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On the Synthesis and Analysis of Steryl Glucosides

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Acknowledgements

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Abstract

Steryl glucosides (SG) and acylated steryl glucosides (ASG) are natural components of plant cell membranes and are therefore present in seeds and oils used in the biodiesel production. During the transesterification reaction of conventional biodiesel production, soluble ASG are converted into SG, which are insoluble in the non-polar fuel matrix. Especially after prolonged storage times at low temperatures, the formation of precipitates is observed, leading to the obstruction of filters, valves or pipes which may cause severe engine performance problems. Analysis of the compounds is difficult, commercially available pure standard materials are costly and their synthesis often requires time-consuming work-up procedures.

In the present master thesis, a new five-step preparation method for cholesteryl glucosides is evaluated and selected steps of the synthesis pathway are further investigated with regard to the used staring material as well as reaction conditions. The synthesised SG can be used as reference or standard material for the quantitative analysis of phytosteryl glucosides in biodiesel or other plant material. APCI-MS-TOF is intensively used in order to monitor the reaction progress and to identify formed intermediates, by-products and end-products.

Zusammenfassung

Sterylglucoside (SG) und acylierte Sterylglucoside (ASG) sind natürliche Bestandteile von Zellmembranen und kommen in unterschiedlichen Konzentrationen auch in vielen Saaten und Pflanzenölen, die Verwendung in der Biodieselproduktion finden, vor. Während der Umesterungsreaktion bei der Biodieselproduktion werden die im Biodiesel löslichen ASG in SG umgewandelt, die allerdings aufgrund ihrer höheren Polarität vor allem nach längerer Lagerung als Feststoff ausfallen. Diese feinen Partikel können Filter, Ventile oder Leitungen verstopfen und somit ein ernstes Problem hinsichtlich Motorschäden darstellen. Auch die Analytik dieser Verbindungen ist aufwändig, reine Standards für die Quantifizierung sind teuer bzw. bedeutet deren Herstellung einen hohen Zeitaufwand und teilweise den Einsatz teurer Chemikalien.

Im Rahmen der vorliegenden Masterarbeit wird eine neue, fünfstufige Synthesemethode für Cholesteryl- glucoside ausgehend von Disacchariden, evaluiert und einzelne, ausgewählte Stufen der Synthese näher untersucht bzw. deren Reaktionsbedingungen variiert sowie unterschiedliche Edukte eingesetzt. Die synthetisierten SG können als Referenz- oder Standardmaterial in der Analytik verwendet werden. APCI-TOF-MS wird eingesetzt, um gezielt den Fortschritt einer Reaktion zu verfolgen und um entstandene Intermediate, Nebenprodukte und Reaktionsprodukte zu identifizieren.

Contents

List of Abbreviations

APCI	Atmospheric pressure chemical ionization
ASG	Acylated steryl glucosides
BSA	N,O-bis(trimethylsilyl) acetamide
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
COC	Cool on column
DAG	Diacyl-glycerol
DCM	Dichloromethane
ELSD	Evaporative light scattering detector
EN	Europäische Norm, European Standard
eq.	equivalents
FAME	Fatty acid methyl esters
FBT	Filter blocking tendency
FID	Flame ionization detector
FS	Free sterol
GC	Gas chromatography
HPLC	High performance liquid chromatography
LDL	Low density lipoprotein
MAG	Monoacyl-glycerol
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PG	Protecting group
R_f	Retention factor
r.t.	room temperature
SE	Esterified sterol
SG	Steryl glucoside
SIM	Single ion monitoring
SPE	Solid phase extraction
TCA	Trichloroacetimidate
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TMSOTF	Trimethylsilyl trifluoromethanesulfonate
TOF	Time of flight
UDP	Uridine diphosphate
UV	Ultraviolet
vol	Volume

1 Introduction

Steryl glucosides (SG) and acylated steryl glucosides (ASG) are naturally present in cell membranes where they fulfil important biological functions and determine the properties of membranes. [1] Many seeds exhibit high SG and ASG concentrations in different ratios and therefore plant oils used in the biodiesel industry may also have significant levels (300-2300 ppm [2]) of these compounds. During the transesterification reaction in biodiesel production (fatty acid methyl esters, FAME), the fat soluble ASG are converted into SG, which are insoluble in this non-polar matrix and precipitate especially after prolonged storage times at low temperatures. The formed particles can lead to filter blocking or plugging of valves and pipes which subsequently may lead to serious engine performance problems. [3] Haze can be observed in FAME already at low concentration levels (ppm region) and even at room temperatures. [2], [4] In addition, the particles can act as 'seed crystals', promoting the crystallization of other substances present in the matrix. [5] Methods to remove SGs from biodiesel like filtration [6], [7], adsorption [7], distillation [2], [7] or enzymatic hydrolysis [8]–[10] are described in the literature. However, simple, technically feasible and economically profitable solutions have not yet been found in a satisfactory manner. Due to the increased demand in alternative fuels, there exists a high interest in solving the above mentioned problems related to biodiesel.

In addition, analysis of these compounds is complex and their synthesis requires time-consuming preparation methods respectively the use of costly chemicals. Commercially available internal standards are expensive, e.g. 10 mg of cholesteryl-β-glucopyranoside with a purity of ≥97% can be bought for 173.50 € from Sigma-Aldrich [11] .

The working group of Martin Mittelbach could already work out a new synthesis route starting from disaccharides for the preparation of SG. The displayed synthesis comprises a five-step reaction sequence whereof selected steps were further investigated in the present master thesis. The synthesised compounds can be used as reference or standard material for the challenging analysis of these molecules.

Furthermore, APCI-TOF-MS is extensively used as an analysis method due its excellent performance for the high sensitive analysis of steryl glucosides.

2 Review of Literature

2.1 Phytosterols and Their Sterol Conjugates

2.1.1 Structure

Sterols and their derivatives belong to an important class of natural compounds which can be found in plants, animals, bacteria and fungi. They are biogenetically classified as triterpenes and are derivatives from androstane which possesses a tetracyclic backbone structure of condensed aliphatic rings with additional methyl groups at C10 and C13. The aliphatic rings are named with letters from A to D and the C- atoms are numbered serially (see Figure 1). Sterols are additionally characterized by a hydroxy group at C3, a hydrocarbon side chain at C17 and a double bond either between C5 and C6 (Δ^5-sterol) or C7 and C8 (Δ^7-sterol) in ring B. [12], [13]

Triterpenes are naturally synthesized by the assembling of six mevalonate units into squalene via a two-step reaction process. Subsequent enzymatic ring closure steps and further transformations lead to the formation of various triterpenes. [14]

Due to variations in length of the side chain, position and number of double bonds and the presence of stereoisomers, over 250 different compounds have been reported. [1], [15]

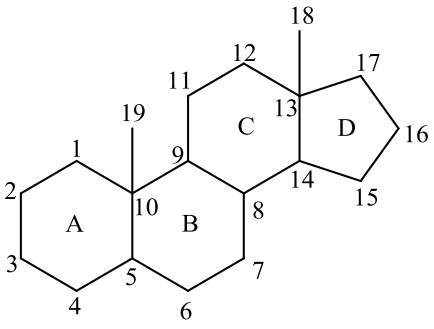


Figure 1: The structure of androstane

Cholesterol is the most common zoosterol in nature as it plays a central role in the physiology of animals. It is an essential part of cell membranes, helps to absorb fatty acids in the intestine and serves as starting material for the synthesis of other sterols. It is a Δ^5 sterol having 8 carbon atoms in the branched side chain. In plants little or no cholesterol is found but instead they contain different types of phytosterols. Their chemical structure is similar to cholesterol but they possess additional methyl- or ethyl groups at C-24 in the side chain. [14]

Sterols are further classified into three different groups, namely 4-desmethyl sterols, 4α-monomethyl sterols and 4,4-dimethyl sterols whereat the latter two serve as sterol precursors and are usually minor components in plant materials. The majority of plant sterols have a double bond between C-5 and C-6 and frequently trans-Δ^{22} double bonds are found in the

side chain. Compounds saturated at the ring B are called stanoles e.g. sitostanol. The most common sterols representatives found in nature are sitosterol, stigmasterol and campesterol (see Figure 2). [1], [13]

