving group or methyl protection group in the anomeric position. The obtained building blocks were used in glycosylation reactions for the synthesis of a deviant HMO, 6′-galactosyllactose (6′-GL).



Scheme 1 Deacetylation of **1** with N435.

Results and discussion

While searching for a quick way to make glycosyl acceptors we studied the deacetylation of peracetylated monosaccharides found in HMOs. The reaction of peracylated D-glucose **1** (a mixture of α- and β-anomers in a ratio of 81 : 19) with N435 was run in methyl *tert*-butyl ether (MTBE) in the presence of 3.5 equivalents of *n*-butanol[35,36] at 45 °C for 19 h. The 4,6-dideacetylated glucose **2** as a dominant product was obtained in 70% yield together with minor amounts of other mono- or trideacetylated side products **2a–d** (Scheme 1).

The solvent screening was carried out at 55 °C (Table 1). Compared to MTBE only cyclopentyl methyl ether (CPME) showed comparable results with 69% isolated yield (Table 1, entry 7). In all other solvents, except toluene, the formation of the side-product **2a** increased. MTBE was chosen as the solvent of choice for further reactions as a slightly higher yield was obtained at a lower temperature.

Next, further optimisation reactions were performed. Peracylated D-glucose **1** (100 mg) and N435 (100 mg) were stirred in MTBE (10 mL) and *n*-BuOH (3.5 equiv.) at 45 °C for 23 h. The conversion of **1** was determined by ^{1}H NMR after 4, 6 and 23 hours (Table 2, entry 1). Either an increase or decrease in the concentration decreased the reaction rate slightly although reactions reached the full conversion of **1** in 23 h (Table 2, entries 2 and 3). At lower concentration, the isolated yield slightly increased to 74% (Table 2, entry 3). Increasing the amount of enzyme led to a faster reaction but decreased the yield of the target to 63% (Table 2, entry 4). Decreasing the amount of enzyme increased the reaction time

Table 1 Solvent screening for the deacetylation of **1** with N435[a]

Entry	Solvent	**1**[b] (%)	**2**[b] (%)	**2a**[b] (%)
1	MTBE[c]	0	70[d]	6[d]
2	Acetone	nd[e]	Traces	Traces
3	Toluene	52	32	16
4	MeCN	77	8	15
5	EtOH	75	2	23
6	$CHCl_3$	77	Traces	23
7	CPME	0	69[d]	nd[e]

[a] Reaction conditions: **1** (0.1 mmol), 1 : 1 **1** : N435(w/w), 0.026 M, 55 °C, *n*-BuOH (3.5 equiv.). [b] Relative ratio of the starting compound and products determined by ^{1}H NMR after 19 h. [c] Reaction at 45 °C. [d] Isolated yield. [e] nd – not determined.

Table 2 Optimisation of deacetylation of **1** with N435

Entry	Conc. of **1** (M)	Ratio **1** : N435 (w/w)	Temp. (°C)	Conv. of **1**[b] (%) 4 h	6 h	23 h	Yield of **2**[c] (%)
1[a]	0.026	1 : 1	45	75	87	100	70
2	0.05	1 : 1	45	63	79	100	69
3	0.013	1 : 1	45	55	71	100	74
4	0.026	1 : 2	45	86	96	100	63
5	0.026	2 : 1	45	45	59	96	82
6	0.026	1 : 1	R.T.	35	42	75	54
7	0.026	1 : 1	30	52	62	93	77
8	0.026	1 : 1	55	92	98	100	58
9	0.026	1 : 2	55	100	—	—	63
10[d]	0.026	1 : 1	45	59	67	82	20

[a] Reaction conditions: **1** (100 mg), N435 (100 mg), MTBE (10 mL), 45 °C, *n*-BuOH (3.5 equiv.), 23 h. [b] Determined by ^{1}H NMR. [c] Isolated yield. [d] No *n*-BuOH added.

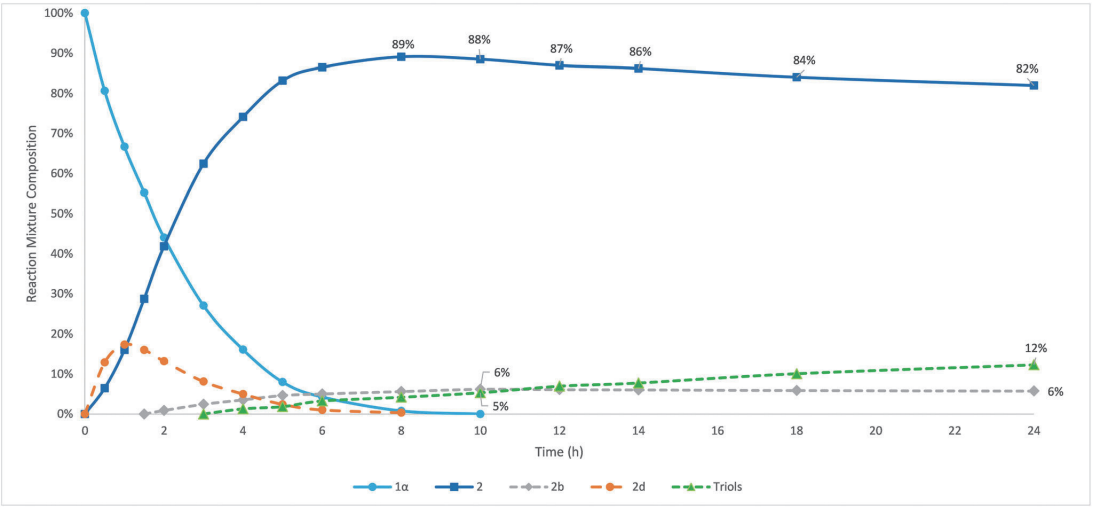


Fig. 1 Kinetic study of N435 deacetylation reaction with **1α**. Reaction conditions: **1α** (100 mg), N435 (100 mg), MTBE (10 mL), 45 °C, *n*-BuOH (3.5 equiv.)

and led to a higher yield of 82% (Table 2, entry 5). The effect of temperature on the yield was also studied (Table 2, entries 6–9). This revealed that the optimal temperature of the deacetylation was 45 °C. In the absence of the nucleophilic reagent the reaction still proceeded, but more side product **2a** was formed and **2** was formed only in 20% isolated yield (Table 2, entry 10). The optimal conditions for the selective deacetylation of **1** were found to be with a 2 : 1 ratio of **1** : N435 (w/w) (Table 2, entry 5).

For a closer look at the process, the deacetylation reaction was studied over time using ^{1}H NMR to determine the ratio of the starting compound and formed products. First, deacetylation reaction was carried out with α-anomer (**1α**) of **1** (Fig. 1). After 30 min two products were detected. One corresponding to 4,6-deacetylated D-glucose **2**, while the second one turned out to be **2d**[37,38] – an intermediate where the sixth position had been deacetylated. The existence of **2d** indicates a stepwise deacetylation process for the formation of **2**, which has been reported previously *via* acyl migration from the fourth to the sixth position.[39] The amount of monodeacetylated product **2b** remained constant after 10 hours meaning that the deacetylation did not follow the acyl migration pathway. The formation of **2** climaxed close to full conversion of **1α** and disappearance of **2d** around 8 h after which a slight decline was seen where **2** deacetylated further to several different triols. The formation of triols did not accelerate after full consumption of **1α** but remained on the same level throughout the reaction.

Using the same method, the deacetylation reaction was studied with β-anomer (**1β**) of **1** as well (Fig. S1†). The reaction was much faster as after 1 h full conversion of **1β** was reached forming the first position deacetylated product **2a**. Similarly to the previous study, further deacetylation happened in the fourth and sixth positions leading to the formation of triol **2c**. The amount of **2c** increased steadily reaching close to full conversion of **2a** after 24 h.

The results from the two kinetic studies show that N435 seems to favour the fourth and the sixth position deacetylation with only traces of other deacetylation products.

The different behaviour of α and β anomers can be further revealed by analysing the interactions in the CAL-B protein (1tca) binding pocket. This analysis shows that **1α** has less steric clashes in the complex with the active site of CAL-B than **1β**, given the better fit of the acetylated groups once the sixth position is placed in the anion hole (Fig. 2). In addition, **1α** can form an additional hydrogen bond with His224 with the acetyl group in the third position that is not available for the β-anomer. Due to these observations **1α** forms a more stable geometry with the catalytic centre of CAL-B than **1β** that may explain the formation of **2**. Spatial representation shows both anomers in the catalytic centre with the sixth position acetyl and ring superposed, their binding poses and hydrogen bonds with amino acid residues (Fig. 3).

The scope and limitations of the CAL-B mediated deacetylation reaction were explored with different peracetylated HMO's building blocks (Scheme 2). The deacetylation of the α-anomer of the peracylated D-glucose **1α** mirrored the outcome of the kinetic study with no **2a** and only small amount of **2b** detected in the crude mixture. The triacetate **2** was obtained in 82% yield. Peracetylated D-galactose **3**, a mixture of pyranose (pyr) and furanose (fur) (52 : 30 : 18 α : β(pyr) : fur) showed after 3 days mainly the deacetylation of the first position while 87% of the α-isomer of **3** remained unreacted and was isolated. No clear product of α-isomer was characterised. On the other hand, similarly to **1β** kinetic study peracetylated β-D-galactopyr-

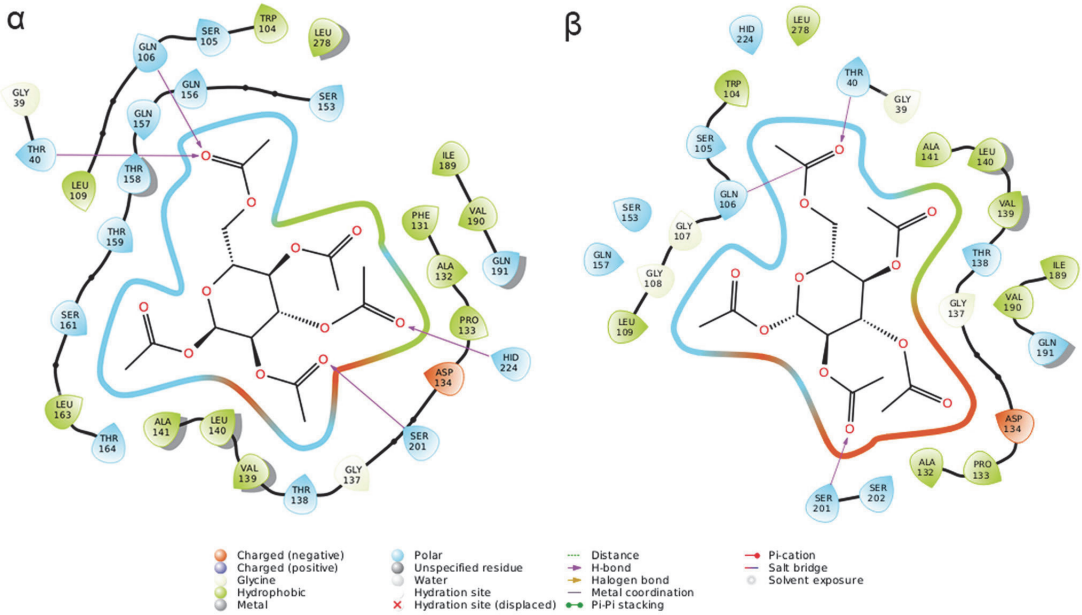


Fig. 2 Interactions of α and β anomers of **1** with residues forming the CAL-B (1tca) binding site.

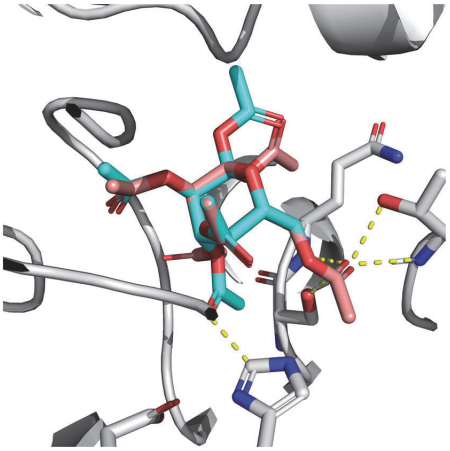
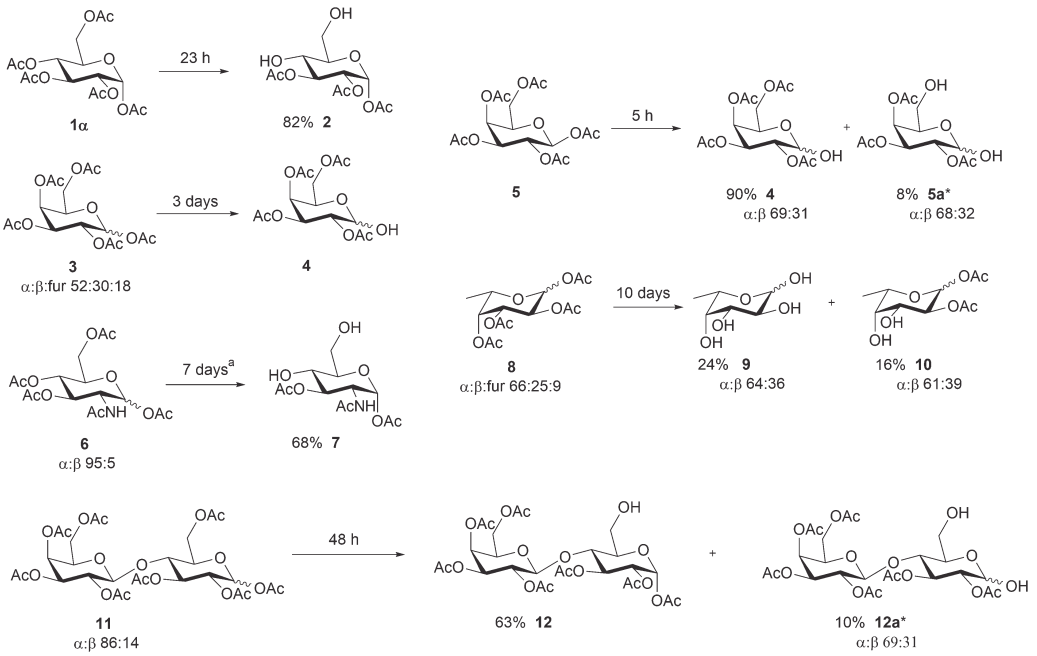


Fig. 3 Spatial representation of α (cyan) and β (pink) anomers of **1** in CAL-B binding site.

anoside **5** reacted rapidly and full conversion was achieved in 5 h affording monodeacetylated compound **4** as the major product in 90% yield, and some minor triacetates (**5a** was the dominant side-product). Peracetylated *N*-acetyl-D-glucosamine **6** (95 : 5 α : β) showed poor solubility in MTBE, after diluting and sonicating the mixture still remained heterogeneous. During the reaction (~2 h) the starting monosaccharide **6** was fully dissolved. The reaction proceeded rapidly at first as 50% of conversion was estimated after 6 h by ^{1}H NMR but full conversion was not achieved even after 7 days according to the TLC. Nevertheless, triacetate **7** was isolated in 68% yield. Peracetylated L-fucose **8** (66 : 25 : 9 α : β(pyr) : fur) reacted very poorly as after 6 h only 2% of conversion was estimated by ^{1}H NMR. After 10 days, the reaction was stopped without reaching full conversion and 27% α-isomer of **8** was isolated together with peracetylated furanose of L-fucose. During the reaction, precipitation occurred, which turned out to be fully deacetylated L-fucose **9** isolated in 24% yield. Another product was a diacetate **10** isolated in 16% yield as a mixture of anomers. Peracetylated D-lactose **11** (86 : 14 α : β) reached full conversion in 48 h affording a monodeacetylated **12** as the main product in 68% yield. Several dideacetylated side-products were also detected in an inseparable mixture where **12a** was the dominant one. D-Glucose moieties containing saccharides **1**, **6** and **11** reacted similarly as the products were dominantly α-anomers and triacetates of 4,6-diols or monohydroxy 6-OH in the case of **11**. D-Galactose (compounds **3** and **5**) mainly formed monodeacetylated products at the anomeric position, which indicates that the equatorial configuration of the fourth position is more favourable than the axial for selective deacetylation. Similarly, β-anomers (**1β** and **5**) seemed to prefer to react at the first position while α-anomers were less reactive overall.