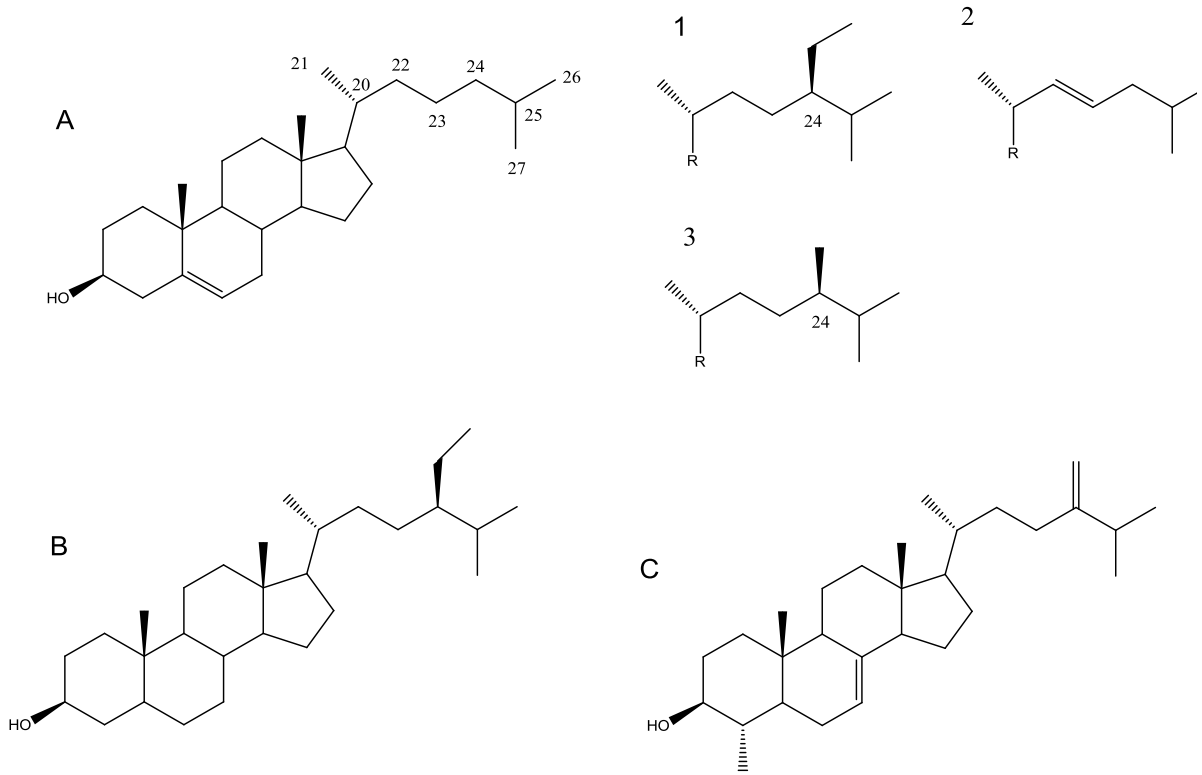


Figure 2: Structure of different sterols; A: cholesterol and with a similar sterol backbone (R): 1: sitosterol, 2: stigmasterol, 3: campesterol; B: sitostanol; C: gramisterol

2.1.2 Chemical Properties of Sterols

The apolar hydrocarbon side chain at one end and a hydroxy group at the other end of the ring system accounts for the amphiphilic character of sterols. The longer the side chain, the more hydrophobic it becomes. Double bonds in the side chain, on the other hand, increase their hydrophilicity. Exposure to heat, light, metal contaminants and oxygen initiate an autocatalytic free radical chain reaction by the attack of preformed radicals at the double bond in ring B. Major reaction products like 7-hydroxy or 7-keto compounds (see Figure 3) are formed after epimerization of the 7-hydroperoxide intermediate. This oxidation reaction is the most important chemical reaction of sterols which helps to control certain processes and determines their storage stability. [1]

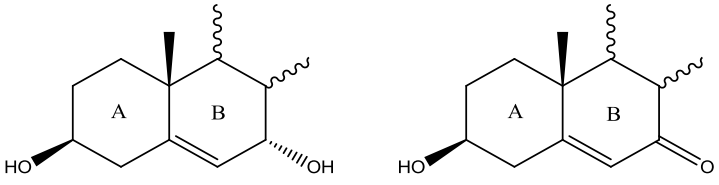


Figure 3: 7α-hydroxy oxidation product (left) and 7-ketone oxidation prouct (right)

2.1.3 Sterol Conjugates

Plant tissue contains different forms of phytosterols: free sterols (FS), esterified sterols (SE), steryl glycosides (SG) and acetylated steryl glycosides (ASG) (see also Figure 4). Unlike in the free sterol, the C3 OH-group at ring A of the other conjugates is covalently bound to other molecules. In the case of SE, the OH-group is esterified to a fatty acid and, in SG, the sterol is bound to a hexose via a 1-O-glycosidic linkage. ASG possess an additional ester bond with a fatty acid at the 6-OH of the sugar moiety. [1], [14]

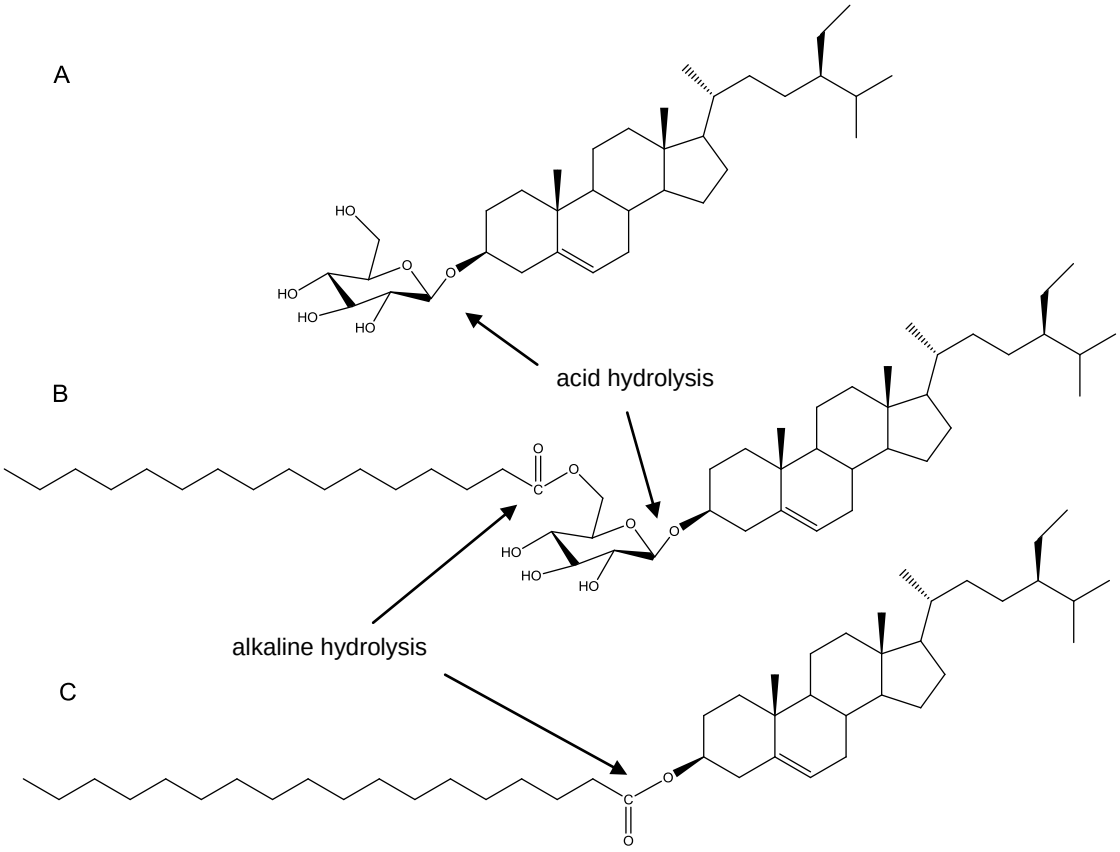


Figure 4: Sitosteryl β-D-glucoside (A), sitosteryl (6'-O-palmitoyl) glucoside (B) and sitosteryl stearate (C) The sites of cleavage via acid and alkaline hydrolysis are marked with arrows

2.1.3.1 Steryl Glycosides and Acylated Steryl Glycosides

SG have four different sites of variation as they can possess various sterols as well as different types and numbers of sugar moieties. The glycosidic linkage can adopt either α- or β-configuration and acylation with fatty acids (mostly palmitic- and oleic-acid) of the carbohydrate is also possible. The most common sugar in monoglycerides is the pyranose

form of D-glucose being linked to the sterol in β-configuration. Other sugars in SGs involve β-D-galactopyranose, β-D-glucopyranuronic acid or α-D-ribofuranose and β-D-xylopyranose. Also, di- and oligoglycosyl sterols can occur in different configurations. [12] Kovganko and Kashkan published a review describing 49 different types of SG and ASG. [16]

2.1.3.2 Synthesis of Steryl Glycosides

The biosynthesis of steryl glycosides is carried out by so called sterol glycosyltransferases which can catalyze the conversion of free sterols to steryl glycosides. The enzymes require an activated sugar donor which is commonly UDP-glucose but other sugars like UDP-rhamnose or UDP-galactose can act as a donor as well. Also more than one sugar moiety can be attached resulting in the formation of steryl di- and oligoglycosides. The mechanism is supposed to involve a ternary complex. Thus, both, the sugar donor as well as the sterol substrate need to bind to the enzyme simultaneously. [12], [17]
In the lab, the glycosylation of sterols can be carried out via the Koenigs-Knorr-reaction using acetobromoglucose and the corresponding sterol as starting material. [15] After the formation of the glycosidic linkage, the protecting groups of the sugar moiety have to be removed. Also, other glycosidation reactions like the trichloroacetimidate-method can be applied; again the use of protecting groups of the sugar moiety are necessary. For further details on glycosidation reactions see Chapter 2.2. A biocatalytic approach is also possible, but due to the requirement of costly activated UDP-sugar donors, large scale synthesis using steryl glycosyltransferases is of minor importance. Münger describes the use of glycolases under reverse hydrolysis conditions in organic solvent systems as these enzymes normally catalyze the cleavage of glycosidic bonds. [15]