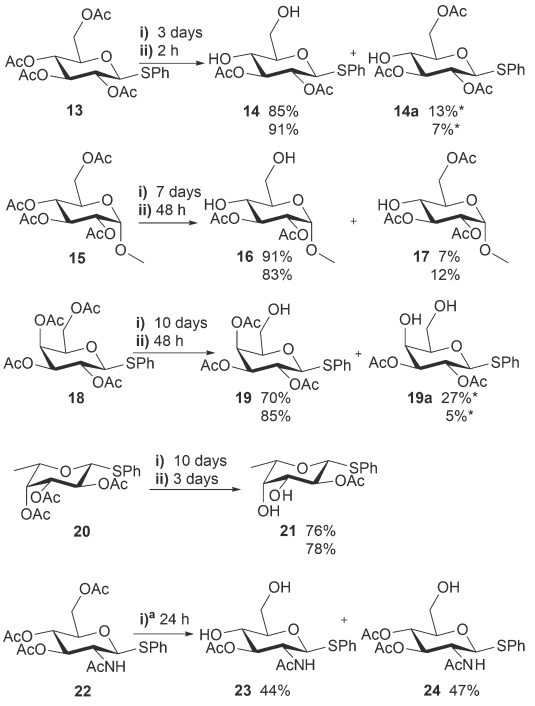
As in many cases, the deacetylation of the anomeric position occurred, and thus it was protected either with thiophenol



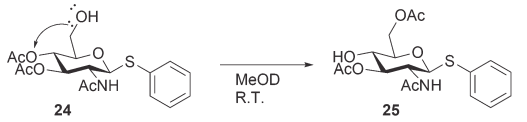
Scheme 2 N435 deacetylation reactions with different peracetylated saccharides. Reaction conditions: saccharide (100 mg), N435 (100 mg), MTBE (10 mL), 45 °C, *n*-BuOH (3.5 equiv.); [a]MTBE (20 mL); *mixture of products, dominant shown.

which could act as a potential leaving group or with a methyl group (Scheme 3). Thioglycosides were deacetylated using two different conditions – condition **(i)** the same as for the peracetylated saccharides and condition **(ii)** double the amount of N435 under reflux to increase the activity and decrease the reaction time (Table 2, entry 9). Phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **13** reacted similarly to **1α** resulting in triacetate **14** in 85% yield, while being less reactive, as full conversion was reached after 3 days. Side-products included a mixture of monodeacetylated thioglycosides with the fourth position deacetylated derivative **14a** being the dominant one. Using **(ii)** resulted in a decrease in reaction time to 2 h, a higher yield (91%) and less monodeacetylated side-products (7%). Surprisingly, the methyl protection group in the anomeric position of D-glucose (compound **15**) decreased the rate of the reaction even further compared to the other D-glucose derivatives **1α** and **13**. Full conversion was reached in 7 days under **(i)** and in 2 days under **(ii)**. This indicates that the anomeric position and its protecting group heavily influences the reactivity of the deacetylation with N435. As in the rest of the D-glucose series the major product of the reaction was a triacetate **16** and a side-product was monodeacetylated **17**. Similarly to the D-glucose derivative **13**, phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside **18** reacted much more slowly than its peracetylated counterpart **5**. Only after 10 days was the full conversion achieved with **(i)** affording monodeacetylated **19** as the main product in 70% yield and a mixture of triacetates, where **19a** was the dominant one. **(ii)** decreased the time to 2 days, increased the yield to 85% and decreased the amount of side-products to 5%. As in the case of peracetylated L-fucose **8**, the phenyl 2,3,4-tri-*O*-acetyl-6-deoxy-1-thio-β-L-galactopyranoside **20** deacetylation reaction was slow under **(i)** but reached full conversion after 10 days. Under **(ii)** the reaction time shortened to 3 days and the yield of triacetate **21** was slightly increased (78%). No side-products were successfully characterised. Phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside **22** exhibited similar solubility issues to **6**, and once again double the amount of MTBE was used together with sonication. Surprisingly, full conversion was reached in 24 h with **(i)**, resulting in triacetate **23** and monodeacetylated **24** in the sixth position, the latter had a small amount (~5%) of another tetraacetate product according to ^{1}H NMR in CD_3OD. Unexpectedly, the amount of unknown product had increased to 27% by the time ^{13}C was taken. After 4 days in an NMR tube 12% of **24** was left, the rest being the product of acyl migration **25** from the fourth to sixth position (Scheme 4). The 4 → 6 acyl migration is known to happen for *N*-acetyl-D-glucosamine compounds generally in buffer/organic solvent mixtures,[40,41] with the aid of an acid[32] or with *n*-BuLi[42] but not spontaneously.

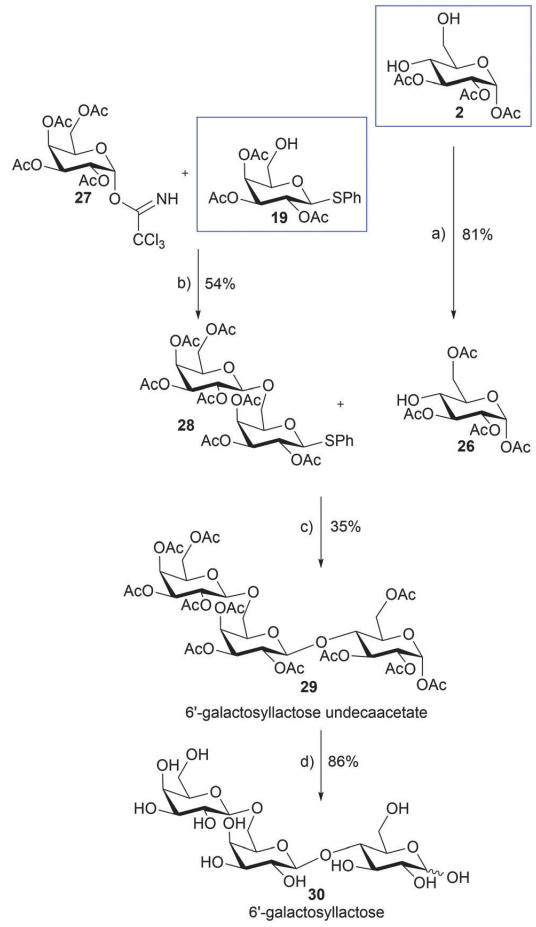
Utilising the products from the N435 deacetylation reactions in a relatively short pathway the synthesis of 6′-galactosyllactose **30** was performed (Scheme 5). First, the primary hydroxyl group of the main product **2** from D-glucose deacetyla-



Scheme 3 Deacetylation reactions with peracetylated thioglycosides and methyl a-D-glucose. Reaction conditions: **(i)** saccharide (100 mg), N435 (100 mg), MTBE (10 mL), 45 °C, *n*-BuOH (3.5 equiv.); **(ii)** saccharide (100 mg), N435 (200 mg), MTBE (10 mL), reflux, *n*-BuOH (3.5 equiv.); [a]**22** (50 mg), N435 (50 mg), MTBE (10 mL); *mixture of products, dominant shown.



Scheme 4 Acyl migration from fourth to sixth position.



Scheme 5 6′-GL synthesis pathway from N435 deacetylated products, which are shown in blue boxes. (a) AcCl, Pyridine, DCM, −40 °C → room temperature, 4 h; (b) $BF_3{\cdot}Et_2O$, DCM, 4 Å MS, −20 °C, 60 min; (c) TfOH, NIS, DCM, 4 Å MS, −20 °C, 30 min; (d) (i) NaOMe, MeOH, room temperature, 1 h (ii) Amberlite 120 H+, 1 h.

tion was acetylated selectively with AcCl to form an acceptor **26** in high yield (81%). Next, product **19** from the thioglycoside **18** deacetylation reaction can be used both as an acceptor and a donor, as the thiophenyl leaving group needs additional activation to use it as a donor. Thus a glycosylation reaction between trichloroacetimidate donor **27** with acceptor **19** catalysed by $BF_3{\cdot}Et_2O$ was performed resulting in disaccharide **28** in moderate yield (54%). The disaccharide donor **28**, with a βGal1 → 6βGal glycosidic bond was then activated by *N*-iodosuccinimide and TfOH for the final glycosylation reaction with **26** resulting in 6′-GL undecaacetate **29** in 35% yield. Finally, **29** was deprotected by Zemplén transesterification[43] with full conversion and a high yield. Unfortunately, after addition of Amberlite 120 H+, the reaction mixture had turned slightly pinkish and after concentrating *in vacuo* 6′-GL **30** was found to have some aromatic impurities. Purification using recrystallisation did not achieve further improvements. A normal phase silica gel column chromatography was successfully performed using a $MeCN:H_2O$ (4 : 1 → 2 : 1) gradient resulting in 37 mg (86%) of 6′-GL.

Conclusions

In conclusion, the deacetylation reaction with N435 was studied with several peracetylated pyranose saccharides and thioglycosides. The deacetylation reaction products varied for

different saccharides, but for glucose-containing moieties mostly fourth and sixth position deacetylation occurred. Partially deprotected saccharides may be used as building blocks in order to synthesise different oligosaccharides, for example a deviant HMO, 6′-GL, was synthesised.

Experimental

General information

Full assignment of ^{1}H and ^{13}C chemical shifts were based on the 1D and 2D FT NMR spectra measured with a Bruker Avance III 400 MHz instrument. Residual solvent signals were used ($CDCl_3$: δ = 7.26 ^{1}H NMR, δ = 77.2 ^{13}C NMR; CD_3OD: δ = 3.31 ^{1}H NMR, δ = 49.0 ^{13}C NMR; D_2O: δ = 4.79 ^{1}H NMR) as internal standards. High-resolution mass spectra were recorded with an Agilent Technologies 6540 UHD Accurate-Mass QTOF LC/MS spectrometer by using AJ-ESI ionisation. Optical rotations were obtained with an Anton Paar GWB Polarimeter MCP 500. Melting points were determined using polarising optical microscope Nagema-K8. Precoated Merck silica gel 60 F_{254} plates were used for TLC and column chromatography was performed with Merck 60 (0.040–0.063 mm) mesh silica gel. Commercial reagents and solvents were generally used as received. DCM was distilled over CaH, EtOAc over phosphorus pentoxide and MeOH over sodium. Petroleum ether (PE) had a boiling point of 40–60 °C. 1,2,3,4,6-Penta-*O*-acetyl-α-D-glucopyranoside (**1α**), 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranoside (**1β**) and 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranoside (**5**) were from Acros Organics. Immobilised *Candida antarctica* Lipase-B, Novozyme N435, was a kind gift from Novozymes A/S. Peracetylated sugars (**1**, **3**, **6**, **8**, **11**, **15**),[41,42] thiophenyl glycosides (**13**, **18**, **20**, **22**)[41,43] and 2,3,4,6-tetra-*O*-acetyl-α-D-glycopyranosyl trichloroacetimidate (**27**)[44] were synthesised by known literature procedures.

General procedures

General procedure for the deacetylation reactions with Novozyme N435. Acetylated saccharide (100 mg), MTBE (10 mL) and *n*-BuOH (3.5 equiv.) were mixed at 45 °C after which N435 (100 mg) was added. The reaction was followed by TLC and upon completion the reaction mixture was filtered, immobilised enzymes were rinsed with DCM (~50 mL) and concentrated *in vacuo*. The crude mixture was purified by silica gel column chromatography (PE : EtOAc 2 : 1 → 1 : 4) unless specified otherwise.

1,2,3-Tri-*O*-acetyl-α-D-glucopyranoside (2). Prepared according to general procedure with **1** and N435 (50 mg) in 23 h gave white solid, yield 64 mg (82%); TLC – PE : EtOAc 1 : 2, R_f = 0.19; mp 130–132 °C (from DCM); $[\alpha]_D^{20}$ +116.3 ($CHCl_3$, c 0.10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.28 (d, J = 3.7 Hz, 1H, H-1), 5.31 (dd, J = 9.0, 10.0 Hz, 1H, H-3), 5.00 (dd, J = 3.7, 10.2 Hz, 1H, H-2), 3.77–3.91 (m, 4H, H-4,5,6a,6b), 3.09 (d, J = 4.4 Hz, 1H, OH-4), 2.26 (t, J = 6.2 Hz, 1H, OH-6), 2.16 (s, 3H), 2.12 (s, 3H), 2.02 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.9, 170.1, 169.4, 89.5 (C-1), 74.0 (C-5), 72.9 (C-3), 69.4 (C-2), 69.1 (C-4), 61.7 (C-6), 21.1, 21.0, 20.7; HRMS (AJS-ESI): calculated for $C_{12}H_{18}O_9Na$ $[M + Na]^+$ 329.0843, found 329.0843.

2,3,4,6-Tetra-*O*-acetyl-D-glucopyranose (2a). Colourless syrup, α : β 3 : 1, side-product from the general procedure with **1**, together with **2b**; TLC – PE : EtOAc 1 : 2, R_f = 0.53; NMR data for α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.54 (t, J = 9.8 Hz, 1H, H-3), 5.47 (d, J = 2.7 Hz, 1H, H-1), 5.05–5.11 (m, 1H, H-4), 4.91 (dd, J = 3.6, 10.2 Hz, 1H, H-2) 4.11–4.30 (m, 3H, H-5,6a,6b), 3.15 (br.s, 1H, OH), 2.10 (s, 3H), 2.08 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H).

NMR data for β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.26 (t, J = 9.6 Hz, 1H, H-3), 5.05–5.11 (m, 1H, H-4), 4.88 (m, 1H, H-2), 4.74 (br.t, 1H, H-1), 3.76 (ddd, J = 2.3, 4.8, 10.1 Hz, H-5), 2.09 (s, 6H), 20.3 (s, 3H), 20.2 (s, 3H); NMR matches with literature data.[44,45]

1,2,3,6-Tetra-*O*-acetyl-α-D-glucopyranoside (2b). Colourless syrup, side-product from general procedure with **1**, together with **2a**; TLC – PE : EtOAc 1 : 2, R_f = 0.53; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.29 (d, J = 3.7 Hz, 1H, H-1), 5.33 (t, J = 9.8 Hz, 1H, H-3), 5.04 (dd, J = 3.7, 10.3 Hz, 1H, H-2), 4.53 (dd, J = 3.6, 12.5 Hz, 1H, H-6a/6b), 4.24 (dd, J = 2.2, 12.5 Hz, 1H, H-6a/6b), 3.95 (td, J = 2.8, 10.1 Hz, 1H, H-5), 3.59 (dt, J = 4.9, 9.7 Hz, 1H, H-4), 3.06 (d, J = 5.0 Hz, 1H, OH), 2.17 (s, 3H), 2.14 (s, 3H), 2.11 (s, 3H), 2.02 (s, 3H); NMR matches with **27**.

2,3-Di-*O*-acetyl-D-glucopyranose (2c). Colourless syrup, α : β 63 : 37, side-product from general procedure with **1**, yield 4 mg (6%); TLC – PE : EtOAc 1 : 2, R_f = 0.05; NMR data for α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.41 (br.s, 1H, H-1), 5.36 (t, J = 9.7 Hz, 1H, H-3), 5.12 (s, 1H, OH-1), 4.77–4.83 (m, 1H, H-2), 3.91–4.10 (m, 2H, H-5,6a/6b), 3.72–3.90 (m, 1H, H-6a/6b), 3.58 (ap.t, J = 9.3 Hz, 1H, H-4), 2.10 (s, 3H), 2.09 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 170.9, 90.1 (C-1), 72.9 (C-3), 71.6 (C-2), 71.5 (C-5), 69.9 (C-4), 62.2 (C-6), 21.0, 20.9.

NMR data for β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (br.s, 1H, OH-1), 5.08 (t, J = 9.3 Hz, 1H, H-3), 4.77–4.83 (m, 2H, H-1,2), 3.91–4.10 (m, 1H,H-6a/6b), 3.72–3.90 (m, 2H, H-4,6a/6b), 3.50 (ap.d, J = 9.0 Hz, 1H, H-5), 2.10 (s, 3H), 2.08 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.6, 171.2, 95.2 (C-1), 76.1 (C-5), 75.5 (C-3), 73.4 (C-2), 68.7 (C-4), 61.4 (C-6), 21.1, 21.0; HRMS (AJS-ESI): calculated for $C_{10}H_{16}O_8Na$ $[M + Na]^+$ 287.0737, found 287.0735.

2,3,4,6-Tetra-*O*-acetyl-D-galactopyranose (4). Prepared according to general procedure with **5** in 5 h gave colourless syrup, α : β 70 : 30, yield 81 mg (90%); TLC – PE : EtOAc 1 : 2, R_f = 0.53; NMR data for α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.51 (t, J = 3.5 Hz, 1H, H-1), 5.46 (dd, J = 1.0, 3.3 Hz, 1H, H-4), 5.38–5.43 (m, 1H, H-3), 5.14 (ddd, J = 0.8, 3.5, 10.8 Hz, 1H, H-2), 4.46 (td, J = 0.8, 6.6 Hz, 1H, H-5), 4.04–4.16 (m, 2H, H-6a,6b), 3.44 (d, J = 3.2 Hz, 1H OH-1), 2.14 (s, 3H), 2.09 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H).

NMR data for β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.43 (m, 1H, H-4), 5.05–5.08 (m, 2H, H-2,3), 4.66–4.72 (m, 1H, H-1), 4.04–4.16 (m, 2H, H-6a,6b), 3.95 (td, J = 1.0, 6.6 Hz, 1H, H-5), 3.84 (d, J = 8.9 Hz, 1H, OH-1), 2.15 (s, 3H), 2.09 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H); NMR matches with literature data.[28]

2,3,4-Tri-*O*-acetyl-D-galactopyranoside (5a). Slightly yellow syrup, α : β 68 : 32, dominant side-product from general procedure with **5**, yield of mixture 7 mg (8%); TLC – PE : EtOAc 1 : 2, R_f = 0.15; NMR data for α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.52 (d, J = 3.6 Hz, 1H, H-1), 5.44 (dd, J = 1.0, 3.6 Hz, 1H, H-4), 5.42 (dd, J = 3.5, 10.4 Hz, 1H, H-3), 5.17 (dd, J = 3.6, 10.3 Hz, 1H, H-2), 4.34 (t, J = 6.4 Hz, 1H, H-5), 3.66 (dd, J = 7.3, 11.6 Hz, 1H, H-6a/6b), 3.54 (dd, J = 5.6, 11.6 Hz, 1H, H-6a/6b), 2.16 (s, 3H), 2.10 (s, 3H), 2.00 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.2, 170.7, 170.3, 90.7 (C-1), 69.2 (C-4), 69.0 (C-5), 68.7 (C-2), 67.6 (C-3), 61.3 (C-6), 21.0, 20.8, 20.8.