2.1.4 Biological Functions of Sterols and Conjugates

Sterols are integrated in membranes of plant and animal cells where they play an important role in determining their physical properties. As a sterol molecule exhibits the same length as a phospholipid monolayer (ie 2.1 nm), it can well be incorporated into membranes. The hydrophobic side chain reaches into the core of membrane bilayers, whereas the free hydroxy-group points towards the aqueous phase to interact with phospholipids and proteins. [1] Sterols are found in the plasma membrane, the outer membrane of mitochondria and the endoplasmatic reticulum and take part in the regulation of the fluidity and permeability of said membranes and of membrane-associated metabolic processes, such as simple diffusion, carrier-mediated diffusion and active transport. Also, cell division, cellular differentiation and proliferation are influenced by the presence of sterols which explains their accumulation in seeds and oils. During greening, maturation, aging or ripening of plant tissue, a change in the

sterol composition can be observed which also affects membrane functions as different sterols have different efficiencies in regulating the membrane fluidity. [1], [14]
Moreau et al. stressed the importance of a fast conversion of FS to SE, SG and ASG in membrane lipid metabolism. As a control mechanism of these interconversions, phytohormone levels as well as environmental factors like light, temperature and water stress are described which indicates the role of sterol conjugates in adaption of membranes on changing conditions. SG and ASG are important components of cell membranes with their sugar moiety orientated into the aqueous region, whereas SEs are poorly soluble in phospholipid bilayers. [1], [14] Having a sugar moiety attached to the sterol, the hydrophilic part of the molecule increases which leads to a change in its biophysical properties. [12] The exchange of SG and ASG between the phospholipid bilayer is slower than the exchange rate of free sterols which helps to regulate sterol content and distribution within the membrane. [17] There are several indications that SGs inherit important biophysical properties although only a few conclusions between them and any biological phenomenon could have been drawn so far. [12] Chaturvedi et al. described the role of steryl glycosides in plants to respond to heat and cold stress due to their influence on membrane functions (e.g. fluidity and phase transition). SG production is involved in the signal transduction system of heat shock protein triggering, which helps to adapt to environmental heat stress in plants. Heat shock experiments on human foetal fibroblast cells indicate the participation of cholesteryl glycoside to heat stress in mammalian cells, as well. [17]

2.1.5 Health Promoting Effects of Sterols and Conjugates

Representing a risk for cardiovascular disease, lowering elevated serum cholesterol levels is of high importance and is being intensively investigated. [14] Plant steryl glucosides and sterols show the ability to suppress the intestinal absorption of dietary and biliary cholesterol which results in a decrease of serum total and LDL (low-density lipoprotein) cholesterol concentrations. Therefore, these compounds are incorporated into functional foods, for example margarine or salad dressings. [12], [14] In addition, further beneficial health effects of plant sterols such as anti-cancer and antioxidant activities are reported. [14]

2.1.6 Food Sources

Highest concentrations of sterols are found in seeds and oils followed by cereal grains or nuts. The intake values of humans depend largely on the type of food consumed as well as living on a certain diet. Vegetarians, for example, can have a daily intake of up to three [1] or four [13] times higher than non-vegetarians whose total daily intake of phytosterols per person per day is estimated to range from 100-500 mg. [1], [13]

Table 1: Food sources of common sterols derived from animals and plants, [13]

Sterol	Food source
cholesterol	animal fats, beef and pork, egg yolks
β- sitosterol	fruit and vegetables, soybeans
stigmasterol	coconuts-, rapeseed-, soybeans-, sunflower- oil, mature tomatoes, various vegetables
campesterol	rapeseed-, soybeans- oil, radishes and other types of vegetables

Also genetic factors, growing and storage conditions as well as processing conditions can have an impact on the sterol content and composition in plants, which together with variations in analytical data can lead to variations and inconsistency of the results. [1] Up to 60% of the total sterols in plant foods derive from glycosylated sterols. High amounts of fresh matter were found in cocoa powder, chestnuts, cashews and soybeans. Higher contents in dry matter were observed in some vegetables like cauliflower, zucchini, broccoli or cucumbers, but the dilution in the actual food has to be considered. Depending on the belonging of food samples to different plant families, different ASG/SG ratios and sterol compositions can be observed. [18]

2.2 Carbohydrate Chemistry

Carbohydrates represent the majority of organic matter on the earth's surface, 10^{11} t cellulose is synthesized by plants per year. In the past, molecules with the formula $C_n(H_2O)_m$ were named a carbohydrate, but today the definition also includes oxidized and reduced derivatives as well as molecules with other atom- types. Carbohydrates are classified according to the number of their subunits as mono-, di-, tri-, tetra-, penta-, oligo- and polysaccharides. [13], [19]
A detailed description of the classifications, structure and nomenclature of carbohydrates can be found in the literature (e.g. [13], [19]) and will not be further discussed in this thesis.

2.2.1 Protecting Groups

The knowledge of using protecting groups is of great importance when performing organic synthesis. Generally, in carbohydrate chemistry, common protecting groups for hydroxy

groups are used, but the high amount of OH-groups already present in a monosaccharide has to be kept in mind. Another important aspect is the regioselective and orthogonal introduction or removal of the protecting groups. The influence of the used groups on the reactivity of the protected molecule respectively the active participation of the protecting groups themselves in further reactions needs to be considered, as well. [19] Attention must be paid to the different reactivity of the various OH-groups within one molecule of a carbohydrate. The anomeric hydroxy group possesses a unique rank as it is embedded in a hemiacetal structure. The other hydroxy groups represent the function of an alcohol, but the differences in reactivity between primary and secondary groups have to be considered as well. [19] Esters, acetals and ethers represent the most common groups for the protection of hydroxy groups of carbohydrates. Selected representatives of the classes are described in more detail below.

2.2.1.1 Esters

Esters are very popular, due to the ease of their synthesis with acid anhydrides or acid chlorides as well as their simple removal. The formation of esters reduces the polarity of the sugar, leading to an improved dissolution in organic solvents. Furthermore, their possible participation in chemical reactions has to be considered. Benzoates can be formed by adding benzoyl chloride in pyridine as a solvent. Using less reactive reagents (e.g. N-benzoylimidazole) or carefully controlling the synthesis conditions can successfully lead to selective benzoylation. The removal can be achieved by transesterification in methanol and the addition of sodium for prolonged reaction times. Acetates are less robust protection groups compared to benzoates, the regeneration to the parent alcohol can be rapidly and quantitatively achieved by sodium in methanol. The introduction can be carried out in different ways: using acetic anhydride in pyridine gives the same anomeric ratio as in the free sugar and acid catalysis leads to the thermodynamic reaction product. Adding sodium acetate leads to an anomerization reaction of the free sugar and, as a consequence, to the preferred acetylation of the more reactive β-anomer (see also overview in Figure 5). Selective protection can be achieved by low reaction temperatures or special reagents. Other ester protection groups are found within the groups of carbonates, borates, phosphates, chloroacetates or sulfates and nitrates. [19]

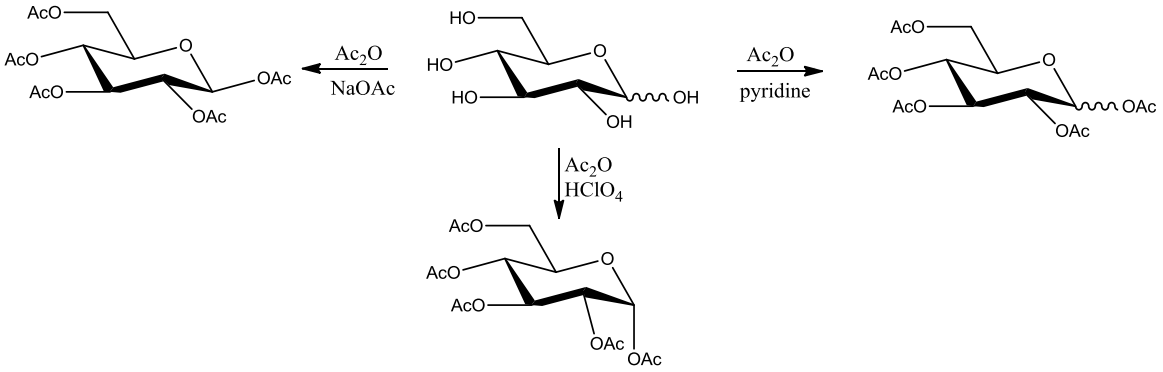


Figure 5: Different reaction conditions and reaction outcomes for the protection of D-glucose with acetyl protecting groups

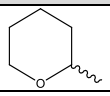
2.2.1.2 Ethers

Ethers are much more robust protecting groups than esters, being stable in mildly acidic conditions and also under basic conditions. Methyl ethers are used for structure elucidation rather than for the protection of hydroxy groups, as they are too stable and not easily removed. Benzyl and allyl ethers can be introduced under neutral, basic or acidic conditions. Removal of benzyl groups can also be carried out via different pathways using hydrogenolysis, catalytic transfer hydrogenolysis, birch reaction conditions or anhydrous ferric chloride. Allyl ethers can be removed by isomerization to prop-1-enyl and versatile following procedures. Silyl ethers are not only used for chemical modification but also as protection groups, as they are very inert concerning most of the common synthetic conditions but can be removed by the supply of fluoride ions (e.g. HF/pyridine) or under acidic conditions (e.g. aqueous HF). [19]

2.2.1.3 Acetals

Acetals are useful groups for the protection of the anomeric center as their introduction can be designed in such a way that only the anomeric hydroxy group is protected. Thus, this allows the reaction of the remaining free OH- groups with other protecting groups. Selectively removing the acetal group at the anomeric center leads to the formation of protected sugars with a free hydroxy group at C1. But also the protection of simple alcohol groups can be performed with acetals which can be easily removed under given conditions (see Table 2). [19]

Table 2: Different acetal protecting groups and reaction conditions for their removal [19]

Protection group	Cleavage conditions
	H^+
CH_3OCH_2-	H^+
$CH_3OCH_2CH_2OCH_2$-	$ZnBr_2$, CH_2Cl_2 or H^+

Using cyclic acetals enables the protection of diol systems. Commonly benzaldehyde and acetone are used as reactants which leads to the formation of 1,3-dioxane and 1,3-dioxolane derivatives. Generally, ketones lead to the formation of cyclic 5-membered acetals whereas aldehydes build six-membered rings which is due to the avoidance of 1,3–diaxial interactions (see Figure 6) . [13], [19]

Benzylidene groups can easily be removed (acid treatment, birch reaction) leading to the reversion to the parent alcohol, but the conversion to other functional groups, e.g. benzyl ethers, can also be performed. Isopropylidene protection groups can be removed for example with trifluoroacetic acid–water systems, the selective removal of one group is possible using methanol and acidic conditions or immobilized acids on silica. [19]

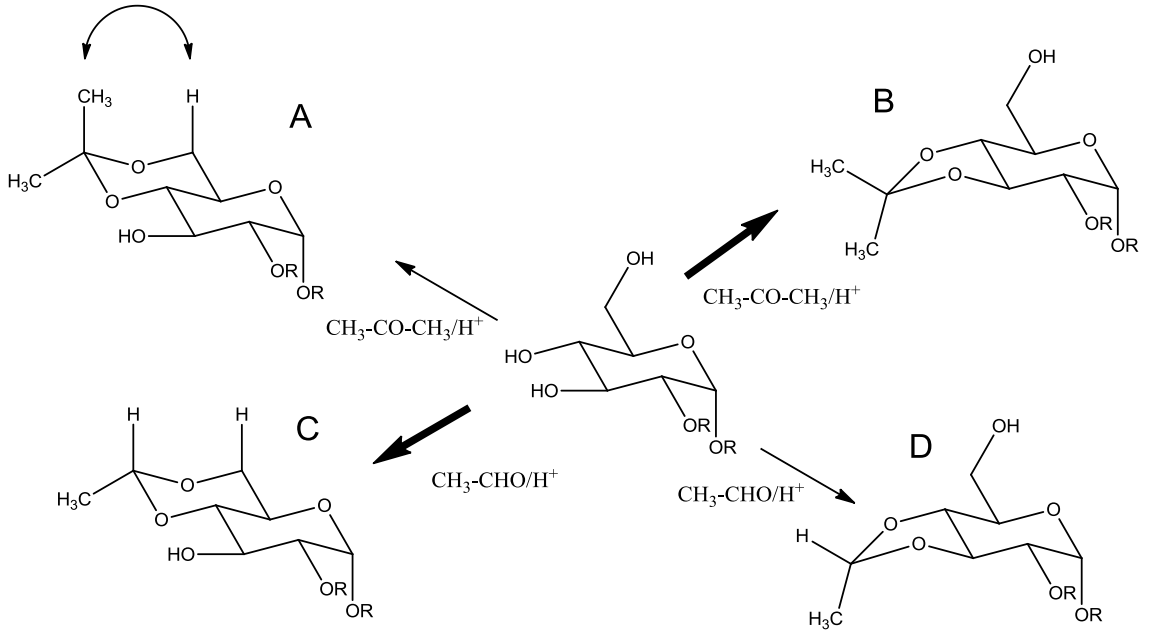


Figure 6: Using acetone as protecting group, mainly product B can be observed due to 1,3 diaxial interactions in the dioxane ring; the use of an aldehyde as protecting group leads primarily to the formation of compound C due to the higher stability of dioxane rings compared to dioxolane rings

As previously mentioned the anomeric OH-group is very reactive and forms glycosides with alcohols under acid catalysis. The sugar acts as a glycosyl donor and the glycosyl acceptor is called aglycon in the formed reaction product (see Figure 7). The glycosidic linkage is cleaved under acidic conditions with e.g. diluted acids. If the heteroatom forming the

glycosidic linkage is oxygen, the products are called O-glycosides. Structures having other heteroatoms are named analogously e.g. N-glycosides or S-glycosides. Glycosides can be numerously found in nature. Their preparation, however, can be tricky. Activation of the glycosyl donor is necessary, protecting groups have to be carefully chosen and introduced to address the desired OH-groups of the donor as well as of the acceptor, and the required stereoselectivity of the product needs to be considered during the synthesis scheme. [13], [19]

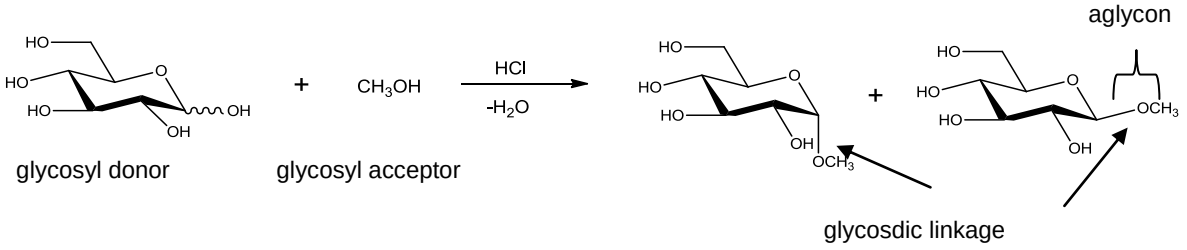


Figure 7: Glycosidation reaction of glucose with methanol under acid catalysis to yield methyl α- (left) and β- (right) D-glucopyranoside

As seen in Figure 8, proposed mechanisms of the glycosidation reaction suggest a species with significant positive charge, most commonly at the anomeric carbon respectively oxocarbenium ion intermediates. [19] The reactivity and stability of these intermediates depend on various factors, some of which are further discussed in this chapter. The solvent has influence on the spatial tightness of the ion pair (contact ion pair or solvent-separated ion pair). The anion may shield the oxocarbenium ion or the opposite face if anion exchange is possible. Furthermore, solvents with a high dielectric constant or an electron lone pair (e.g. diethyl ether) can have a stabilizing effect on the built positive charge. Another important aspect is the influence of the substituent, usually a protected hydroxy group, at C2 of the glycosyl donor on the glycosidation reaction. Ethers represent inert protecting groups being electronically passive and in the case of benzyl ethers, steric hindrance to the acceptor molecule is installed. Esters do not only have an influence concerning steric hindrance but they represent electron withdrawing groups, discouraging the positive charge at C1 to be built-up. Sometimes, the glycosyl acceptor has to be activated, for example, by introducing trityl, silyl or stannyl ethers which increase the polarization of bonding electrons towards oxygen and therefore leading to a higher nucleophility of the oxygen atom. [19]

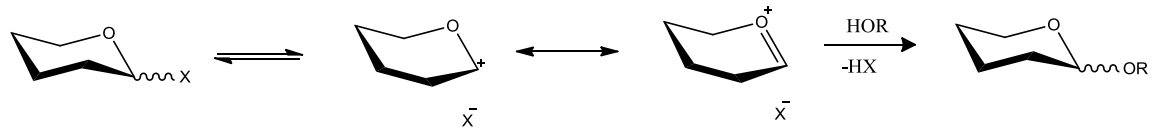


Figure 8: Proposed mechanism and occurring intermediates during the formation of a glycosidic linkage

The synthesis of glycosides can be classified into different methods:

a) Fischer-Helferich- method
b) Koenigs-Knorr-reaction
c) Trichloroacetimidate (TCA) method
d) C-1-O alkylation

A detailed description of those methods can be found in the literature, b) and c) are discussed in more detail below. [13]