NMR data for β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38 (dd, J = 0.8, 3.0 Hz, 1H, H-4), 5.10 (m, J = 6.5 Hz, 2H, H-2,3), 4.72 (d, J = 7.4 Hz, 1H, H-1), 3.81 (t, J = 6.3 Hz, 1H, H-5), 3.74 (dd, J = 7.1, 11.5 Hz, 1H, H-6a/6b), 3.55 (m, 1H, H-6a/6b), 2.17 (s, 3H), 2.10 (s, 3H), 2.01 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.3, 171.1, 170.2, 96.1 (C-1), 74.0 (C-5), 71.4 (C-2/3), 70.6 (C-2/3), 69.0 (C-4), 61.0 (C-6), 21.0, 20.8, 20.7; HRMS (AJS-ESI): calculated for $C_{12}H_{18}O_9Na$ $[M + Na]^+$ 329.0843, found 329.0838.

2-Acetamido-1,3-di-*O*-acetyl-2-deoxy-α-D-glucopyranoside (7). Prepared according to general procedure with **6** in 7 days gave white solid from column chromatography (DCM : MeOH 100 : 0 → 90 : 10), yield 53 mg (68%); TLC – DCM : MeOH 90 : 10, R_f = 0.25; mp 83–87 °C (from DCM); $[\alpha]_D^{20}$ +86.0 ($CHCl_3$, c 0.09); ^{1}H NMR (400 MHz, CD_3OD) δ 8.00 (d, J = 9.2 Hz, 1H, NH), 6.08 (d, J = 3.6 Hz, 1H, H-1), 5.17 (dd, J = 8.9, 11.0 Hz, 1H, H-3), 4.27–4.34 (m, 1H, H-2), 3.66–3.87 (m, 4H, H-4,5,6a,6b), 2.18 (s, 3H), 2.07 (s, 3H), 1.91 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.8, 172.6, 171.2, 92.1 (C-1), 76.0 (C-5), 74.5 (C-3), 69.2 (C-4), 62.0 (C-6), 52.3 (C-2), 22.3, 20.9, 20.9.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.14 (d, J = 3.6 Hz, 1H, H-1), 5.93 (d, J = 8.8 Hz, 1H, NH), 5.12 (dd, J = 9.3, 10.9 Hz, 1H, H-3), 4.32 (ddd, J = 3.6, 8.8, 11.0 Hz, 1H, H-2), 3.91 (t, J = 9.6 Hz, 1H, H-4), 3.83–3.86 (m, 2H, H-6a,6b), 3.77 (br.s, 1H, OH), 3.72 (td, J = 3.2, 9.8 Hz, 1H, H-5), 2.95 (br.s, 1H, OH), 2.16 (s, 3H), 2.12 (s, 3H), 1.93 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.5, 170.8, 169.5, 91.1 (C-1), 73.8 (C-5), 73.4 (C-3), 67.9 (C-4), 61.5 (C-6), 51.5 (C-2), 23.2, 21.1, 21.1; HRMS (AJS-ESI): calculated for $C_{12}H_{19}NO_8Na$ $[M + Na]^+$ 328.1003, found 328.0998; NMR matches with literature data.[46]

L-Fucose (9). Prepared according to general procedure with **8** in 10 days gave white solid, yield 12 mg (24%); NMR matches with the commercial one.

1,2-Di-*O*-acetyl-6-deoxy-L-galactopyranoside (10). Prepared according to general procedure with **8** in 10 days gave slightly yellowish white solid, α : β 61 : 39; yield 12 mg (16%); TLC – PE : EtOAc 1 : 2, R_f = 0.15; mp 105–107 °C (from DCM); NMR data for α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.25 (d, J = 3.8 Hz, 1H, H-1), 5.15 (dd, J = 3.8, 10.3 Hz, 1H, H-2), 4.11 (q, J = 6.6 Hz, 1H, H-5), 4.00 (dd, J = 3.3, 10.2 Hz, 1H, H-3), 3.87 (d, J = 2.6 Hz, 1H, H-4), 2.12 (s, 3H), 2.08 (s, 3H), 1.31 (d, J = 6.6 Hz, 3H, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.3, 169.3, 90.1 (C-1), 72.0 (C-4), 70.0 (C-2), 68.7 (C-3), 68.5 (C-5), 21.0, 20.9, 16.2.

NMR data for β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.60 (d, J = 8.3 Hz, 1H, H-1), 5.04 (dd, J = 8.4, 9.6 Hz, 1H, H-2), 3.75–3.82 (m, 2H, H-4,5), 3.71 (dd, J = 3.4, 9.7 Hz, 1H, H-3), 2.11 (s, 3H), 2.10 (s, 3H), 1.36 (d, J = 6.5 Hz, 1H, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.6, 169.5, 91.9 (C-1), 73.1 (C-3), 72.2 (C-2) 71.6 (C-4/5), 71.5 (C-4/5), 21.0, 20.9, 16.1; HRMS (AJS-ESI): calculated for $C_{10}H_{16}O_7Na$ $[M + Na]^+$ 271.0788, found 271.0783.

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-1,2,3-tri-*O*-acetyl-α-D-glucopyranoside (12). Prepared according to general procedure with **11** in 48 h gave white solid, yield 59 mg (63%); TLC – PE : EtOAc 1 : 2, R_f = 0.29; mp 91–93 °C (from DCM); $[\alpha]_D^{20}$ +45.0 (CH_2Cl_2, c 0.10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.26 (d, J = 3.7 Hz, 1H, H-1), 5.47 (t, J = 9.8 Hz, 1H, H-3), 5.35 (dd, J = 0.8, 3.4 Hz, 1H, H′-4), 5.12 (dd, J = 7.9, 10.4 Hz, 1H, H′-2), 5.00 (dd, J = 3.4, 10.4 Hz, 1H, H′-3), 4.96 (dd, J = 3.7, 10.3 Hz, 1H, H-2), 4.62 (d, J = 7.9 Hz, 1H, H′-1), 4.14 (dd, J = 6.5, 11.1 Hz, 1H, H′-6a/6b), 4.08 (dd, J = 7.1, 11.1 Hz, 1H, H′-6a/6b), 3.97 (t, J = 9.7 Hz, 1H, H-4), 3.91 (t, J = 6.8 Hz, 1H, H′-5), 3.71–3.88 (m, 3H, H-5,6a,6b), 2.16 (s, 3H), 2.15 (s, 3H), 2.05 (s, 3H), 2.05 (s, 6H), 2.01 (s, 3H), 1.96 (s, 3H), 1.93 (dd, J = 4.3, 9.4 Hz, 1H, OH-6); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 170.3, 170.2, 170.1, 169.9, 169.3, 169.3, 101.2 (C′-1), 89.3 (C-1), 74.6 (C-4), 73.1 (C-5), 71.1 (C′-3), 70.8 (C′-5), 69.8 (C-3), 69.7 (C-2), 69.4 (C′-2), 66.9 (C′-4), 61.0 (C′-6), 60.0 (C-6), 21.1, 21.0, 20.9, 20.8, 20.7; HRMS (AJS-ESI): calculated for $C_{26}H_{36}O_{18}Na$ $[M + Na]^+$ 659.1794, found 659.1784; NMR matches with literature data.[9]

Phenyl 2,3-di-*O*-acetyl-1-thio-β-D-glucopyranoside (14). Prepared according to general procedure with **13** in 3 days gave white solid, yield 69 mg (85%); with N435 (200 mg) and reflux in 2 h gave white solid, yield 74 mg (91%); TLC – PE : EtOAc 1 : 1, R_f = 0.12; mp 103–105 °C (from DCM); $[\alpha]_D^{20}$ −29.9 ($CHCl_3$, c 0.10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42–7.48 (m, 2H), 7.28–7.35 (m, 3H), 5.06 (t, J = 9.3 Hz, 1H, H-3), 4.93 (t, J = 9.6 Hz, 1H, H-2), 4.75 (d, J = 10.0 Hz, 1H, H-1), 3.93 (dd, J = 3.2, 12.1 Hz, 1H, H-6a/6b), 3.81 (dd, J = 4.6, 12.1 Hz, 1H, H-6a/6b), 3.72 (t, J = 9.5 Hz, 1H, H-4), 3.45 (ddd, J = 3.4, 4.6, 9.7 Hz, 1H, H-5), 3.10 (br.s, 1H, OH-4), 2.30 (br.s, 1H, OH-6), 2.08 (s, 3H), 2.07 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.6, 169.7, 132.6 (2× C), 132.2, 129.2 (2× C), 128.4, 85.9 (C-1), 79.8 (C-5), 77.2 (C-3), 70.2 (C-2), 69.2 (C-4), 62.3 (C-6), 21.0, 20.9; HRMS (AJS-ESI): calculated for $C_{16}H_{20}O_7SNa$ $[M + Na]^+$ 379.0822, found 379.0825; NMR matches with literature data.[47]

Phenyl 2,3,6-tri-*O*-acetyl-1-thio-β-D-glucopyranoside (14a). Dominant side-product from general procedure with **13** in 3 days gave colourless syrup, mixture yield 12 mg (13%); with N435 (200 mg) and reflux in 2 h gave white solid, yield 6 mg (7%); TLC – PE : EtOAc 1 : 1, R_f = 0.33; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46–7.52 (m, 2H), 7.28–7.34 (m, 3H), 5.03–5.09 (m, 1H, H-3), 4.93 (t, J = 9.6 Hz, 1H, H-2), 4.69 (d, J = 10.0 Hz, 1H, H-1), 4.45 (dd, J = 4.0, 12.2 Hz, 1H, H-6a/6b), 4.37 (dd, J = 1.2, 12.1 Hz, 1H, H-6a/6b), 3.53–3.58 (m, 2H, H-4,5), 2.93 (br.s, 1H, OH-4), 2.12 (s, 3H), 2.09 (s, 3H), 2.08 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.7, 171.5, 169.6, 133.0 (2× C), 132.2, 129.0 (2× C), 128.4, 86.0 (C-1), 78.2 (C-5), 76.7 (C-3), 69.9 (C-2),

68.9 (C-4), 63.1 (C-6), 21.0 (2× C), 20.9; HRMS (AJS-ESI): calculated for $C_{18}H_{22}O_8SNa$ $[M + Na]^+$ 421.0928, found 421.0929.

Methyl 2,3-di-*O*-acetyl-α-D-glucopyranoside (16). Prepared according to general procedure with **15** gave clear syrup in 7 days, yield 70 mg (91%); with N435 (200 mg) and reflux in 48 h gave clear syrup, yield 64 mg (83%); TLC – PE : EtOAc 1 : 2, R_f = 0.13; $[\alpha]_D^{20}$ +132.5 ($CHCl_3$, c 0.13); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.27–5.33 (m, 1H, H-3), 4.90 (d, J = 3.6 Hz, 1H, H-1), 4.81 (dd, J = 3.6, 10.2 Hz, 1H, H-2), 3.82–3.92 (m, 2H, H-6a,6b), 3.67–3.74 (m, 2H, H-4,5), 3.39 (s, 3H, CH_3), 3.21 (ap.d, J = 2.5 Hz, 1H, OH-4), 2.37 (br.t, J = 5.5 Hz, 1H, OH-6), 2.09 (s, 3H), 2.07 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 170.5, 96.9 (C-1), 73.5 (C-3), 71.3 (C-4), 70.9 (C-2), 69.9 (C-5), 62.1 (C-6), 55.4 (CH_3), 21.0, 20.9; HRMS (AJS-ESI): calculated for $C_{11}H_{18}O_8Na$ $[M + Na]^+$ 301.0894, found 301.0888; NMR matches with literature data.[48]

Methyl 2,3,6-tri-*O*-acetyl-α-D-glucopyranoside (17). Prepared according to general procedure with **15** gave yellow syrup in 7 days, yield 7 mg (7%); with N435 (200 mg) and reflux in 48 h gave yellow syrup, yield 11 mg (12%); TLC – PE : EtOAc 1 : 2, R_f = 0.48; $[\alpha]_D^{20}$ +118.8 ($CHCl_3$, c 0.03); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.29 (dd, J = 9.4, 9.9 Hz, 1H, H-3), 4.90 (d, J = 3.7 Hz, 1H, H-1), 4.86 (dd, J = 3.7, 10.1 Hz, 1H, H-2), 4.48 (dd, J = 4.3, 12.2 Hz, 1H, H-6a/6b), 4.30 (dd, J = 2.3, 12.2 Hz, 1H, H-6a/6b), 3.82 (ddd, J = 2.3, 4.3, 10.0 Hz, 1H, H-5), 3.57 (dt, J = 5.1, 14.5 Hz, 1H, H-4), 3.40 (s, 3H, CH_3), 2.96 (d, J = 5.2 Hz, 1H, OH-4), 2.13 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.7, 171.5, 170.3, 96.9 (C-1), 72.9 (C-3), 70.6 (C-2), 69.7 (C-5), 69.4 (C-4), 62.8 (C-6), 55.3 (CH_3), 20.9, 20.8, 20.8; HRMS (AJS-ESI): calculated for $C_{13}H_{20}O_9Na$ $[M + Na]^+$ 343.1000, found 343.0995.

Phenyl 2,3,4-tri-*O*-acetyl-1-thio-β-D-galactopyranoside (19). Prepared according to general procedure with **18** in 10 days gave white solid, yield 64 mg (70%); with N435 (200 mg) and reflux in 48 h gave white solid, yield 77 mg (85%); with **18** (2.49 mmol, 1.098 g), N435 (2.2 g) and reflux in 48 h gave white solid, yield 892 mg (90%); TLC – PE : EtOAc 1 : 2, R_f = 0.54; mp 93–96 °C (from DCM); $[\alpha]_D^{20}$ +19.2 ($CHCl_3$, c 0.10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.52 (m, 2H), 7.28–7.35 (m, 3H), 5.39 (d, J = 3.2 Hz, 1H, H-4), 5.26 (t, J = 9.9 Hz, 1H, H-2), 5.08 (dd, J = 3.3, 9.9 Hz, 1H, H-3), 4.75 (d, J = 10.0 Hz, 1H, H-1), 3.78–3.85 (m, 1H, H-5), 3.73–3.78 (m, 1H, H-6a/6b), 3.51 (ddd, J = 5.6, 7.5, 11.2 Hz, 1H, H-6a/6b), 2.26 (dd, J = 7.4, 6.1 Hz, 1H, OH-6), 2.13 (s, 3H), 2.09 (s, 3H), 1.99 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.2, 170.1, 169.6, 132.5, 132.4 (2× C), 129.1 (2× C), 128.3, 86.6 (C-1), 77.5 (C-5), 72.1 (C-3), 68.2 (C-4), 67.7 (C-2), 61.0 (C-6), 21.0, 20.8, 20.7; HRMS (AJS-ESI): calculated for $C_{18}H_{22}O_8SNa$ $[M + Na]^+$ 421.0928 found 421.0930; NMR matches with literature data.[49]

Phenyl 2,3-di-*O*-acetyl-1-thio-β-D-galactopyranoside (19a). Dominant side-product from general procedure with **18** in 10 days gave yellow syrup, mixture yield 22 mg (27%); with N435 (200 mg) and reflux in 48 h gave yellow syrup, mixture yield 4 mg (5%); TLC – PE : EtOAc 1 : 2, R_f = 0.18; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.51 (m, 2H), 7.28–7.34 (m, 3H), 5.32 (t, J = 9.9 Hz, 1H, H-2), 4.98 (dd, J = 3.1, 9.8 Hz, 1H, H-3), 4.74 (d, J = 10.0 Hz, 1H, H-1), 4.19 (d, J = 2.8 Hz, 1H, H-4), 3.94 (dd, J = 5.8, 11.9 Hz, 1H, H-6a/6b), 3.86 (dd, J = 4.5, 12.0 Hz, 1H, H-6a/6b), 3.64 (ap.t, J = 5.1 Hz, 1H, H-5), 3.03 (br.s, 1H, OH), 2.67 (br.s, 1H, OH), 2.08 (s, 3H), 2.08 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 169.8, 132.7, 132.3 (2× C), 129.2 (2× C), 128.2, 86.5 (C-1), 78.0 (C-5), 74.6 (C-3), 68.5 (C-4), 67.8 (C-2), 62.7 (C-6), 21.0, 21.0; HRMS (AJS-ESI): calculated for $C_{16}H_{20}O_7SNa$ $[M + Na]^+$ 379.0822, found 379.0814.

Phenyl 2-*O*-acetyl-6-deoxy-1-thio-β-L-galactopyranoside (21). Prepared according to general procedure with **20** gave white solid in 10 days, yield 60 mg (76%); with N435 (200 mg) and reflux in 3 days gave white solid, yield 61 mg (78%); TLC – PE : EtOAc 1 : 2, R_f = 0.25; mp 142–144 °C (from DCM); $[\alpha]_D^{20}$ – 18.3 ($CHCl_3$, c 0.11); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.53 (m, 2H), 7.26–7.34 (m, 3H), 4.95 (t, J = 9.7 Hz, 1H, H-2), 4.61 (d, J = 10.0 Hz, 1H, H-1), 3.78 (dd, J = 3.3, 6.1 Hz, 1H, H-4), 3.62–3.71 (m, 2H, H-3,5), 3.12 (d, J = 8.4 Hz, 1H, OH-3), 2.55 (d, J = 6.4 Hz, 1H, OH-4), 2.15 (s, 3H), 1.37 (d, J = 6.5 Hz, 3H, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.3, 133.1, 132.4 (2× C), 129.0 (2× C), 128.0, 86.1 (C-1), 74.8 (C-5), 74.1 (C-3), 72.1 (C-4), 72.4 (C-2), 21.2, 16.7 (CH_3); HRMS (AJS-ESI): calculated for $C_{14}H_{18}O_5SNa$ $[M + Na]^+$ 321.0767, found 321.0760.