2.2.1.4 Koenigs-Knorr-Reaction

The Koenigs-Knorr reaction uses glycosyl halides such as acetobromoglucose as starting material, which can easily be prepared from fully acylated glucose by the reaction with HBr/HOAc. The reaction yields α-acetobromoglucose in a stereospecific manner due to the anomeric effect and the electronegativity of the bromine atom. [13] Heavy metal salts are used as promoters of the reaction; they can either be used in a heterogeneous process as insoluble salts or in a homogeneous reaction mode if soluble salts are used which influences the reaction mechanism. Using heterogeneous promoters, the reaction operates as a 'bimolecular', 'push-pull' mechanism which leads to the inversion of the stereochemistry at the anomeric center. During the homogeneous reaction, a S_N1- like transition state is proposed under participation of the neighbouring acetyl protecting group at C2 (see Figure 9). [13], [19]

As the introduced alcohol faces two possible sites to attack, not only the formation of the glycoside is observed but also the formation of an orthoester which can rearrange into the glycoside under the prevailed acidic conditions (see Figure 9). [19] Using acetyl esters as protecting groups, the formation of the β-glycoside is preferred. Compounds lacking this neighbouring-group effect (e.g. the use of benzyl ethers) give a mixture of α- and β-glycosides. [13] The formation of orthoesters leads to a diminution of yield and in addition, large-scale synthesis using the Koenigs-Knorr reaction is not advisory due to the use of toxic, heavy metals. Today, reactions with glycosyl fluorides are gaining more and more attraction. [19]

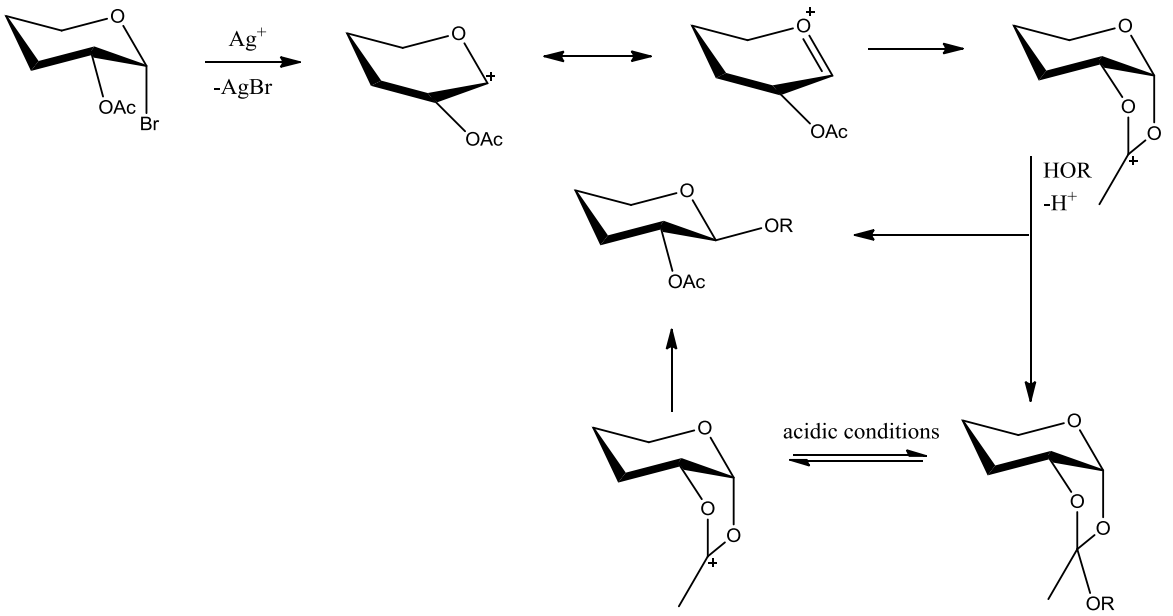


Figure 9: Proposed mechanism of the Koenigs-Knorr reaction with participation of the protecting group at C2. As side-product, the formation of ortho-esters can be observed which can rearrange to the oxocarbenium intermediate under acidic conditions

2.2.1.5 Trichloroacetimidate-Method

O-Glycosyl-trichloroacetimidates have good glycosyl donor properties; they are stable and can be stored for a longer period of time and heavy metal reagents can be avoided. Therefore, it is nowadays the preferred method compared to the Koenigs-Knorr reaction. [13], [19] The method is based on the addition of alcohol anions (alcoholates) to electron-poor nitriles, also known as Pinner reaction [20]. The reaction is carried out under basic conditions using dichloromethane (DCM) as a solvent and choosing the suitable base leads to the formation of anomerically pure compounds, as can be seen in Figure 10.

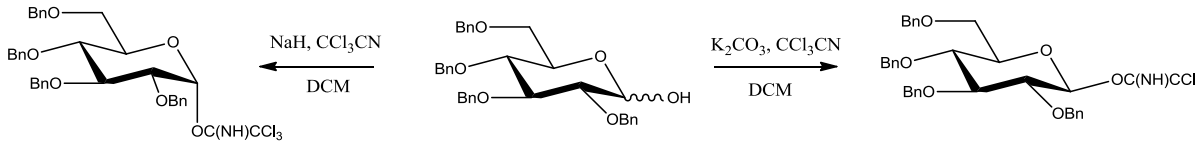


Figure 10: Reaction of tetra-benzylated glucose with trichloroacetonitril gives the trichloroacetimidate derivative of the sugar. Using NaH results in the formation of the α-anomer, the addition of potassium carbonate yields in the formation of the β-compound

In the presence of sodium hydride in combination with prolonged reaction times, the formation of *O*-α-glucopyranosyl-TCA is favoured whereas with potassium carbonate the β-anomer can be obtained. In the first step, the β-oxide adds faster to the nitrile which is due to the higher nucleophility of the β-oxide based on steric and stereoelectronic effects. The

repulsion between the lone pairs of the ring oxygen and the free electron pairs of the anomeric oxygen is more severe in the β-position than in the α-position leading to a higher reactivity of the β-anomer (see Figure 11).

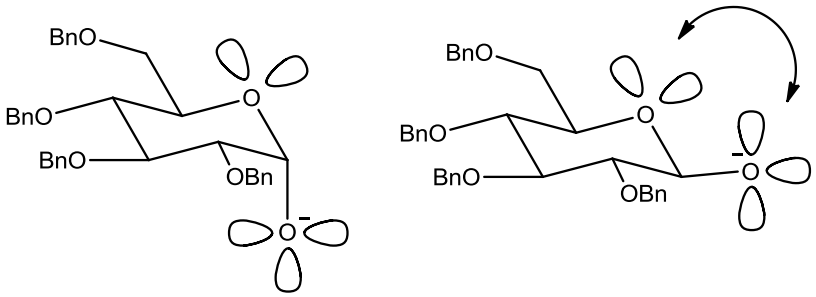


Figure 11: Repulsion between free electron pairs is higher in the β-oxide

Thus, β-TCA is primarily formed which can be converted into the thermodynamically more stable α-compound during a slow, base catalyzed reaction. Potassium carbonate is a weaker base than NaH and can only catalyze the reaction of the β-imidate, but not the reverse reaction leading to the anomerization of the compound and is therefore used in the kinetically controlled reaction mode. During the second step, the TCA-derivative can further react to form a glycosidic bond with a glycosyl acceptor. A promoter like boron trifluoride diethyl etherate or trimethylsilyl triflate is used, and with careful selection of the reaction conditions, compounds can be obtained stereoselectively. Using a mild promoter and donors with non-participating protection groups at C2 leads to products with an inverted configuration at the anomeric center, whereas the configuration of the reaction product with stronger promoters (like trimethylsilyl triflate) depends on the solvent. Dichloromethane gives the α-D-glycoside, the β-anomer is formed when acetonitrile or propionitrile is used. [19]

2.3 Biodiesel and Problems Related to Steryl Glucosides

2.3.1 Basics and Production of Biodiesel

Fatty acid methyl esters (FAME), derived from natural fats and oils, are the dominant alternative fuel used for diesel engines in the transport sector. Each year about 16 million tons are produced whereof vegetable oils (first generation) such as rape seed oil, soybean oil and palm oil are the most important feedstocks. Also, other feedstocks like used cooking oil, animal fat or oils derived from microorganisms like microalgae (second and third generation biodiesel) are used or are under investigation. [21] FAMEs are classically produced by transesterification of triacylglycerols with methanol using homogeneous catalysts like sodium methylate and sodium or potassium hydroxide under release of glycerol. [21], [22] The

resulting biodiesel can be directly used as neat fuel for vehicles with diesel engines or, more commonly, it is used in a blend with conventional diesel fuel. [3], [23] In Europe, according to EN 590, it is permitted to use up to 7 vol/vol% of biodiesel in blend with conventional diesel. The added biodiesel must meet the standards specified in EN 14214. [3], [24] One of the key goals of Europe 2020, formulated by the European Commission, includes the reduction of green house gas emissions by 20% compared to the amount in 1990. Not only that but also an increase of the share of renewable energies on the total energy consumption of up to 20% by the year 2020. This will, thus, increase the demand on fuels derived from sustainable resources even further. [25]

2.3.2 SG in Biodiesel

As already discussed in Chapter 2.1, SG and ASG are natural components of plant membranes and are therefore present in vegetable oils and fats. ASG, which are soluble in vegetable oils, are converted into insoluble SG during the transesterification reaction used in conventional biodiesel production (see Figure 12). High contents of ASG in the feedstock lead to a biodiesel with a high amount of SG. Severe problems of the fuel arise due to the poor solubility of SG in FAME. They occur as dispersed fine solid particles which can cause filter plugging, the obstruction of valves, pipes or sensors and fuel system clogging. This leads to an increased risk of engine performance problems or engine failure. [3], [4], [5] Even at room temperature and low concentration levels in the ppm region, the presence of haze, which consists of particles from 10 to 15 microns, can be observed. [2], [4] Also, the promotion of crystallization of other components like monoacylglycerols and the formation of aggregates exacerbates above mentioned issues. Storage at low temperatures accelerates the formation of precipitates. Due to the high melting point of steryl glycosides (240°C), heating to achieve transit through a blocked filter is not easily feasible. At high levels and sufficient storage time, the precipitates settle to the bottom of tanks and vessels ‘hiding’ their filter plugging potential. [4] [5] As the feedstock of biodiesel originates from natural material, the composition of the final product depends not only on processing parameters for vegetable oils and biodiesel production but also on the type of oil used, environmental and climatic conditions, growing area and the properties of different plant species, which results in high fluctuations of SG and ASG levels in the used oils and fats. [3] [5] Also, the formed precipitates in biodiesel may contain no or only little amounts of SG. Additionally other compounds like untransesterified partial glycerides (MAG or DAG) can be present in the solid material. [5] According to Tang et al. [7] or Lee [2], the amount of ASG in plants is approximately two to ten times greater than the concentration of SG, both being present in the mg/kg range in crude oils. About 2300 ppm SG is found in crude soybean oil and palm oil but the amount in corn and sunflower oil is lower, being about 500 and 300 ppm,

respectively. [2] The processed biodiesel contains 28 to 270 ppm steryl glucosides if derived from soybean oil, 480 ppm if originated from corn oil and 140 ppm if made out of palm oil. [26] According to EN 14214, the upper limit of the amount of total contamination in biodiesel is 24 ppm. [24]

Using the so called „filter blocking tendency test", the filter blocking capabilities of fuel is measured. The fuel sample is passed through a specified filter at 20°C and the pressure difference across the filter is measured. If solid matter is present in the sample, it shows a low performance in the test. [4]

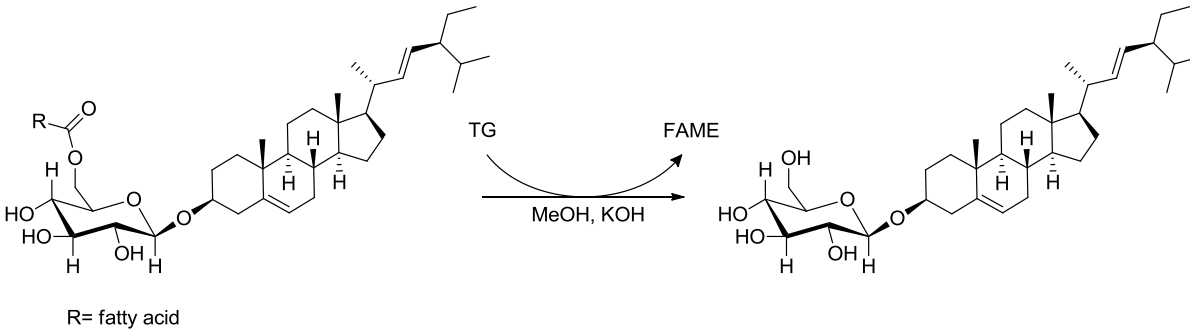


Figure 12: Alkaline hydrolysis of acylated steryl glucosides to steryl glucosides during the transesterifiaction reaction in FAME production

2.3.3 Removal of SG from Biodiesel

2.3.3.1 Filtration, Adsorption, Centrifugation

Filtration, adsorption and centrifugation methods are able to reduce the SG content in biodiesel with high concentrations thereof, hence being present as solid particles. [7] Filtration is a simple and direct method to reduce precipitates formed in biodiesel. Tang et.al [7] investigated the effect of room temperature (r.t.) soak filtration and cold soak filtration using different types of membrane filters on the concentration of SG in biodiesel. Lower temperature treatment showed a higher removing efficiency than filtration at r.t., furthermore, the addition of different types of adsorbents improved filtration performance significantly. Also different adsorbent and centrifuge treatments were able to reduce SG contents markedly from 161 ppm to a threshold value of about 20 ppm in soybean oil based biodiesels. A reduction below this threshold value is not achieved by these methods as they do not have an impact on the soluble SG fraction. [7] Danzer et al. suggested a cold filtration step as clean up treatment directly after the transesterification during the biodiesel production process in order to obtain biodiesel with improved qualities. Therefore, the biodiesel is cooled from generally 143°C to about 21°C and a filter aid (e.g. diatomaceous earth, silica, clay,

cellulose, magnesium silicate) is added after a certain residence time (1-1.5 h) to form a slurry which is then filtered to remove the particulates. The added adsorbent acts as a filter aid, building up a filter cake which helps to remove the precipitates of the biodiesel at a sub-micron level. [6] Others applied magnesium silicate and bleaching earth as adsorbent material at a temperature range from 65 to 80°C reducing SG contents from 97.6 ppm to 20 ppm. [27] However, filtering techniques as well as the addition of adsorbents does involve an additional step during biodiesel production, which is not only expensive but also time-consuming. Furthermore, filtration and centrifugation methods require the presence of solid particles which in turn means waiting for the compounds to precipitate respectively to form aggregates before those techniques can be applied. Also, losses of biodiesel can be observed leading to a reduced overall yield of the produced biodiesel. [9] Another patent describes the removal of precipitates in biodiesel and the prevention of their formation by subjection to ultrasound energy e.g. in an ultrasonic bath or a flow through an ultrasonic apparatus. This can happen in any location like the storage tank at the refinery, a fuel station pump or a fuel line of a vehicle. Typically, between 5000 and 7000 Joules of energy are applied. To prevent overheating of the fuel due to high levels of thermal energy generated by intensive ultrasound, the energy is pulsed. Dissociation of the present precipitates from 50% up to 90% can be achieved. Treating a biodiesel sample to prevent the formation of solid particles leads to a reduction of 70 to 90% of precipitates compared to untreated control samples. [23]

2.3.3.2 Distillation

Distillation is a conventional method for treating biodiesel in order to reduce its filter blocking tendency (FBT). Tang et. al [7] reported a reduction of the SG content to a non-detectable level. Although purification by distillation is the most effective method for the removal of SG and ASG content in biodiesel, the simultaneous reduction of natural antioxidants has a negative impact on the oxidation stability of the fuel. Furthermore, from an economical point of view, this method is expensive and inefficient as a lot of energy needs to be invested in order to heat and cool the biodiesel. [2], [7]

2.3.3.3 Enzymatic Reactions

Brask et al. [8] describe the possibility of removing SG in biodiesel by converting the compounds back to their acylated form using enzymatic catalysis achieving SG contents below 50 ppm to below 5 ppm, whereas the initial amount of SG present ranges from 30 to over 300 ppm. Enzymes that are able to transfer an acyl group to the sugar show lipase or acyltransferase activity and should preferably use a fatty acid alkyl ester as acyl donor. Using

immobilized enzymes has several advantages compared to free enzyme powder or liquid formulations like: the easy recovering of the catalyst, the possibility to implement a continuous process, higher operation stability and the operation under low-water conditions. There are various suitable ways of immobilizing the enzymatic catalyst e.g. using inorganic carriers (diatomaceous earth, silica), synthetic resins or natural polysaccharide carriers. [8] Menzella et al. [9] claim that many steryl glycosidases and lipases do not work in an efficient way and are not able to effectively reduce SG levels in biodiesel. Therefore, they presented thermostable enzymes that can hydrolyse the glycosidic linkage between the sterol and the sugar moiety of SG and ASG. Also, they provide the generation of 'designer enzymes' which can be used for the elimination of key impurities in biodiesel that are responsible for the formation of precipitates such as saturated monoacylglycerols or SG. Enzymatic reactions work best when the substrates are in solutions because enzymes cannot efficiently access compounds being fixed in agglomerates. The solubility of SG and their accessibility rises with increasing temperature which in turn reduces enzymatic activity and therefore can cause denaturation of the enzymes. The solubility of SG in biodiesel lies around 50 ppm at 50°C, which is the optimal reaction temperature of the enzymes presented by Brask et al. Being that the steryl glucoside content of crude biodiesel ranges from 10 to 300 ppm, a significant amount of compounds may not be addressable by the enzymes. The designed thermostable enzymes are capable of working at high temperatures between 50 and 110°C. The incubation time of the proposed method ranges from 30 minutes to several hours, depending on process parameters and the properties of the sample like viscosity, mixing rates, temperature, amount of SG present or the amount and the activity of enzymes added. A reduction of 20 to more than 80% of initial SG content can be achieved, leading to better quality fuel and, due to the cost-effectiveness, to a fuel which is competitive in the market. [9] Other groups describe the hydrolysis of SG as well, leading to the formation of in biodiesel completely soluble sterols and glucose which can be eliminated during water-washing steps. Optimization of the reaction conditions lead to the successful scale of up to 20 tons where 7 g of enzyme per ton of fuel were stirred at 65°C leading to a complete removal of steryl glucosides, compared to an initial concentration of 75 ppm, within approximately 2 h. Furthermore, a continuous stirred-tank reactor is under development, which due to recycling processes, prevents loss of biodiesel. The losses are lower than losses during the distillation method (>3%) or the removal with magnesium silicate (1.27%). After enzymatic treatment the resulting biodiesel is comparable to distilled fuels concerning its performance. According to the group, the presented method is able to reduce the SG content efficiently at affordable costs. [28] Münger and Nyström presented a method of enzymatic hydrolysis and removal of ASG as well as SG in crude oils before the transesterifiaction step of biodiesel production. The used enzymes carry out hydrolysis of the glycosidic bond in ASG and SG and favour