Phenyl 2-acetamido-3-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside (23). Prepared according to general procedure with **22** (50 mg), N435 (50 mg) and MTBE (10 mL) in 24 h gave white solid from column chromatography (DCM : EtOAc 1 : 2 → 0 : 100), yield 18 mg (44%); TLC – EtOAc 100%, R_f = 0.15; mp 78–81 °C (from DCM); $[\alpha]_D^{20}$ –52.6 ($CHCl_3$, c 0.11); ^{1}H NMR (400 MHz, CD_3OD) δ 7.47–7.54 (m, 2H), 7.24–7.33 (m, 3H), 5.02 (t, J = 9.6 Hz, 1H, H-3), 4.89 (d, J = 10.5 Hz, 1H, H-1), 3.83–3.91 (m, 2H, H-2,6a/6b), 3.71 (dd, J = 5.5, 12.2 Hz, 1H, H-6a/6b), 3.54 (t, J = 9.5 Hz, 1H, H-4), 3.40 (ddd, J = 2.2, 5.5, 9.8 Hz, 1H, H-5), 2.03 (s, 3H), 1.91 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.1, 172.4, 135.0, 132.7 (2× C), 130.0 (2× C), 128.5, 87.6 (C-1), 82.0 (C-5), 78.3 (C-3), 69.4(C-4), 62.5 (C-6), 54.4 (C-2), 22.8, 20.8; HRMS (AJS-ESI): calculated for $C_{16}H_{21}NO_6SNa$ $[M + Na]^+$ 378.0982, found 378.0973.

Phenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside (24). Prepared according to general procedure with **22** (50 mg), N435 (50 mg) and MTBE (10 mL) in 24 h gave white solid, yield 21 mg (47%); TLC – EtOAc 100%, R_f = 0.28; ^{1}H NMR (400 MHz, CD_3OD) δ 7.48–7.55 (m, 2H), 7.26–7.35 (m, 3H), 5.23 (t, J = 9.7 Hz, 1H, H-3), 4.97 (t, J = 9.5 Hz, 1H, H-4), 4.96 (d, J = 10.5 Hz, 1H, H-1), 3.93 (t, J = 10.3 Hz, 1H, H-2), 3.54–3.70 (m, 3H, H-5,6a,6b), 2.00 (s, 3H), 1.97 (s, 3H), 1.92 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.2, 171.9, 171.4, 134.7, 133.0 (2× C), 130.1 (2× C), 128.8, 87.6 (C-1), 79.8 (C-5), 75.6 (C-3), 70.3 (C-4), 62.1 (C-6), 54.4 (C-2), 22.8, 20.7, 20.6.

Phenyl 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside (25). Product of acyl migration with **24** in CD_3OD in 4 days (85% conversion); ^{1}H NMR (400 MHz, CD_3OD) δ 7.46–7.54 (m, 2H), 7.25–7.35 (m, 3H), 5.02 (dd, J = 9.1, 9.9 Hz, 1H, H-3), 4.87 (d, J = 10.6 Hz, 1H, H-1), 4.42 (dd, J = 2.0, 11.9 Hz, 1H, H-6a/6b), 4.23 (dd, J = 6.2, 11.9 Hz, 1H, H-6a/6b), 3.86 (t, J = 10.3 Hz, 1H, H-2), 3.59 (ddd, J = 2.0, 6.3, 9.9 Hz, 1H, H-5), 3.51 (t, J = 9.4 Hz, 1H, H-4), 2.05 (s, 3H), 2.04 (s, 3H), 1.92

(s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.1, 172.5, 172.3, 134.5, 133.2 (2× C), 129.9 (2× C), 128.8, 87.2 (C-1), 79.9 (C-5), 78.0 (C-3), 69.7 (C-4), 64.6 (C-6), 54.3, (C-2), 22.8, 20.8, 20.8; HRMS (AJS-ESI): calculated for $C_{18}H_{23}NO_7SNa$ $[M + Na]^+$ 420.1087, found 420.1078.

1,2,3,6-Tetra-*O*-acetyl-α-D-glucopyranoside (26). Procedure from literature[50] modified for **2**: to a solution of **2** (300 mg, 0.98 mmol) and pyridine (2 equiv., 158 μL) in dry DCM (10 mL) under argon at −40 °C acetyl chloride (1.2 equiv., 84 μL) was added dropwise. The reaction was stirred at −40 °C for 3 h, warmed up to room temperature and further stirred for 1 h. Upon completion, the reaction mixture was transferred into separation funnel, washed with aq. sat. $NaHCO_3$, 1 M HCl, brine and dried using phase separator. Crude mixture was purified by flash silica gel column chromatography (PE : EtOAc 2 : 1 → 1 : 2) resulting in white solid, yield 281 mg (82%). The reaction was followed by TLC – PE : EtOAc 1 : 2, R_f = 0.45; mp 87–90 °C (from DCM); $[\alpha]_D^{20}$ +59.2 ($CHCl_3$, c 0.11); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.28 (d, J = 3.7 Hz, 1H, H-1), 5.32 (t, J = 9.8 Hz, 1H, H-3), 5.02 (dd, J = 3.7, 10.3 Hz, 1H, H-2), 4.51 (dd, J = 3.7, 12.4 Hz, 1H,H-6a/6b), 4.24 (dd, J = 2.2, 12.4 Hz, 1H, H-6a/6b), 3.94 (qd, J = 2.0, 10.1 Hz, 1H, H-5), 3.59 (dt, J = 5.1, 9.7 Hz, 1H, H-4), 3.14 (d, J = 5.2 Hz, 1H, OH-4), 2.16 (s, 3H), 2.12 (s, 3H), 2.10 (s, 3H), 2.01 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 171.6, 170.0, 169.1, 89.5 (C-1), 72.6(C-5), 72.2 (C-3), 69.2 (C-2), 68.7 (C-4), 62.6 (C-6), 21.0, 21.0, 21.0, 20.6; HRMS (AJS-ESI): calculated for $C_{14}H_{20}O_{10}Na$ $[M + Na]^+$ 371.0949, found 371.0942; NMR matches with literature data.[33]

Phenyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 → 6)-2,3,4-tri-*O*-acetyl-1-thio-β-D-galactopyranoside (28). Procedure from literature:[51] **27** (2 mmol, 985.4 mg, 2 equiv.), **19** (1 mmol, 398.4 mg) and activated 4 Å molecular sieves (1 g) in dry DCM (10 mL) was stirred at −20 °C under argon for 1 h and then $BF_3{\cdot}Et_2O$ (2 equiv., 247 μL) was added dropwise to the mixture, which was stirred for another 60 min. Solid was filtered and rinsed with DCM (~75 mL). The combined filtrate was washed with sat. aq. $NaHCO_3$ and brine. The organic phases were separated, dried using phase separator and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (PE : EtOAc 2 : 1 → 47 : 53) and gave white solid, yield 394 mg (54%). The reaction was followed by TLC – PE : EtOAc 1 : 1, R_f = 0.35; mp 72–75 °C (from DCM); $[\alpha]_D^{20}$ −10.9 ($CHCl_3$, c 0.13); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44–7.52 (m, 2H), 7.28–7.36 (m, 3H), 5.40 (d, J = 2.8 Hz, 1H, H-4), 5.36 (d, J = 2.9 Hz, 1H, H′-4), 5.23 (t, J = 10.0 Hz, 1H, H-2), 5.17 (dd, J = 8.2, 10.3 Hz, 1H, H′-2), 5.03 (dd, J = 3.2, 9.9 Hz, 1H, H-3), 4.96 (dd, J = 3.3, 10.4 Hz, 1H, H′-3), 4.74 (d, J = 10.0 Hz, 1H, H-1), 4.49 (d, J = 8.0 Hz, 1H, H′-1), 4.15 (dd, J = 6.7, 11.3 Hz, 1H, H′-6a/6b), 4.10 (dd, J = 6.9, 11.4 Hz, 1H, H′-6a/6b), 3.93 (t, J = 5.7 Hz, 1H, H-5), 3.87 (t, J = 6.5 Hz, 1H, H′-5), 3.81 (dd, J = 4.9, 11.0 Hz, 1H, H-6a/6b), 3.76 (dd, J = 7.4, 10.7 Hz, 1H, H-6a/6b), 2.15 (s, 3H), 2.12 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 1.97 (s, 3H), 1.96 (s, 3H), 1.96 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 170.4, 170.3, 170.2, 170.1, 169.6, 169.6, 133.2, 131.9 (2× C), 129.3 (2× C), 128.1, 100.9 (C′-1), 87.0 (C-1), 76.2 (C-5), 72.2 (C-3), 71.0 (C′-3), 70.9 (C′-5), 68.6 (C′-2), 67.9 (C-4), 67.4 (C-2,C-6), 67.1 (C′-4), 61.3 (C′-6), 21.0, 20.8 (3xC), 20.7 (2× C); HRMS (AJS-ESI): calculated for $C_{32}H_{40}O_{17}SNa$ $[M + Na]^+$ 751.1878, found 751.1864; NMR matches with literature data.[52]

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 6)-2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-1,2,3,6-tetra-*O*-acetyl-α-D-glucopyranoside (29). Procedure from literature:[53] thiophenyl donor **28** (0.395 mmol, 287.9 mg, 1.32 equiv.), acceptor **26** (0.3 mmol, 104.5 mg) and activated 4 Å molecular sieves in dry DCM (3 mL) was stirred at room temperature under argon for 1 h and then cooled to −20 °C. NIS (101.2 mg, 1.5 equiv.) and TfOH (10.6 μL, 0.4 equiv.) were added. After being stirred at −20 °C for 30 min, the reaction was quenched with a few drops of triethylamine and warmed to room temperature. The resulting mixture was filtered, washed with sat. aq. NaHCO3, 5% aq. $Na_2S_2O_3$ solution, water, brine, dried using phase separator and concentrated *in vacuo*. Purification by column chromatography on a silica gel column (PE : EtOAc 3 : 2 → 1 : 4) afforded white solid 140 mg (45%), which was further purified by recrystallisation in Et_2O to afford white solid, yield 101 mg (35%). The reaction was followed by TLC – PE : EtOAc 1 : 1, R_f = 0.12; mp 111–114 °C (from Et_2O); $[\alpha]_D^{20}$ +11.5 ($CHCl_3$, c 0.10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.26 (d, J = 3.7 Hz, 1H, H-1), 5.46 (t, J = 9.8 Hz, 1H, H-3), 5.39 (d, J = 2.9 Hz, 1H, H″-4), 5.37 (d, J = 3.4 Hz, 1H, H′-4), 5.16 (dd, J = 8.0, 10.4 Hz, 1H, H″-2), 5.10 (dd, J = 7.8, 10.3 Hz, 1H, H′-2), 5.07 (dd, J = 3.7, 10.4 Hz, 1H, H-2), 5.04 (dd, J = 3.4, 10.6 Hz, 1H, H″-3), 4.95 (dd, J = 3.4, 10.4 Hz, 1H, H′-3), 4.58 (d, J = 7.9 Hz, 1H, H″-1), 4.47 (d, J = 7.9 Hz, 1H, H′-1), 4.45 (dd, J = 1.5, 12.2 Hz, 1H, H-6a/6b), 4.19 (dd, J = 6.5, 11.3 Hz, 1H, H″-6a/6b), 4.06–4.12 (m, 2H, H-6a/6b, H″-6a/6b), 4.01 (ddd, J = 1.6, 4.6, 10.0 Hz, 1H, H-5), 3.93 (t, J = 6.6 Hz, 1H, H″-5), 3.83 (t, J = 9.6 Hz, 1H, H-4), 3.74–3.80 (m, 3H, H′-5,6a,6b), 2.18 (s, 3H), 2.16 (s, 3H), 2.14 (s, 3H), 2.13 (s, 3H), 2.07 (s, 6H), 2.05 (s, 6H), 2.00 (s, 3H), 1.97 (s, 3H), 1.95 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5 (2× C), 170.3, 170.2, 170.2, 170.1, 170.0, 169.5, 169.4, 169.3, 169.0, 101.2 (C′-1), 100.3 (C″-1), 89.2 (C-1), 75.7 (C-4), 72.5 (C′-5), 71.2 (C′-3), 70.9 (C-5, C″-3, C″-5), 70.1 (C-3), 69.4 (C-2), 69.3 (C′-2), 68.6 (C″-2), 67.2 (C′-4), 67.1 (C″-4), 65.5 (C′-6), 61.7 (C-6), 61.3 (C″-6), 21.1, 21.0 (2× C), 20.8, 20.8 (4xC), 20.7, 20.6 (2× C), HRMS (AJS-ESI): calculated for $C_{40}H_{54}O_{27}Na$ $[M + Na]^+$ 989.2745, found 989.2759.

6′-Galactosyllactose or β-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose (30). Prepared using Zemplén transesterification:[43,54,55] **29** (81.5 mg) was dissolved in anhydrous MeOH (1.8 mL) equipped with $CaCl_2$ tube. 0.3 mL of 0.01 M NaOMe solution was added, and the reaction was vigorously stirred at room temperature. After completion (1 h) water (1 mL), Amberlite 120 H+ (1.5 g) were added and the reaction mixture was stirred for 60 min. The reaction mixture was filtered and washed with MeOH/H_2O 2 : 1 mixture. The filtrate was concentrated and MeOH/EtOAc mixture is added to crash out the product. MeOH traces is codistilled with EtOAc. Resulting in pinkish solid, yield 39.1 mg (92%), which was unsuccessfully tried to crystallise. It was then purified with column chromatography – normal phase MeCN : H_2O 4 : 1 → 2 : 1. Resulting in slightly beige solid, yield 37 mg

(86%), α : β 38 : 62. The reaction was followed by TLC – PE : EtOAc 2 : 1 and MeCN : H_2O 2 : 1 R_f (for MeCN : H_2O) = 0.38; mp 165–168 °C (sinters), 185–188 °C (from H_2O); ^{1}H NMR (400 MHz, D_2O) δ 5.23 (d, J = 3.8 Hz, 1H, αH-1), 4.68 (d, J = 8.0 Hz, βH-1), 4.43–4.53 (m, 4H, βH′-1, βH″-1, αH′-1, αH″-1), 4.03–4.12 (m, 2H), 3.52–4.00 (m, 33H), 3.28–3.33 (m, 1H, βH-2); ^{13}C NMR (101 MHz, D_2O, EtOH used as internal standard) δ 104.0, 103.8 & 103.8 (4× C, 2× C′-1, 2× C″-1), 96.4 (βC-1), 92.5 (αC-1), 80.1, 79.9, 75.8 (2× C), 75.4, 75.2, 74.7 (2× C), 74.4, 73.3, 73.3, 73.1 (2× C), 72.3, 71.7, 71.5 (2× C), 71.5 (2× C), 70.6, 69.7 (2× C), 69.3 (2× C), 69.1 (2× C), 61.7 (2× C), 60.8, 60.7. HRMS (AJS-ESI): calculated for $C_{18}H_{32}O_{16}Na$ $[M + Na]^+$ 527.1583, found 527.1576.

Computational studies

The structure of CAL-B in 1TCA (Protein Databank) was downloaded and processed in Maestro v12.1 (Schrödinger 2021). Structures of **1α** and **1β** were used from PubChem accession numbers 2723636 and 2724702, respectively. The complexes were formed manually, according to the geometry in structure 1LBS (Protein Databank). Structures were characterized with OPLS4 force-field.[52] 500 steps of conjugate gradient minimization were performed in nonpolar solvent ($CHCl_3$) in MacroModel v12.5 (Schrödinger 2021) constraining the important interactions in the anion hole in the active site.

Conflicts of interest

There are no conflicts to declare.

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Appendix 2

Publication II

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Interplay of Monosaccharide Configurations on the Deacetylation with *Candida antarctica* Lipase-B

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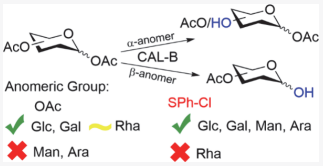
ABSTRACT: Configurational differences in monosaccharides determine the products and selectivity of the transesterification reaction with *Candida antarctica* lipase-B (CAL-B). The *β*-anomers of peresterified pyranose monosaccharides tend to yield anomeric deprotection products, while the *α*-anomers preferentially react at the sixth or fourth position. CAL-B differentiates between enantiomers, either reacting more rapidly with D-enantiomers of monosaccharides or having a different selectivity based on the enantiomer. Pivaloylated and benzoylated saccharides are the limits of the CAL-B transesterification reaction, while lower boiling point alcohols such as MeOH and EtOH can replace *n*-BuOH as the nucleophilic reagent. Finally, CAL-B can be successfully recycled in both long and short reaction time reactions.



INTRODUCTION

Naturally occurring oligosaccharides, their derivatives, and glycoconjugates possess glycosidic bonds in specific positions and with particular stereochemistry. Consequently, the total synthesis of natural carbohydrate-containing compounds typically involves the use and transformations of several different protecting groups in multistep synthesis to enable the formation of glycosidic bonds at the precise positions and with specific stereochemistry.[1−5]

To minimize the number of different protecting groups and thereby reduce the number of steps required to achieve selectively deprotected carbohydrates, several methods are available. Chemical procedures with pyranose monosaccharides primarily affect the first and/or the sixth positions, as these are the most easily manipulated due to their reactivity, compared to other positions.[6] However, for both positions, there are several drawbacks to chemical processes like overreaction leading to a mixture of products,[7−9] protecting group migration,[10,11] very harsh conditions, or dangerous reagents.[12−14] Enzymatic methods generally employ mild conditions and avoid hazardous chemicals, although they can also suffer from overreaction. Similar to chemical methods, enzymes preferably also target the first and the sixth positions (Scheme 1A).[15] Enzymes are mostly used in aqueous buffer solutions,[15] which introduces issues such as solubility of starting materials/products, complex workup procedures due to aqueous and mixed solvents, and relatively narrow conditions for the enzyme activity.[16]

It has been shown that *Candida antarctica* lipase-B (CAL-B) can overcome some of the problems previously listed as it can work in organic solvents,[17] regains its activity in a wide temperature range,[18,19] and, when immobilized, facilitates relatively easy workup procedures.[20,21] Ester groups are substrates for hydrolytic enzymes, are commonly used to protect hydroxyl groups in carbohydrates, and are thus common in chemical and enzymatic methods of synthesis. We have previously shown that immobilized CAL-B Novozyme N435 can deacetylate a variety of acetyl-protected pyranose saccharides, with a general preference for deacetylation at the fourth and sixth positions in D-glucose-based compounds (D-glucose (D-Glc), D-glucosamine, and D-lactose (Lac)) (Scheme 1B).[20]

This work focuses on investigating how different core structures of carbohydrates influence the activity of CAL-B, how anomeric substituents and their configuration affect the catalytic activity, and the limitations of CAL-B's tolerance for various protecting groups (Scheme 1C). The differences in configurations were tested on different pyranoses: D/L-Glc pentaacetate, its epimers D-galactose (Gal) and *α*-D-mannose pentaacetates, and peracetylated D/L-arabinose (Ara) enantiomers and L-rhamnose (Rha). Although the thiophenol leaving group (SPh) in the anomeric position is tolerated by CAL-B, the transesterification reaction takes 2 days with increased temperature and amounts of enzyme.[20] Therefore, other substituents in the anomeric position: chloride, aliphatic cyclic phosphate esters, diarylphosphate esters, and thiophenol derivates, were also tested. Several bulkier protecting groups such as benzoyl (Bz), pivaloyl (Piv), and *n*-butyryl (But) esters for CAL-B were used to determine the enzyme's limitations. Different alcohol nucleophiles were checked to replace *n*-butanol (*n*-BuOH) and concurringly the side product of *n*-butyl acetate with one that has a lower boiling point. Finally,

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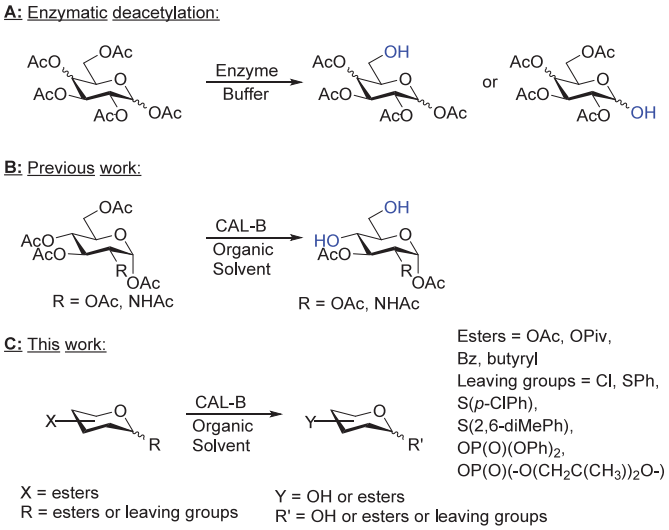
Scheme 1. (A) Enzymes Have Been Used to Deprotect Mainly the First or the Sixth Position of Pyranose Monosaccharides; (B) Previously, We Have Shown That CAL-B in Organic Media Deprotects Mainly the Fourth and the Sixth Position in D-Glucopyranose-Based Monosaccharides; (C) in This Work, Several Different Protecting Groups Were Used, and the Influence of Configuration on the Deacetylation Reaction Were Investigated

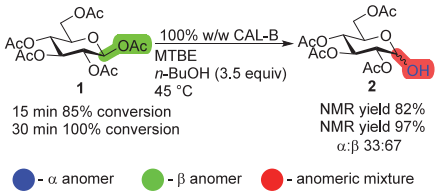


immobilized CAL-B's recyclability was tested in two different reactions with varying reaction times.

RESULTS AND DISCUSSION

Anomeric Position. Transesterification reaction with β-D-Glc pentaacetate **1**, immobilized CAL-B (w/w saccharide/CAL-B; Novozyme N435), and *n*-BuOH in methyl *tert*-butyl ether (MTBE) was complete in 30 min, resulting in the selectively deprotected first position (Scheme 2, Figure S1).

Scheme 2. Transesterification Reaction with β-D-Glc Pentaacetate 1 with CAL-B[a]

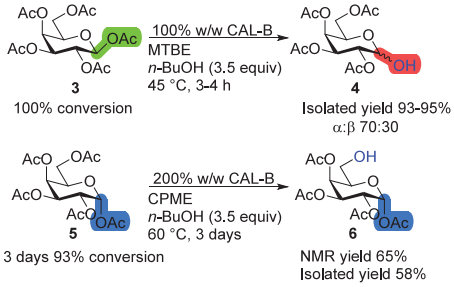


[a]Below the reaction is the color code used throughout the article: anomeric substituents highlighted in blue are α-anomers, green are the β-anomers, and red corresponds to the α/β-mixture; reaction conditions: β-D-Glc **1** pentaacetate (100 mg), 100% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE (10 mL), 45 °C.

Please note that the w/w ratio was used to determine only the selectivity pattern of the reaction. To compare the reactions of saccharides with different molecular weights, the ratio of molar mass to enzyme activity units should be used. Based on these and previous results,[20] we have shown that CAL-B targets different positions depending on the anomer of D-Glc pentaacetate with a higher catalytic activity toward β-D-Glc pentaacetate.

Similar to D-Glc, the β-anomer of D-Gal pentaacetate **3** reacts much faster than its α-anomer **5**, achieving full conversion within 3−4 h with a very high selectivity toward the main product of the first position deacetylated **4**. In contrast, the α-anomer **5** requires 3 days of reaction time, an increased amount of immobilized enzyme, cyclopentyl methyl ether (CPME) as a solvent, and an elevated temperature (60 °C) (Scheme 3). CAL-B has shown similar catalytic activity in

Scheme 3. Difference in Regioselectivity for D-Gal Anomers[a]



[a]Reaction conditions: monosaccharide (100 mg), 100% or 200% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE or CPME (10 mL), 45 or 60 °C.

CPME compared to MTBE.[20] As such, when increased temperatures were used, CPME was used interchangeably with MTBE. The isolated yield of the sixth position deacetylated Gal tetraacetate **6** was 58%, with no single clear main side product, but rather multiple further deacetylated products. Once again, CAL-B shows a preference for different

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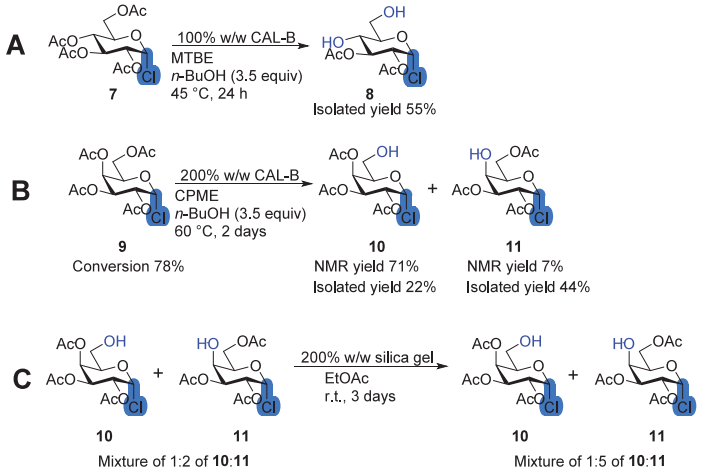
Scheme 4. Influence of the Chloride Leaving Group on CAL-B Transesterification Reaction: (A) Reaction of α-D-Glc-Cl Tetraacetate 7 with CAL-B; (B) α-D-Gal-Cl Tetraacetate 9 with CAL-B; NMR yields for Products 10 and 11 Are from the Crude Reaction Mixture Being Heavily in Favor of Product 10, while after Purification and Isolation, Product 11 Appeared as the Main Product; (C) Mixture of Products Was Stirred with Silica Gel to Check Its Influence on the Acyl Migration, Reaction Conditions: Monosaccharide (100 mg), 100% or 200% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE or CPME (10 mL), 45 or 60 °C; Acyl Migration: Mixture of 10 and 11 (30 mg), Silica Gel (60 mg), EtOAc (1 mL), Room Temperature



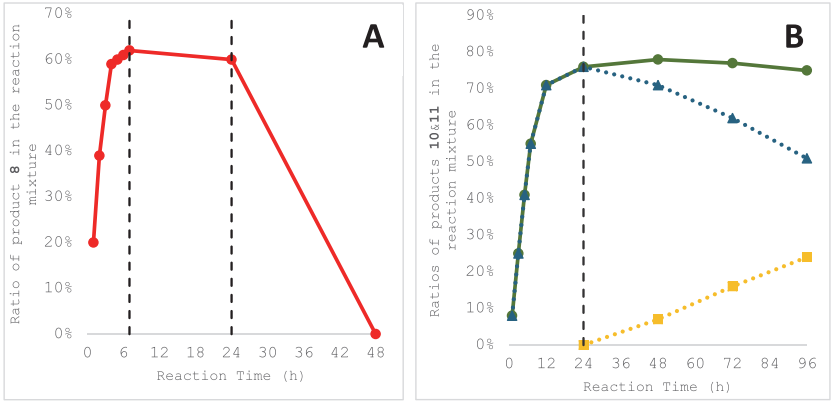


Figure 1. (A) CAL-B transesterification reactions with D-Glc-Cl tetraacetate **7**. The red circles show the NMR yield of product **8**. The three steps of the transesterification reaction profile are separated by dashed lines. Reaction conditions: D-Glc-Cl tetraacetate **7** (100 mg), 100% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE (10 mL), and 45 °C. (B) CAL-B transesterification reaction with D-Gal-Cl tetraacetate **9**. The blue triangles show the NMR yield of product **10**, yellow squares show the NMR yield of product **11**, and green circles are the sum of the two product NMR yields. The two steps of the transesterification reaction are separated by a dashed line. Reaction conditions: D-Gal-Cl tetraacetate **9** (100 mg), 200% CAL-B (w/w), *n*-BuOH (3.5 equiv), CPME (10 mL), and 60 °C.

positions depending on the Gal pentaacetate anomeric configuration.

Anomeric Substituents. The reaction of α-D-Glc-Cl tetraacetate **7** with CAL-B (Scheme 4A) proceeded similar to peracetylated α-D-Glc **42** with one major product of fourth and sixth positions deacetylated **8**. After 7 h, the conversion reached approximately 62% according to ^{1}H NMR. After 24 h, the conversion remained the same with small amounts (<5%) of side products appearing. After 48 h, product **8** was no longer detected by NMR and several overreaction products were observed, while approximately 38% of the unreacted starting material remained in the reaction mixture. The transesterification reaction has stopped after 7 h, indicating that the formed product **8** inhibits the access of the starting material **7** to the catalytic pocket of CAL-B. The observed progress of the initial slow formation of overreaction products (<5% in 17 h) followed by a rapid increase in overreaction and then full conversion from **8** in 24 h matches well with the product activation reaction characteristics.[22] It is supposed that the reaction profile of D-Glc-Cl **7** with CAL-B consists of three steps: an initial selective transesterification, followed by the inhibition and a lag phase, and finally a second nonselective transesterification (Figure 1A). The reaction of D-Gal-Cl tetraacetate **9** with CAL-B has the same regioselectivity with

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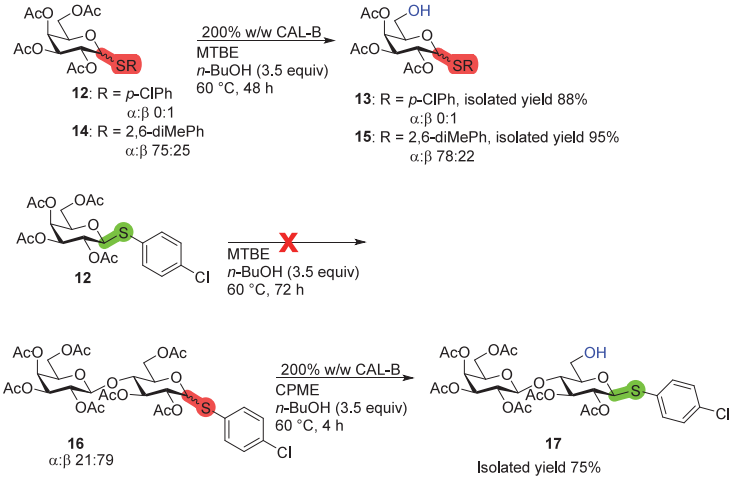
Scheme 5. Influence of Different Thiophenol Derivatives on the CAL-B Deacetylation Reaction[a]



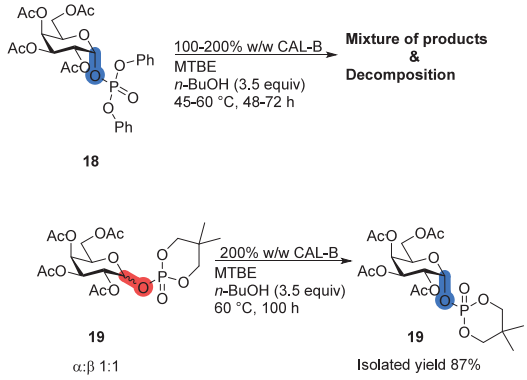
[a]Blank reaction, where no enzyme was added, showed no reactivity; reaction conditions: saccharide (100 mg), 200% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE or CPME (10 mL), 60 °C.

D-Gal pentaacetate **5** (Scheme S1), while the reaction profile was similar to that of D-Glc-Cl tetraacetate **7**. The first step, initial selective transesterification, happened during 24 h and afforded the sixth position deacetylated product **10** in 76% NMR yield (Scheme 4B, Figure 1B). The second step, the inhibition and lag phase, started after 24 h. It continued for the 4 days during which the reaction was followed as only <5% overreaction products were detected by NMR. The second step was accompanied by acyl migration, which might have inhibited any further transesterification and prolonged the second step. After 48 h, the fourth position deprotected product **11** was detected, and by the fourth day, its NMR yield had increased to 24%. Following purification by column chromatography on silica gel, the overall ratio of products **10**:**11** had changed to 1:2. To prove that acyl migration continues during column chromatography, a portion of the isolated mixture of products was stirred with 200% silica gel (w/w) for 3 days. The acyl migration was carried out successfully, resulting in a final ratio of 1:5 in favor of tetraacetate **11**. Prolonged stirring with a higher amount of 1000% silica (w/w) led to decomposition.

Thioglycoside **12**, equipped with a *p*-chlorothiophenol leaving group, was selectively deacetylated in the sixth position, yielding product **13** in 88% yield in 48 h (Scheme 5). Compared to a thiophenol leaving group, there was a slight increase in the yield, but the reaction time remained 48 h.[20] *p*-Chlorothiophenol is a solid and less foul-smelling compared to thiophenol; as such, other carbohydrates of interest were used with *p*-chlorothiophenol as the sulfur-containing leaving group instead of thiophenol due to its practical advantages. A blank reaction of thioglycoside **12** and *n*-BuOH was checked in the absence of an enzyme. There was no reaction with thioglycoside **12** in 72 h at 60 °C according to ^{1}H NMR. The anomeric mixture of peracetylated D-Gal with the 2,6-dimethylthiophenol leaving group **14** reacted similar to previously mentioned thioglycosides, producing the sixth position deacetylated product **15**. After 48 h, close to full conversion was reached, no clear anomeric preference was seen, very selective with only an unreacted starting material leftover, and even higher yield was achieved. Peracetylated D-Lac has been shown to undergo deacetylation in the sixth position of the D-Glc ring with CAL-B. This was also observed when using peracetylated D-Lac **16** with *p*-chlorothiophenol as the leaving group. The selective sixth position deacetylation product **17** was the major outcome of the reaction with the *β*-anomer, while most of the *α*-anomer of D-Lac **16** did not react. The reaction of D-Lac **16** with CAL-B reacted similar to peracetylated *β*-D-Glc-SPh rather than D-Gal thioglycosides. This suggests that the saccharide equipped with a leaving group has a larger influence on CAL-B catalytic activity than the nonreducing terminal saccharide.[20]

Next, Gal with phosphate leaving groups, which are the native substrates in enzymatic glycosylations,[23] were under the study (Scheme 6). Acyclic phosphate ester **18** and its products

Scheme 6. Influence of the Phosphorous Leaving Group on the CAL-B Deacetylation Reaction[a]



[a]Reaction conditions: monosaccharide (100 mg), 200% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE (10 mL), 60 °C.