temperatures between 30 and 55°C; the incubation time ranges preferably between 15 and 25 hours. The reaction can be carried out at an early stage of biodiesel production, during the degumming step of vegetable oils and therefore prohibits later precipitation of SG in biodiesel. According to the patent, the enzymes are able to completely hydrolyse present ASG and SG. [10]

2.3.3.4 Pretreatment

Being that the inclusion of extra steps after the transesterification during the production of biodiesel increases process complexity and costs, the removal of ASG from the feedstock represents yet another interesting option. Degumming and bleaching steps have shown to diminish the SG and ASG content of vegetable oils. Enhancing and optimizing these steps could help to avoid the introduction of additional clean- up treatments in the transesterification chain. [29]

2.4 Analysis of Steryl Glucosides

The problems related to formed precipitates in biodiesel as well as the interest in biological functions and quantification in food requires applicable analytical tools and methods for the determination of SG and ASG, often within a complex matrix. Sampling, sample preparation and the choice of appropriate internal standards play a crucial role during the development of analysis methods. [30] Sensitivity, selectivity and the estimation of recoveries (sample preparation) are important factors for the quantification of SG. [15]

Hexane, which is also commonly used for the extraction of lipids, is also suitable for the quantitative extraction of free and esterified sterols whereas more polar solvents, e.g. a chloroform-methanol mixture, are needed for a complete extraction of glycosylated sterols. To separate the different sterol classes, liquid chromatography, solid phase extraction (SPE) or preparative thin-layer chromatography (TLC) can be used. Silver ion chromatography separates sterols according to the total number of C-C double bonds in the molecule. [14], [31] Hydrolysis of acylated species (SE, ASG) to the non-acylated ones (FS, SG) using alkaline hydrolysis can simplify the sample pre-treatment. [31]

2.4.1 Liquid Chromatographic Methods

High Performance Liquid Chromatography (HPLC) can be used for both, qualitative separation and quantitative analysis of phytosterol classes. The separation of the different sterol conjugates can be performed using normal phase HPLC columns (e.g. silica, DIOL) whereas reversed phase columns (e.g. C18, phenyl) are used to distinguish between the different species within one class. For quantification, separation into polar and non polar classes is recommended. [14] However, some SG are co-eluting at most proposed methods due to their structural similarity (e.g. stigmasteryl and campesteryl glucoside) and would therefore require a mass spectrometry detector for their individual detection. [15] Different universal detectors are applicable for the HPLC measurements. UV-detection (200 nm) enables the use of free sterols standards for the quantification of SG as the sugar moiety does not affect the UV absorption at this wavelength, avoiding the requirement of standard materials. However, to verify chemical integrity, recovery efficiency and to test the accuracy and precision of the analytical method, appropriate standards are a necessity. Furthermore, the mobile phase has to be selected carefully to guarantee high sensitivity at this wavelength region. [31] Murui and Wanaka (1993) described the measurement of SG in vegetable oils using HPLC under isocratic conditions and UV detection. SG and ASG were isolated from an oil sample using SPE (silica gel cartridge) and the two compound classes were then separated using TLC. ASG were converted into SG by alkaline hydrolysis followed by derivatization to the corresponding UV-sensitive 1-anthroylnitrile steryl glucosides, which enables detection at 254 nm and increases the sensitivity allowing the detection of trace amounts of SG. [31], [32] Using an evaporative light scattering detector (ELSD) permits the use of all common HPLC-solvents in isocratic and gradient mode. The use of a standard material is necessary due to the mass dependency of the detection, but as already

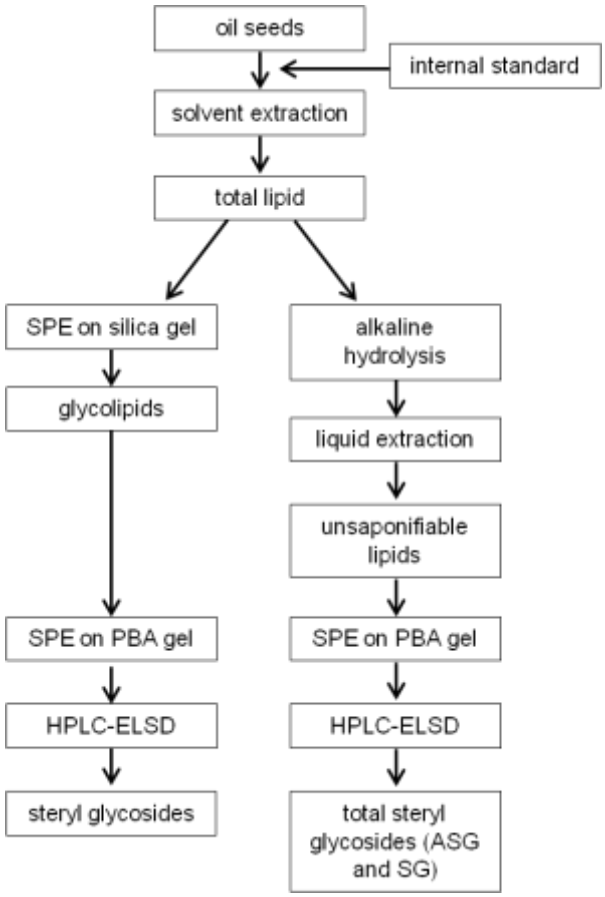


Figure 13: Sample preparation and analysis scheme (adapted from [31]) for the quantification of SG and ASG from oil seeds using HPLC-ELSD

discussed, appropriate standards are essential for a total analysis protocol. [31] The method of Moreau et al. (2002) injects a prepared total lipid extract in two different HPLC systems in order to quantify the non-polar class of sterol conjugates (FS, SE) and the polar class (SG, ASG) separately. Both systems use a Diol column and an ELSD detector, but different solvent gradient programs for the two classes (hexane based phase for the non polar classes, hexane/isopropanol/water mixtures for SG and ASG) are applied. [14] The HPLC-ELSD method of [31] aims at the determination of Δ7- and Δ5-steryl glycosides without the need of derivatization or acidic hydrolysis steps prior to analysis. Sample preparation included extraction of the lipid fraction from pumpkin seeds, SPE on modified silica gel linked to phenylboronic acid and mild alkaline hydrolysis of ASG to SG. Figure 13 gives an adapted and simplified overview of the described analysis method.

2.4.2 Gas Chromatographic Methods

The analysis using GC methods is commonly used for the qualitative and quantitative determination of SG and FS due to its high sensitivity, simplicity and accurateness. [15], [31] The separation of structurally similar sterol species as well as the sensitivity of GC methods is generally higher than the HPLC procedures. [14] Flame ionization detectors (FID) are most frequently used which have the advantage of almost equal responses for all free sterol species. [15] SG can be measured as intact conjugates but more frequently, acidic hydrolysis of the glycosidic linkage prior to GC analysis to free sterols and glucose is applied. Due to the limited volatility of the intact compounds, cleavage of the glycosidic bond and further chemical alteration improves the measurement. [15] The main drawback of acid hydrolysis is the lability of some SG species to the applied acidic conditions leading to isomerizations and the generation of *de novo* sterol species (artefacts). Affected sterol species are for example Δ7-phytosterols, Δ5-avenasterol or lathosterol. [14], [15], [31] Using enzymatic hydrolysis can overcome the mentioned problems. Often derivatization to form trimethylsilyl (TMS) ethers is performed for a higher resolution and stabilization of thermo-labile sterols (prevention of dehydration and decomposition). [14], [15] As an alternative to acid hydrolysis, the hydroxy groups of intact SG are directly silylated and the TMS ether derivatives are subjected to GC measurement avoiding therefore the formation of artefacts. High temperature ramps up to 370°C are necessary due to the high boiling point of SG, often cool on column (COC) injection is used. [30] The high costs of commercial standard material however is a problem as well as the uncertainty of equal detector responses for all compounds have not yet been tested. [15] A complete derivatization of the hydroxy groups is critical in order to avoid broad and overlapping signals which depends on appropriate silylation agents and is influenced by the matrix. [30] An overview of the different sample preparation methods is given in Figure 14.