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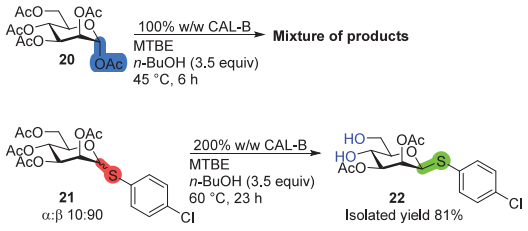
were too unstable to give reasonable and practically applicable results (Scheme S2, Table S1). Therefore, the more stable cyclic aliphatic phosphate ester **19** was tested for the deacetylation with CAL-B instead. Surprisingly, with 200% CAL-B and at 60 °C almost no products of the transesterification reaction were detected. Instead, anomeric enrichment had happened. The ratio of α/β anomers in compound **19** was initially approximately 1:1; after 4 days, it had fully converted to the α-anomer. Phosphate and phosphonate esters are known to inhibit lipases,[24−26] and the latter compounds have been crystallized with CAL-B, showing their interactions with the catalytic triad.[27] As for monosaccharide **19**'s phosphate leaving group, it does not inhibit CAL-B completely nor does it form an irreversible covalent bond with the catalytic serine-105. Instead, it seems to interact in the catalytic site and inhibit the transesterification reaction. We speculate that the β-anomer of **19** is preferred over the α-anomer, and as it interacts with the catalytic triad, it temporarily breaks the bond between the Gal first position's oxygen and the phosphate leaving group's phosphorus atom. The formed anion then epimerises into the α-configuration, which is thermodynamically favored due to the anomeric effect. Finally, the bond is formed between Gal and the phosphate leaving group, thus forming the α-anomer of **19** as the sole product. An alternative nonenzymatic approach consisting of dissociation/association mechanism cannot also be excluded.

To conclude, acetate is a specific protecting group in the anomeric position, enabling regioselective deacetylation with CAL-B depending on the relative configuration of the substituent. Sterically more demanding groups, such as thiophenol derivatives, direct the attack to other positions or block it completely (as with phosphates). A smaller chloride substituent forms products that inhibit the activity of CAL-B and initiate acyl migration. Either due to different positions being deacetylated or the due to the characteristics of the substituents (steric influence, electronic effects, etc.), the reactions were significantly slower, especially with nonacetate β-anomeric substituents, demanding higher temperatures and more enzyme to be used.

Second Position. To check the influence of the substituents in the second position on the deacetylation reaction with CAL-B, α-D-mannose pentaacetate **20** was used (Scheme 7). The reaction reached full conversion in 6 h. Unfortunately, TLC showed many products, and crude NMR analysis revealed at least 11 anomeric protons. These analysis results show that α-mannose pentaacetate **20** cannot be selectively deprotected using CAL-B. D-Mannose with the *p*-chlorothiophenol leaving group (compound **21**) did not react at all in 48 h with 45 °C and 100% CAL-B. Increasing the amount of enzyme and using elevated temperatures led to the selective deacetylation of the β-anomer in 12 h in the sixth and the fourth positions affording product **22**, while the α-anomer did not react in 23 h. Compared to peracetylated β-D-Glc-SPh,[20] CAL-B has lower catalytic activity with D-mannose **21** but the same selectivity.

Scheme 7. Influence of the Second Position on the CAL-B Transesterification Reactions[a]

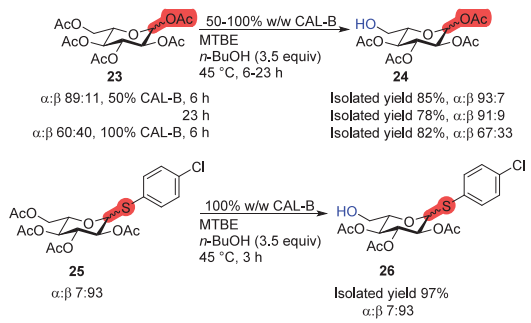


[a]Reaction conditions: monosaccharide (100 mg), 100 or 200% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE (10 mL), 45 or 60 °C.

Fourth Position. Comparing the two epimers, D-Gal and D-Glc, it is clear, that their structural differences influence the catalytic activity of the enzyme and therefore the rate of the reaction with CAL-B. If the substituents in the fourth and in the fifth positions are *trans* to each other (compound **1**, Scheme 2), the reaction is roughly 6−8 times faster compared to when they are cis (compound **3**, Scheme 3). Corresponding α-anomers, compounds **5** (Scheme 3) and **42** (Scheme 12), lead to different products. The sixth position deacetylation is the major product **6** for D-Gal, but for D-Glc, deacetylation of the sixth position is an intermediate through which the main product **43** is formed. Thus, the fourth position's configuration affects whether it is deacetylated during the transesterification reaction. Similar results for α-anomers can be seen with chloride substituents (compounds **7** and **9**, Scheme 4), even though due to the substituent effects, the fourth position can be deacetylated through acyl migration. If the fourth position is deacetylated during the reaction, further deacetylation for both epimers does not happen contrary to the reactions in buffer solution.[20,28]

D- and L-Configurations. To further investigate the influence of configurational differences, CAL-B transesterification reactions were conducted on L-Glc pentaacetate **23** and *p*-chlorothiophenol equipped L-Glc tetraacetate **25** (Scheme 8).

Scheme 8. Influence of L-Configuration of Glc on the Transesterification Reaction with CAL-B[a]



[a]Reaction conditions: monosaccharide (100 mg), 50 or 100% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE (10 mL), 45 °C.

Anomeric enrichment[29,30] was carried out on L-Glc pentaacetate **23** from an α:β ratio of 60:40 to 89:11 (Scheme S3). The reaction of the obtained anomeric mixture with CAL-B deacetylated the sixth position of both anomers in 6 h (product **24**). No anomeric preference was detected. Full conversion was not reached even after 23 h, which indicates that product **24** inhibits CAL-B catalytic activity. For L-thioglycoside **25**, CAL-B showed a very high selectivity toward deacetylating the sixth position and yielding product **26** in almost quantitative yield (97%) with fully retained anomeric

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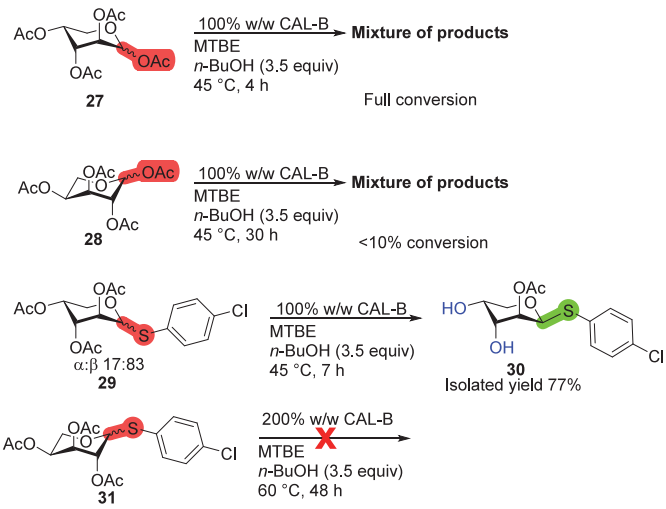
Scheme 9. Influence of D- and L-Configurations of Arabinopyrnoses on the CAL-B Deacetylation Reaction[a]



[a]Reaction conditions: monosaccharide (100 mg), 100% or 200% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE (10 mL), 45 or 60 °C.

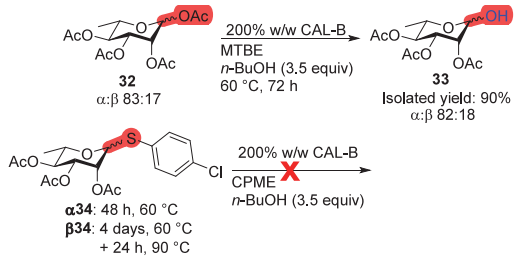
configuration (Scheme 8). For Glc, CAL-B does not prefer one enantiomer over the other, but can deacetylate the D-enantiomer more than once,[20] while showing no anomeric preference for the L-enantiomer.

Our study of the configurational differences was continued on the D- and L-enantiomers of arabinopyranoses (Ara) with CAL-B transesterification reactions. Both peracetylated Ara enantiomers (**27** and **28**, Scheme 9) lacked selectivity, giving multitude of products. Nevertheless, D-Ara tetraacetate **27** reacted much faster than L-Ara tetraacetate **28**, having reached full conversion in 4 h, while L-Ara **28** had <10% conversion in 30 h. When thioglycosides **29** and **31** were used in transesterification reactions with CAL-B, both enantiomeric and anomeric preferences were observed. The reaction of β-D-Ara **29** reached full conversion in 7 h, affording a monoacetate **30** (77% overall yield, 92% comparing β-anomers only), while α-D-Ara **29** remained unreacted after 24 h according to ^{1}H NMR. L-Ara with *p*-chlorothiophenol leaving group **31** did not react even at elevated temperatures and increased amounts of enzyme. These results indicate a clear preference of the D-enantiomer of Ara over the L-enantiomer for CAL-B.

For L-rhamnose (Rha), peracetylated Rha **32** reacted slowly at 45 °C. Increasing the temperature and the amount of enzyme led to the first position deprotected product **33** after 3 days in 90% isolated yield (Scheme 10). There was no anomeric selectivity observed for Rha. Comparing L-Rha **32** (which is 6-deoxy-L-mannopyranose) to the D-mannose pentaacetate **20** reaction with CAL-B (Scheme 7) showed an increase in selectivity. Unfortunately, no further comparison can be made about selectivity, as both anomers of Rha equipped with *p*-chlorothiophenol leaving group **34** did not react even after increasing the temperature to 90 °C.

Different Protecting Groups. Deprotection of benzoylated D-Glc **35** with CAL-B was selective toward the sixth position affording compound **36** but was very slow even after increasing the temperature to 90 °C (Scheme 11). The initial α/β anomeric ratio of D-Glc **35** was 75:25, which did not change throughout the reaction according to crude ^{1}H NMR

Scheme 10. Investigation of L-Rha Transesterification Reaction with CAL-B[a]



[a]Reaction conditions: monosaccharide (100 mg), 200% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE or CPME (10 mL), 60 °C or 60 → 90 °C.

data. Benzoylated D-Gal **37** had the same very slow rate of reaction as compound **35**. After 6 days with 200% CAL-B and 60 °C, 16% conversion was reached according to NMR, but there was no clear selectivity as at least five different products were detected. Continuing with bulky protecting groups, pivaloylated D-Glc **38** gave no reaction even after 3 days at 60 °C with 200% CAL-B. Butyryl ester-protected D-Glc **39** on the other hand showed full conversion after 6 h. Two products, sixth position deprotected **40** and deprotected anomers **41**, were isolated in one fraction with a total yield of 80%. The ratio of the formed products **40**:**41** was 2:1, which coincided with the initial anomeric ratio of the starting material **39**. Since the product **40** was purely an α-anomer, it can be concluded that the sixth position deacetylation occurs with the α-anomer of **39**, while the β-anomer is deacetylated in the first position.

To summarize, CAL-B shows some catalytic activity with benzoylated monosaccharides, but they react rather slowly or give mixtures of products (for D-Gal) to be practically viable. Pivaloylated D-Glc **38** seems to be the limit for CAL-B as there was no reaction at all. Butyrated D-Glc **39** shows similar

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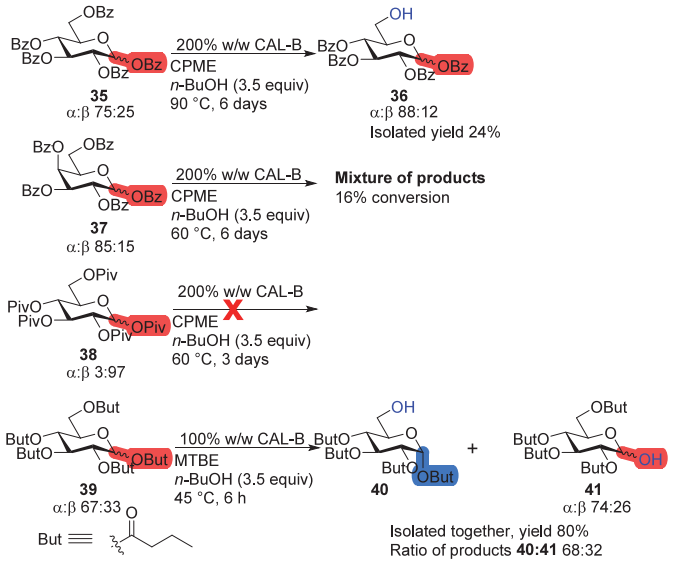
Scheme 11. CAL-B Deacetylation Reaction with Monosaccharides Protected with Various Protecting Groups[a]

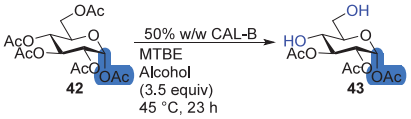


[a]Reaction conditions: monosaccharide (100 mg), *n*-BuOH (3.5 equiv), 100−200% CAL-B, MTBE or CPME (10 mL), 45−90 °C.

anomeric preferences as seen with acetyl protecting groups, although only a monodeacetylated product is formed. As CAL-B's natural substrates are fatty acid esters, it seems to prefer linear aliphatic ester protecting groups.

Nucleophilic Transesterification Reagents. During the deacetylation reaction with CAL-B when *n*-BuOH is used as a nucleophilic reagent, *n*-BuOAc as a side product is formed. To replace it with lower boiling esters, ethanol and methanol were tested as nucleophilic transesterification reagents (Scheme 12). Using MeOH or EtOH would simplify the workup and working with the crude reaction mixtures.

Scheme 12. Different Alcohols for Transesterification with α-D-Glc Pentaacetate 42[a]



Entry	Alcohol	Yield of 43[1]	Formed Ester
1	*n*-BuOH	82-90%	BuOAc
2	MeOH	80%	MeOAc
3	EtOH[2]	85%	EtOAc

[1] Isolated yield; [2] technical grade 96% EtOH

[a]Reaction conditions: α-D-Glc pentaacetate **42** (100 mg), 50% CAL-B (w/w), alcohol (3.5 equiv), MTBE (10 mL), 45 °C, 23 h.

Previously, when 50% CAL-B (w/w) was used in the deacetylation reaction with α-D-Glc pentaacetate **42**, after 23 h, 82% yield was achieved.[20] Using MeOH and EtOH as the nucleophilic reagents did not influence the reaction outcome significantly (Scheme 12, entries 1−3). When EtOH was used with peracetylated β-D-Glc **1**, in 30 min, 95% NMR yield was achieved with the products' anomeric ratio α:β 54:46, compared to the *n*-BuOH outcome of 97% NMR yield with α:β 33:67 (Scheme 2). Crude NMR was clear of any side products of the transesterification reaction (see the SI). The only notable difference when using EtOH instead of *n*-BuOH as the nucleophilic reagent was the anomeric ratio of the products. As such, both alcohols seem to be good alternatives to *n*-BuOH.

Enzyme Recycling. Novozyme N435 has been successfully recycled from organic media reactions and reused by Rodrigues et al.[31] We chose two reactions for testing of the recyclability of immobilized CAL-B: a short reaction time (30 min) with β-D-Glc pentaacetate **1** (Scheme 2) with 100% CAL-B and a long reaction time (23 h) with α-D-Glc pentaacetate **42** with 50% CAL-B (Scheme 12). Five cycles were carried out with short reaction time to mainly test how the washing and filtering would influence CAL-B, while three cycles were done with long reaction time to mainly test the influence of mechanical stirring on the stability of the immobilized enzyme (Figure 2, Table S2). After each reaction, immobilized CAL-B was washed with DCM (∼50 mL) and air-dried for 1 h. The isolated yields of deacetylated tetraacetate **2** varied in the range of experimental errors. Recycling CAL-B in transesterification reactions with α-D-Glc pentaacetate **42** had very stable results, showing that mechanical stirring does not influence the catalytic activity of CAL-B. Based on these results, we conclude that CAL-B can be recycled successfully in both long and short reaction time reactions.

■ CONCLUSIONS

In conclusion, an experimentally simple protocol for the deacetylation of various pyranose monosaccharide derivatives with immobilized CAL-B was elaborated, providing valuable synthetic intermediates for the carbohydrate synthesis. Depending on the configuration and the nature of the

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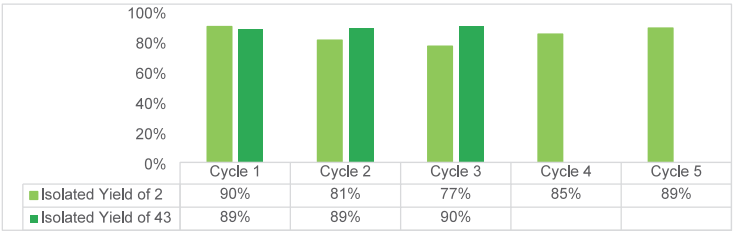

Figure 2. Recycling of immobilized CAL-B. Light green bars show the results of recycling CAL-B in transesterification reactions with β-D-Glc pentaacetate **1**; reaction conditions: β-D-Glc pentaacetate **1** (100 mg), 100% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE (10 mL), 45 °C, 30 min. Dark green bars show the results of recycling CAL-B in transesterification reactions with α-D-Glc pentaacetate **42**; reaction conditions: α-D-Glc pentaacetate **42** (100 mg), 50% CAL-B (w/w), *n*-BuOH (3.5 equiv), MTBE (10 mL), 45 °C, 23 h.

protecting groups, several trends and correlations were identified within CAL-B transesterification reactions.

The anomeric position directs the transesterification reaction outcomes to different positions, affording selectively deprotected products with varying reaction rates. The second position's configuration, for α-pentaacetates, determines whether the transesterification reaction is selective or not. The fourth position's configuration influences the rate of the reaction and whether the main product is a diol or monodeprotected saccharide. CAL-B can differentiate between D- and L-enantiomers of pyranose monosaccharides. We also found that saccharides with a Cl-leaving group do not reach full conversion as they form products that inhibit the transesterification reaction. Meanwhile, the thiophenol leaving group and its derivates give similar results but overall decrease the rate of the reaction. Phosphorus leaving groups seem to interact with CAL-B directly, inhibiting the transesterification reaction, and instead anomeric enrichment happens. Bulkier protecting groups either give a slow reaction (benzoyl), do not react (pivaloyl), or partially follow the same behavior of acetylated counterparts (butyrated). We showed that *n*-BuOH can be replaced by lower boiling point nucleophilic reagents such as MeOH and EtOH. Finally, CAL-B was successfully recycled and reused in two different reactions up to five cycles.

EXPERIMENTAL SECTION

General Experimental Information. Full assignment of ^{1}H and ^{13}C chemical shifts was based on the 1D and 2D (COSY, HSQC and HMBC) FT NMR spectra measured with a Bruker Avance III 400 MHz instrument. Residual solvent signals were used ($CDCl_3$: δ = 7.26 ^{1}H NMR, 77.2 ^{13}C NMR; CD_3OD: δ = 3.31 ^{1}H NMR, 49.0 ^{13}C NMR; $(CD_3)_2SO$: δ = 2.50 ^{1}H NMR, 39.5 ^{13}C NMR; D_2O: δ = 4.79 ^{1}H NMR) as internal standards. High-resolution mass spectra were recorded with an Agilent Technologies 6540 UHD Accurate-Mass QTOF LC/MS spectrometer by using AJ-ESI ionization. Prior to analysis, the instrument was calibrated in a mass range of m/z 50−3200. Optical rotations were obtained with an Anton Paar GWB Polarimeter MCP 500. Melting points were determined using a polarizing optical microscope Nagema-K8. Precoated Merck silica gel 60 F_{254} plates were used for TLC, and column chromatography was performed with Merck 60 (0.040−0.063 mm) mesh silica gel. Commercial reagents and solvents were generally used as received. DCM was distilled over CaH or phosphorus pentoxide, ethyl acetate (EtOAc) and acetone over phosphorus pentoxide, and MeOH and toluene over sodium. Petroleum ether (PE) had a boiling point of 40−60 °C. Immobilized *C. antarctica* lipase-B, on hydrophobic acrylic resin, Novozyme N435, with 10,000 (propyl laurate unit/g) activity was a kind gift from Novozymes A/S.

General Procedure for Preparation of 1,2,3-Tri-*O*-acetyl-α-D-glucopyranoside 43 with CAL-B. In a manner analogous to Kanger et al.,[20] α-D-glucose pentaacetate **42** (100 mg), MTBE (10 mL), and *n*-BuOH (3.5 equiv) were mixed at 45 °C. After saccharide was dissolved, stirring was set to 100 rpm, and 50% CAL-B (50 mg) was added. The reaction was followed by TLC, and upon completion after 23 h, the reaction mixture was filtered, immobilized enzymes were rinsed with DCM (∼50 mL), and the filtrate was concentrate in vacuo. The crude mixture was purified by silica gel column chromatography (PE:EtOAc 2:1 →1:4), yielding a white solid (70 mg, 90%). TLC − PE:EtOAc 1:2, R_f = 0.19; NMR matches with previously reported values.[20]

Gram-Scale Synthesis of 1,2,3-Tri-*O*-acetyl-α-D-glucopyranoside 43. According to the general procedure with α-D-Glc pentaacetate **42** (2.56 mmol, 1 g), MTBE (100 mL), *n*-BuOH (3.5 equiv, 821 μL), 45 °C, and 50% CAL-B (500 mg), the reaction was run for 23 h and resulted in a white solid (705 mg, 90%).

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.4c02582.

Experimental procedures, characterization data, and NMR spectra for all synthesized compounds (PDF)

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Notes

The authors declare no competing financial interest.

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Appendix 3

Publication III

Hunt, K. E.; Miller, A.; Jarg, T.; Kriis, K.; Kanger, T. Selective acetylation of unprotected thioglycosides and fully unprotected monosaccharides with *Candida antarctica* lipase-B. *ACS Omega*, **2025**, *10* (19), 20047–20053. https://doi.org/10.1021/acsomega.5c02467

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Article

Selective Acetylation of Unprotected Thioglycosides and Fully Unprotected Monosaccharides with *Candida antarctica* Lipase-B

Kaarel Erik Hunt, Annette Miller, Tatsiana Jarg, Kadri Kriis, and Tõnis Kanger*

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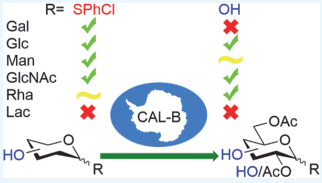
ABSTRACT: A selective enzymatic acetylation method for the protection of the second and the sixth positions of thio-D-galactopyranoside was found using immobilized *Candida antarctica* lipase-B (CAL-B). Unfortunately, it was determined that the immobilized enzyme cannot be recycled effectively. The optimized acetylation method was screened with different thioglycosides and with fully unprotected saccharides. New methods for several new partially protected saccharides were found, while the synthesis of some known saccharides, e.g., the third and the sixth position-protected D-glucose or the fourth position-protected L-rhamnose, was improved. Furthermore, an enzymatic acetal formation between the fourth and the sixth positions was discovered. The main limitation for acetylation reactions with CAL-B has been determined to be the substrate solubility.



INTRODUCTION

Regioselective protection and deprotection of functional groups in carbohydrate chemistry are vital parts of oligosaccharide synthesis. Generally, in total synthesis, several different protecting groups are used in multiple steps to arrive at the targeted specifically protected saccharide.[1−3] Some positions of monosaccharides can be more easily protected than others.[4−6] In pyranoses, the sixth position is a primary hydroxyl group and can be selectively protected and deprotected in high yields.[7−10] Chemical regioselective modifications in the second to fourth positions, which are all secondary hydroxyl groups, are more complicated. It can be achieved by relying on steric interactions, metal complexes, cyclic protecting groups, and shifting reaction conditions toward the formation of the kinetic or thermodynamic product.[11−18] Enzymatic methods for the selective protection and deprotection mostly cover ester-protecting groups, mainly in the sixth or the first position.[19,20] *Candida antarctica* lipase-B (CAL-B) has been shown to work in organic media while exhibiting a good regioselectivity in a wide range of substrates.[21] We have previously used immobilized CAL-B, Novozyme N435, for the regioselective deacetylation of monosaccharides in organic media.[22,23] As for selective acetylation, Riva et al. (1997)[24] have shown that when the anomeric position of various α-monosaccharides is protected, CAL-B can selectively acetylate the sixth position in organic media with 74−99% yield (Scheme 1A), while β-anomers depend more on the anomeric protecting group and can have either the sixth position or the sixth and the second/third positions acetylated, resulting in a mixture of products. Following on their work, Holmstrøm and Pedersen (2020)[25] increased the selectivity toward the third and sixth position acetylations with various anomerically protected monosaccharides (Scheme 1B).

Here, we report a regioselective acetylation of the second and the sixth positions with different D-galactopyranose thioethers (Scheme 1C). Resulting products are soluble in organic media with the third and the fourth positions left unprotected, making them useful intermediates for the synthesis of various natural oligosaccharides, including galactooligosaccharides and human milk oligosaccharides.[26−28] In addition, we have also found conditions for the selective enzymatic synthesis of an acetal between the fourth and the sixth positions, with thio-D-galactopyranoside leaving the second and the third positions unprotected. Furthermore, we report here using the optimized acetylation method to synthesize selectively protected saccharides from fully unprotected pyranose monosaccharides (D-glucose, D-glucosamine, D-mannose, L-rhamnose) and their thioether counterparts. To our knowledge, fully unprotected monosaccharides have not been selectively acetylated in organic media. Finally, some limitations for the acetylation reactions with CAL-B were discovered.

RESULTS AND DISCUSSION

First, we investigated the role of solvent in the selectivity of acetylation. The intention was to obtain selectively protected diols in the second and the sixth positions (Table 1). So far, 2,6-diprotected monosaccharides have been obtained via multistep synthesis[29] or via separation from other re-

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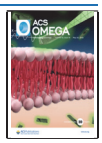

Scheme 1. (A) Riva et al. (1997)[24] Showed That Anomerically Protected Saccharides React Differently Based on the Anomeric Configuration, (B) Holmstrøm and Pedersen (2020)[25] Focused More on the Selective Acetylation of 3,6-*O*-diAc Products, (C) This Work Focuses on 2,6-*O*-diAc Formation for Thio-D-galactopyranosides and Selective Protection of Other Thioglycosides and Fully Unprotected Saccharides

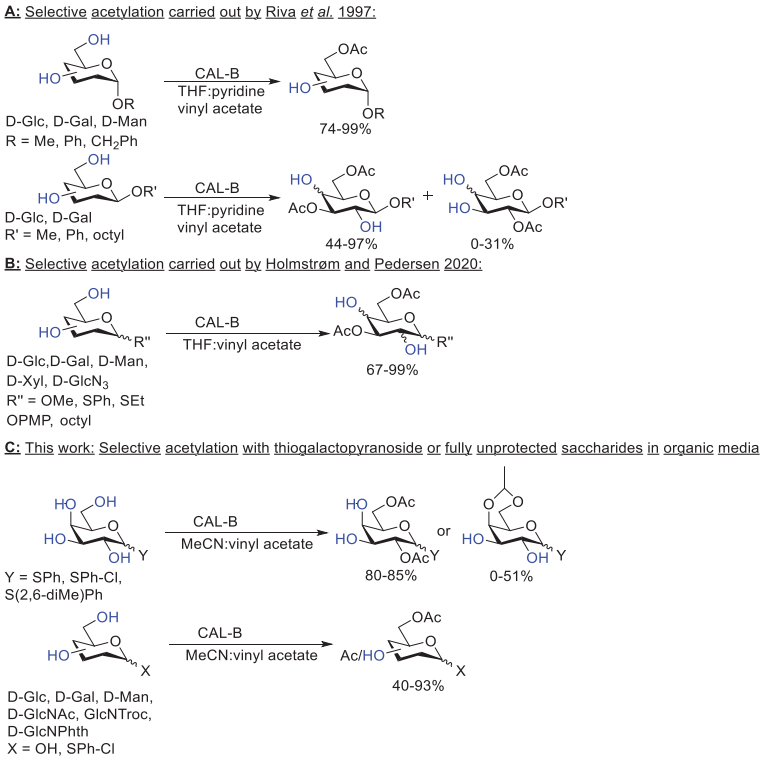

Table 1. Solvent Screening for Acetylation with Thio-D-galactopyranosides 1 and 2 with CAL-B[a]

					products yield (%)[b]			
no.	S.M.	solvent	acylation reagent	time (h)	mono-OAc	**3** or **4**	**5** or **6**	7
1	**1**	THF/pyridine 4:1	Vin.Ac	168	mix	traces	traces	
2	**1**	MTBE/pyridine 4:1	Vin.Ac	48	traces	49	20	
3	**1**	chloroform	Vin.Ac	72	traces			51
4	**1**	MTBE	Vin.Ac	72	traces			49
5	**1**	acetone	Vin.Ac	96	mix	traces	traces	
6	**1**	acetone	Ac_2O	96	mix	traces	traces	
7	**2**	MeCN	Ac_2O	48	mix	18*	50*	
8	**2**	MeCN	Vin.Ac	48	traces	72		
9[c,d]	**2**	THF	Vin.Ac	96	traces	83* (56)	17*	
10[c,d]	**2**	MeCN	Vin.Ac	48		92* (57)	8*	
11[c]	**2**	MeCN	Vin.Ac	24		93* (80)	7*	
12[c,e]	**2**	MeCN	Vin.Ac	96				

[a]Reaction condition: thioglycoside **1** or **2** (60 mg), vinyl acetate (20 equiv), solvent (5 mL), CAL-B (60 mg), 45 °C. [b]Isolated yield shown or in (). [c]Ratio of solvent:vinyl acetate 1:1 (2.4 mL). [d]20% w/w CAL-B used. [e]Reaction without CAL-B; S.M.—starting material, mix—mixture of products, not isolated or characterized; *—determined by ^{1}H NMR from the crude mixture.

gioisomers.[25] CAL-B, like other lipases, is an enzyme that has been used in diverse media, like water, organic solvents, mixtures of various solvents, etc. The main reason is that the hydrophobic component in the solvent system is required for CAL-B to adopt the active form.[21] Clearly, solvent influences the conformation of the active site pocket of CAL-B.[30,31]

Unprotected thio-D-galactopyranoside **1** or **2** was used as a model substrate (Scheme 2). Both thiophenol and *p*-chlorothiophenol pyranosides were tested, with no differences observed in the acetylation reaction with CAL-B. The latter group was still preferred as the less foul-smelling protecting group. Immobilized CAL-B was used as a weight ratio with the

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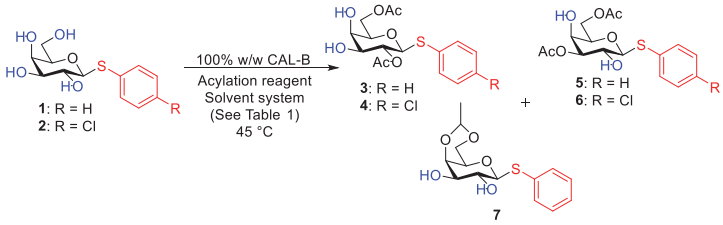
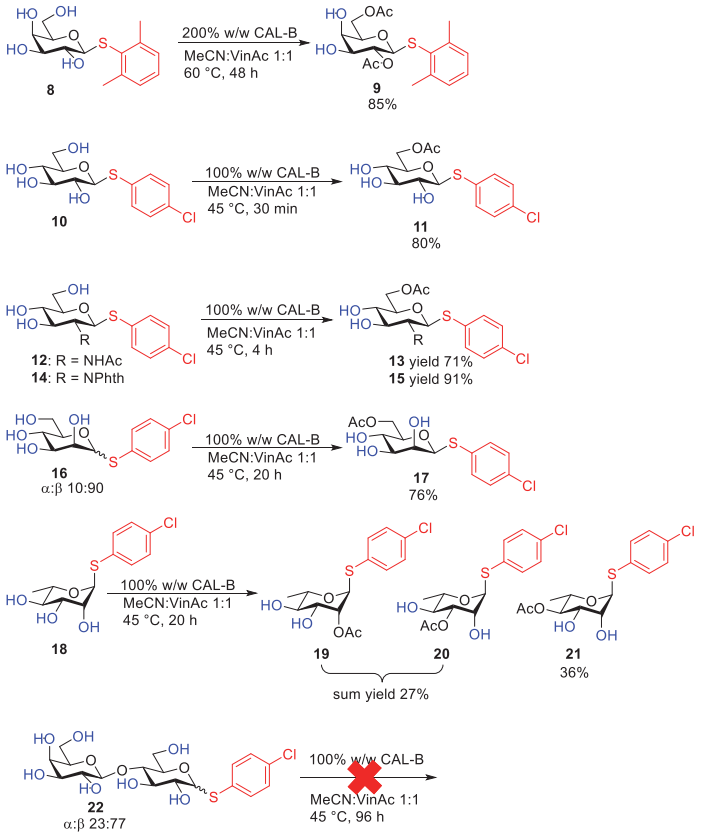
Scheme 2. Acetylation of Thio-D-galactopyranosides 1 and 2 with CAL-B Resulting in 2,6-*O*-diAc (Compounds 3 and 4) and 3,6-*O*-diAc (Compounds 5 and 6) Regioisomers or Formation of 4,6-*O*-ethylidene Acetal 7



Scheme 3. CAL-B Acetylation Reactions with Thioglycosides, Where: Thio-D-Galactose, D-Glucose, D-Glucosamine, and D-Mannose Gave Selective Acetylation Reactions; Thio-L-Rhamnose, a Mixture of Monoacetylated Products, and Thio-D-Lactose Did Not React[a]



[a]Reaction conditions: thioglycoside (60−100 mg), MeCN:vinyl acetate 1:1 (0.08 M of substrate), 100−200% w/w CAL-B, 45−60 °C; yields shown are isolated yields.

substrate as the method of choice following previous examples[24,25,32] instead of mmol and enzyme activity units. The w/w method was used to investigate only the regioselectivity pattern of the acetylation reaction. To compare reactivities with different molecular weight saccharides, a molar ratio to enzyme activity units should be used. First, Riva et al.'s[24] conditions were tested with thio-D-galactopyranoside **1**. Equal amounts of monosaccharide and immobilized enzyme, CAL-B (100% (w/w)), were stirred in the mixture of tetrahydrofuran (THF)/pyridine (4:1) with 20 equiv of vinyl acetate as the acylating reagent at 45 °C (Scheme 2, Table 1 no. 1). The main outcome of the reaction was a mixture of different monoacetates. Different solvents were screened where it was known CAL-B has high activity[26] and/or were polar solvents. Changing THF to methyl *tert*-butyl ether (MTBE), which was the best solvent for the deacetylation,[22] resulted in a

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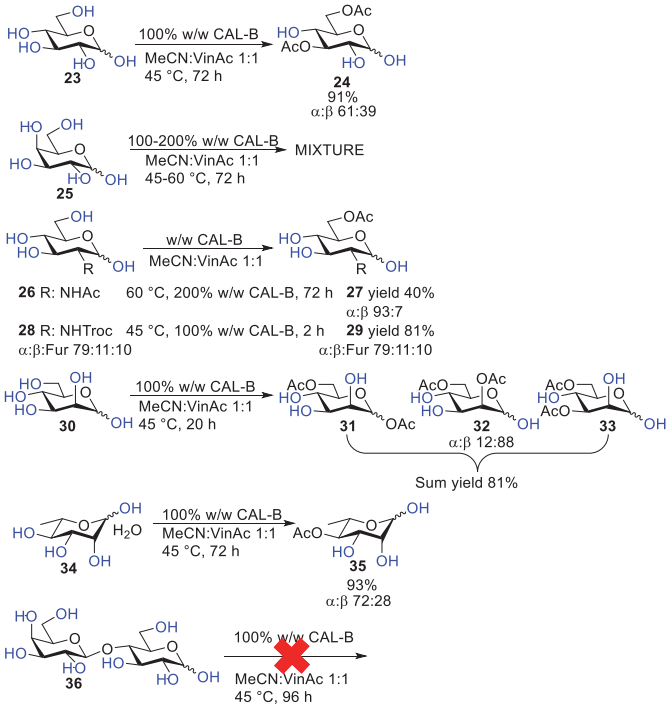
Scheme 4. CAL-B Acetylation Reactions with Fully Unprotected Saccharides, Where: D-Glucose, D-Glucosamines, and L-Rhamnose Gave Selective Acetylation Products; D-Mannose, a Mixture of Diols; D-Galactose an Unpurifiable Mixture; and D-Lactose Did Not React[a]



[a]Reaction conditions: saccharide (60−100 mg), MeCN/vinyl acetate 1:1 (0.08 M of substrate), 100−200% w/w CAL-B, 45−60 °C; yield shown are isolated yields.

~ 2.5:1 mixture of 2,6-*O*-diAc **3** and 3,6-*O*-diAc **5** isomers (Table 1, no. 2). Pyridine has been shown to inhibit enzymatic activity,[24] so reactions were performed in pure chloroform or pure MTBE. Surprisingly, these reactions gave, under unoptimized conditions, a single diastereomer of an ethylidene acetal between the fourth and sixth positions (product **7**) in around 50% yield (Table 1, nos. 3 and 4). Acetals are commonly used protecting groups in oligosaccharide synthesis, although rarely synthesized by enzymatic methods other than glycosidic bond formation. Acetaldehyde needed for acetal formation is a byproduct of the transesterification, but it is also formed via the hydrolysis of vinyl acetate even under dry conditions.[33] To confirm, a reaction with thioglycoside **2** in chloroform and vinyl acetate without CAL-B was run, and it showed no acetal formation. Thus, a new enzymatic method was found for 4,6-*O*-ethylidene acetal formation with vinyl acetate. In further screening of solvents, we found that only traces of the desired 2,6-*O*-diAc product **3** were formed with both vinyl acetate and acetic anhydride as acylating reagents running the reaction in acetone (Table 1, nos. 5 and 6). Acylation with acetic anhydride in MeCN favored the formation of the 3,6-*O*-diAc isomer **6** (Table 1, no. 7). Its selective synthesis has been described previously.[25] Replacing acetic anhydride with vinyl acetate changed the regioselectivity of the acetylation, selectively affording 2,6-*O*-diAc product **4** in high yield (72%) in 48 h (Table 1, no. 8). However, there were some unreacted starting materials and monoacetates still present. It is supposed that the acylating reagent as a cosolvent influences the conformation of the active site pocket of CAL-B as well and thus the regioselectivity.[30,29,25] Increasing the amount of vinyl acetate in THF gave high selectivity toward the wanted 2,6-*O*-diAc **6** (Table 1, no. 9). The reaction time was considerably long (96 h) as only 20% CAL-B was used. The selectivity was increased further, and the reaction time decreased to 48 h by switching THF to MeCN, which had shown the best results with a lower amount of vinyl acetate, still with 20% CAL-B (Table 1, no. 10). Increasing the amount of CAL-B to 100% decreased reaction time to 24 h, and diacetate **4** was isolated in 80% yield (Table 1, no. 11). (For full optimization, see Table S1 in the SI.) Finally, the background reaction was investigated. CAL-B was omitted from the reaction mixture, and no acetylation occurred in 96 h (Table 1, no. 12).

Previously, it has been shown that Novozyme 435 can be recycled successfully in the deacetylation reactions.[23] Three cycles with thio-D-galactopyranoside **2** were conducted using the same recycling conditions (Table S2). After each reaction, the immobilized enzyme was washed with dichloromethane (DCM) (~50 mL) and air-dried for 1 h. The first cycle resulted once again in an 80% isolated yield but started to fall after the second cycle in 50% yield. By the end of the third cycle, monoacetylated products started to dominate; some

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starting material was leftover, and the yield of 2,6-*O*-diAc **4** decreased further to 42%. We surmise that the enzyme or the resin was acetylated, which led to either the enzyme deactivation or inhibited the starting material from reaching the catalytic site.

Next, the acetylation of thioglycosides of various monosaccharides was studied (Scheme 3). When the leaving group in D-galactose was changed to 2,6-dimethyl thiophenol (compound **8**), the reaction efficiency decreased, but the regioselectivity of the reaction remained high. Increased amounts of enzyme, higher temperature, and longer reaction time were needed to get to 2,6-*O*-diAc product **9** in a similar yield (85%) as with thioglycosides **1** and **2**. It is known that changing the stereochemistry of monosaccharides, i.e., switching from one sugar to another, drastically influences regioselectivity and reactivity. That was proven once again as thio-β-D-glucopyranoside (compound **10**) had reached full conversion in just 30 min with 80% yield of the sixth position acetylated product **11**. The results obtained with thioglycoside epimers (D-galactose and D-glucose) **2** and **10** showed that the fourth position influences mainly the rate of the reaction as well as whether the acetylation reaction is producing a diacetate or a monoacetate. Thio-D-glucopyranosamine substrates **12** and **14** reacted for 4 h with acetylation occurring in the sixth position, resulting in products **13** and **15**. The reaction with the *N*-acetyl protecting group (compound **12**) was slightly less selective, affording monoacetate **13** in 71% yield with unselective overreaction products and starting material left, while phthalate had a very high selectivity, and 91% yield was achieved after product **15** was purified by crystallization (Scheme S2). D-Glucose-based compounds (**10**, **12**, and **14**) all reacted similarly; it seems that the amide in the second position does not influence regioselectivity but slightly lowers the rate of the reaction. Thio-D-mannopyranoside **16** gave selectively the sixth position β-product **17** in 76% yield in 20 h. The α-anomer of **16** did not react selectively, giving several products. The reaction with thio-D-mannopyranose **16** further showed the influence of the second position as comparing thioglycoside epimers (D-glucose and D-mannose) **10** and **16**, the rate of the reaction decreased 40 times. Acetylation of thio-L-rhamnopyranoside **18** resulted in the mixture of three different monoacetates **19**−**21**. Only 4-*O*-Ac **21** was managed to be isolated separately from this mixture. D-Lactose with *p*-chlorothiophenol leaving group **22** did not react at all. We surmise that it is due to solubility issues.

Selective derivatization of fully unprotected saccharides is a challenge because of their poor solubility in organic solvents. The solubility greatly influences the reaction outcome of unprotected saccharides and is the main limitation in CAL-B acetylation reactions.

Fully unprotected D-glucopyranose **23** reacted slowly with vinyl acetate in the presence of CAL-B (Scheme 4). Still, the reaction was highly selective, affording 3,6-*O*-diAc isomer **24** in 91% yield. Both increasing the temperature and the amount of enzyme led to a decrease in yield and still needed 3 days of reaction time to reach full conversion (Scheme S1). Unprotected D-galactopyranose **25** did not react selectively and led to a mixture of products. Some compounds were characterized, but they remained in mixtures (Table S3). Surprisingly, the corresponding thioglycosides (**1**, **2**, and **8**) reacted very selectively. For the D-glucopyranosamine series, solubility seemed to influence the outcome. Hydrochloric salt of D-glucosamine and phthalate-protected compounds had very poor solubility and did not react at all after 3 days (Scheme S2). Acetyl-protected glucosamine **26** reacted slowly and needed harsher conditions to get the sixth position-protected product **27** in 40% yield in 3 days, while 2,2,2-trichloroethoxycarbonyl (Troc)-protected glucosamine **28** reacted in just 2 h in 81% yield toward the sixth position-protected product **29**. The large difference in the rate of the reactions between the two D-glucopyranosamine compounds can be attributed to solubility. Similar to the thioglycoside counterparts (**12** and **14**), only the sixth position was acetylated. D-Mannopyranose **30** showed limited selectivity, giving a diacetate fraction with 81% yield including 1,6-*O*-diAc **31** (13%, α-anomer characterized only), 2,6-*O*-diAc **32** (63%, α/β 88:12), and 3,6-*O*-diAc **33** (24%, α-anomer characterized only). Some minor uncharacterized peaks most likely correspond to β-anomers of products **31** and **32**. It is contrary to thio-D-mannopyranoside **16**, which gave selectively the sixth position acetylation. The unprotected first position seems to allow selective diacetate formation for both epimers, D-glucopyranose and D-mannopyranose (compounds **23** and **30**), while the second position's configuration determines the regioselectivity. L-Rhamnopyranose **34** gave a slow reaction as well but with very high selectivity toward the fourth position acetylation with a 93% isolated yield of product **35**. The fourth position-acetylated L-rhamnopyranose had been previously synthesized in four steps, starting with benzyl α-L-rhamnopyranoside.[34,35] Similar to the thioglycoside of lactose, fully unprotected D-lactose **36** did not react within 4 days, most probably because of solubility issues.

Chemically selective acetylation occurs most often in the primary position of the target saccharide. Generally, it is followed by full protection of the saccharide and purification. In most cases, if partially protected sugar is needed, the anomeric position is protected by either a thio or methoxy group. Furthermore, high-boiling-point solvents like pyridine and dimethylformamide are used, which complicate the workup procedures; reactions are generally overnight if not longer, and high temperatures are used, e.g., with trityl chloride up to 100 °C.[36] While in our case most thioglycosides were also protected in the sixth position, the workup is much simpler and reaction times shorter with consistently high yields. For unprotected sugars, acetyl protecting groups cannot be selectively added via chemical means as the first position would be protected as well.

CONCLUSIONS

In conclusion, starting from thio-D-galactopyranoside, a new enzymatic acetylation method for the synthesis of the second and the sixth position-protected diacetate using CAL-B was found and optimized. During optimization, an unprecedented enzymatic acetal-forming method between the fourth and the sixth positions was also discovered. Unfortunately, recycling tests showed that CAL-B cannot be recycled as the yield dropped from 80% to 42% after the third cycle. Several new partially protected thioglycosides were synthesized while testing the CAL-B acetylation reaction. Furthermore, fully unprotected saccharides were used as substrates and acetylated selectively. For known compounds, the previously known synthesis pathways were shortened. To summarize, we have shown that CAL-B can be used to synthesize partially protected saccharides, which can be used further in the total synthesis of natural oligosaccharides.

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EXPERIMENTAL SECTION

General Experimental Information. Full assignment of ^{1}H and ^{13}C chemical shifts was based on the 1D and 2D (COSY, HSQC, and HMBC) FT NMR spectra measured with a Bruker AVANCE III 400 MHz instrument. Residual solvent signals were used ($CDCl_3$: δ = 7.26 ^{1}H NMR, 77.2 ^{13}C NMR; CD_3OD: δ = 3.31 ^{1}H NMR, 49.0 ^{13}C NMR; $(CD_3)_2SO$: δ = 2.50 ^{1}H NMR, 39.5 ^{13}C NMR; D_2O: δ = 4.79 ^{1}H NMR) as internal standards. High-resolution mass spectra were recorded with an Agilent Technologies 6540 UHD Accurate-Mass QTOF LC/MS spectrometer by using AJ-ESI ionization. Prior to analysis, the instrument was calibrated in a mass range of m/z 50−3200. Optical rotations were obtained with an Anton Paar GWB Polarimeter MCP 500. Melting points were determined using a NAGEMA-K8 polarizing optical microscope. Precoated Merck silica gel 60 F_{254} plates were used for TLC, and column chromatography was performed with Merck 60 (0.040−0.063 mm) mesh silica gel. Commercial reagents and solvents were generally used as received. DCM was distilled over CaH or phosphorus pentoxide, ethyl acetate (EtOAc) and acetone over phosphorus pentoxide, and MeOH and toluene over sodium. Petroleum ether (PE) had a boiling point of 40−60 °C. Silicon oil bath on top of a magnetic stirrer with heating was used as a heat source for reactions requiring heating. Immobilized *C. antarctica* lipase-B, Novozyme N435, with 10,000 (propyl laurate unit/g), was a kind gift from Novozymes A/S.

General Procedure for Preparation of 4-Chlorophenyl 2,6-Di-O-acetyl-1-thio-β-D-galactopyranoside ***4*** *with CAL-B.* 4-Chlorophenyl 1-thio-β-D-galactopyranoside **2** (60 mg, 0.196 mmol) was dissolved in MeCN/vinyl acetate 1:1 (2.4 mL), heated to 45 °C, and dissolved or stirred for 10 min. Stirring was set to 100 rpm, and CAL-B (60% w/w, 36 mg) was added. The reaction vessel was equipped with an air cooler with a $CaCl_2$ tube. The reaction was followed by TLC (DCM/EtOAc 1:2, R_f = 0.34), and upon completion, the reaction mixture was filtered. Immobilized enzymes were washed with DCM (∼50 mL), and the filtrate was concentrated in vacuo. The crude mixture was purified by silica gel column chromatography (PE/EtOAc 2:3 → 1:9), yielding a white solid (61 mg, 80%); mp 134−137 °C (from DCM); $[\alpha]_D^{20}$ + 7.1 (acetone, *c* 0.08); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41−7.46 (m, 2H), 7.24−7.30 (m, 2H), 4.98 (t, *J* = 9.7 Hz, 1H, H-2), 4.56 (d, *J* = 10.0 Hz, 1H, H-1), 4.36 (dd, *J* = 5.6, 11.7 Hz, 1H, H-6*a*/6b), 4.29 (dd, *J* = 7.0, 11.7 Hz, 1H, H-6*a*/6b), 3.95 (d, *J* = 3.2 Hz, 1H, H-4), 3.70 (t, *J* = 6.7 Hz, 1H, H-5), 3.68 (dd, *J* = 3.3, 9.4 Hz, 1H, H-3), 3.07 (s, 2H, OH-3,4), 2.15 (s, 3H), 2.08 (s, 3H); $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$): δ 171.3, 171.3, 134.4, 134.0 (2xC), 131.1, 129.1 (2xC), 85.9 (C-1), 76.2 (C-5), 73.4 (C-3), 71.0 (C-2), 68.9 (C-4), 63.1 (C-6), 21.2, 21.0; HRMS (AJS-ESI): $[M + Na]^+$ for $C_{16}H_{19}ClO_7SNa^+$, 413.0432; found, 413.0426.

Note: there is a small amount of leaching of resin occurring during the DCM wash.

Mmol-Scale Synthesis of 2,6-Dimethylphenyl 2,6-Di-O-acetyl-1-thio-β-D-galactopyranoside ***9*** *with CAL-B.* According to the general procedure with 2,6-dimethylphenyl 1-thio-β-D-galactopyranoside **8** (1.78 mmol, 534 mg), MeCN (11 mL), vinyl acetate (11 mL), 60 °C, and 200% CAL-B (1068 mg), the reaction was run for 48 h, resulting in a white solid (581 mg, 85%). TLC—DCM/EtOAc 1:4, R_f = 0.52; column chromatography eluent system DCM/acetone 0% → 15% acetone; mp 190−194 °C (from DCM); $[\alpha]_D^{20}$ + 41.5 (acetone, c 0.09); ^{1}H NMR (400 MHz, MeOD): δ 7.09−7.16 (m, 3H), 5.14 (t, *J* = 9.8 Hz, 1H, H-2), 4.35 (d, *J* = 10.2 Hz, 1H, H-1), 4.31 (dd, *J* = 8.2, 11.5 Hz, 1H, H-6*a*/6b), 4.08 (dd, *J* = 4.2, 11.5 Hz, 1H, H-6*a*/6b), 3.84 (ap.d, *J* = 3.0 Hz, 1H, H-4), 3.65 (dd, *J* = 3.4, 9.5 Hz, 1H, H-3), 3.58 (ddd, *J* = 0.8, 4.2, 8.1 Hz, 1H, H-5), 2.53 (s, 6H, 2xCH_3), 2.16 (s, 3H), 1.94 (s, 3H); $^{13}C\{^{1}H\}$ NMR (101 MHz, MeOD): δ 172.4, 172.1, 145.20 (2xC), 133.0, 130.2, 129.2 (2xC), 90.4 (C-1), 77.6 (C-5), 74.0 (C-3), 72.7 (C-2), 70.5 (C-4), 64.9 (C-6), 22.7 (2xC), 21.1, 20.7; HRMS (AJS-ESI): $[M + Na]^+$ for $C_{18}H_{24}O_7SNa^+$, 407.1135; found, 407.1131.

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c02467.

Experimental procedures, characterization data, and NMR spectra for all synthesized compounds (PDF)

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Notes

The authors declare no competing financial interest.

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Hariduskäik

2019–2025	Tallinna Tehnikaülikool, PhD
2013–2019	Strathclyde Ülikool, MSc First-Class Honours
2007–2013	Tallinna Reaalkool
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Keelteoskus

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2023–	Tallinna Tehnikaülikool, nooremteadur
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2016–2017	Sika Tehnoloogia AG, keemik
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Teaduspreemiad ja tunnustused

2023	Doktorantide lühipresentatsioonide võitja, Tallinna Tehnikaülikool
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2016	Distinction ja Certificate of Excellence in Practical Skills, Strathclyde Ülikool
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Teised publikatsioonid

2015	*Beilstein J. Org. Chem.* **2015,** *11,* 171–2178. https://doi.org/10.3762/bjoc.11.235

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