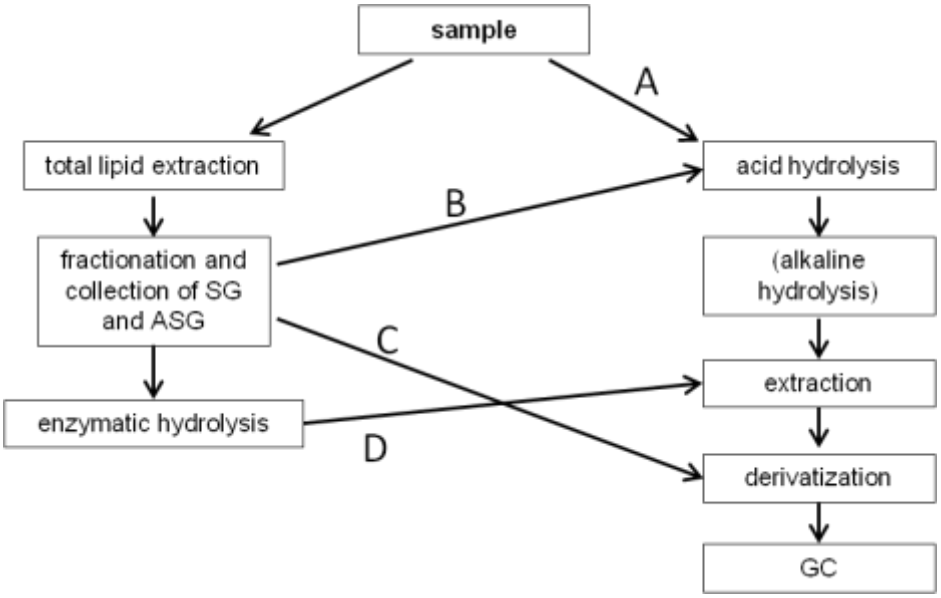


Figure 14: Scheme of GC analysis methods for SG (adapted from [15]); A: total sterol analysis; B:total SG analysis with acid hydrolysis; C: direct total SG analysis without hydrolysis; D: total SG analysis with enzymatic hydrolysis

2.4.3 Mass Spectrometry

Mass spectrometry (MS) is a valuable tool for the identification and quantification of sterols. GC-MS with electron impact (EI) ionization is commonly employed for identification of free phytosterols whose fragmentation patterns are included in online databases. One has to be aware that the published spectra include data for free sterols as well as for their TMS-ether and acetate-ester derivatives. However, the identification of unknown compounds is only possible by comparison with library spectra of compounds of the same form. [14] LC-MS methods are not yet well-established but promising techniques for sterol analysis [15] and high-resolution MS allows a non-destructive and direct measurement of complex lipids [33]. [15] Using soft ionization methods like atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) enables the direct measurement of sterols and their intact conjugates. [14], [15] The differences in ionization efficiency of polar and non-polar molecules has to be considered. ESI and APCI are applicable for SG due to their high polarity compared to free sterols. Identification and quantification of SG can be performed with direct infusion MS/MS experiments and a triple-quadrupole (Q) or a Q-TOF detector. [33] SG form different ion species like $[M+NH_4]^+$, $[M+Na]^+$ or $[aglycone+H-H_2O]^+$, depending on the infusion conditions. [15] Time-of-flight mass analyzers provide extremely high resolution and mass accuracy, at present, they achieve a resolving power of $10\text{-}60*10^3$ and a mass accuracy of 1-5 ppm. [15]

2.4.4 SG determination in Biodiesel

Pieber et al. describe the analysis of SG in biodiesel using high temperature GC/MS detection with COC injection for the identification and quantification of the compounds in FAME. A sample of FAME is weighed into a GC vial followed by the addition of silylation reagent (BSA+TMCS) and for quantification an internal standard (solution in pyridine) is added. After incubation at 70°C for 1 h and dilution with pyridine, the sample was subjected to GC measurement. Identification is performed using scan mode with a mass range from 50 to 1000 m/z, for quantification SIM mode m/z values of 147, 204 and 217 are used because these mass to charge ratios represent characteristic ion fragments of the silylated glucose moiety of all steryl glucoside species. Coelution with other biodiesel components is not observed at the retention range of silylated SG. As already mentioned, complete silylation of the glucose moiety is essential, which is especially problematic in the complex FAME matrix due to the presence of other reactive hydroxy groups. Therefore, the choice of an appropriate silylation reagent and the addition thereof in excess is necessary. Using this method, a limit of quantification of 50 µg/kg is possible (SIM mode) and a limit of detection of 40 µg/kg was determined (scan mode). [30] Bondioli et al. purified the obtained biodiesel samples using silica micro-cartridges prior to analysis. To the fraction containing the analyte, BSTFA (silylating agent) is added and after reacting for 30 min. at ambient temperature, the samples are prepared for GC/FID and GC/MS analysis with temperatures reaching 360 respectively 370°C. The limit of detection is estimated to be around 10 mg/kg whereas recovery rates range between 71 and 88%. [22] Moreau and colleagues analyzed SG in precipitates present in biodiesel using HPLC methods. HPLC-ELSD, under normal-phase isocratic conditions, is able to reach a detection limit of 100 ppm and therefore the method can be used for the quantification of SG in precipitates of biodiesel samples, but is not sufficient for the quantification in FAME as already low concentrations < 50 ppm may cause filtration problems. Alternatively, HPLC-APCI-MS can be used for the identification of SG next to common FAMEs. No molecular ion was detected rather the ions of the sterol moieties. [5] Others distilled the biodiesel sample at 180°C and 3 mbar and used the residue (glycerides, sterols, tocopherols and SG) for further analysis. Derivatization of an aliquot of the distillation residue using a silylation reagent is performed and the treated sample is measured using GC. The concentration factor reached by distillation varies between 40 and 50%, the limit of detection is below 15 mg/kg and a high recovery factor of the internal standard (betulin and tricaprin) is obtained (>99%). [34] A method for the quantification of ASG and SG in their native forms in biodiesel was presented by [35]: Sample preparation is carried out using column chromatography and after silylation of the fraction of interest and subsequent measurement using GC/FID is performed. A limit of quantification of 10 mg/kg for SG and ASG and recovery rates between 75% and 90% are stated. Using HPLC-ELSD

with high carbon load C18 columns allows a higher sample load and provides a better resolution of non-polar and polar compounds. Using a sample pre-treatment simply by centrifugation in combination with this HPLC-ELSD method, concentration levels of SG of around 0.01 mg/mL are achieved. However the method is only applicable for biodiesel samples with a level of SG of ≥30 ppm. [36] Identification of different types of SG can be performed using MS with positive APCI ionization, ammonium-adducts of the molecular ions of different SG species as well as H^+ adducts of characteristic sterol fragments are detected. [34]

3 Experimental

3.1 Chemicals

3.1.1 Solvents

Acetone, HPLC, VWR Chemicals
Acetonitrile (CH_3CN), for HPLC, VWR Chemicals
Chloroform ($CHCl_3$), for HPLC, Sigma-Aldrich
Dichloromethane (DCM) (techn.), Institute of Chemistry, University of Graz
Diethyl ether (Et_2O) (techn.), Institute of Chemistry, University of Graz
Dimethylformamide (DMF), for synthesis, Merck
Dioxane, 98%, Institute of Chemistry, University of Graz
Ethanol (EtOH), 96%, VWR Chemicals
Ethanol (EtOH), absolute pro analysis VWR Chemicals
Ethyl acetate (techn.), VWR Chemicals
Methanol (MeOH), for HPLC, VWR Chemicals
n-Hexane, for HPLC, VWR Chemicals
Petrol ether, 40-60°C, DA3, reinst, Roth
Pyridine, ≥99%, Sigma-Aldrich
Toluene, for HPLC, VWR Chemicals

3.1.2 Reagents

Acetic anhydride, for synthesis, Institute of Chemistry, University of Graz
Amberlyst® 15, Merck
Ammonium chloride (NH_4Cl), ≥ 99%, Institute of Chemistry, University of Graz
Benzoylchloride (BzCl), 99%, Institute of Chemistry, University of Graz
Benzylbromide (BnBr), 98%, Institute of Chemistry, University of Graz
Calcium chloride ($CaCl_2$), (techn.), Institute of Chemistry, University of Graz
Celite® 545, 89.6%, Roth
Charcoal, pro analysi, Merck
Cholesterol, ≥ 92.5%, Sigma
Cholesteryl-β-D-glucopyranoside, 97%, Sigma Aldrich
Concentrated hydrochloric acid (HCl conc., 37%), Institute of Chemistry, University of Graz
Concentrated sulfuric acid (H_2SO_4 conc.), 95-98%, Roth
Cyclohexene, for synthesis, Institute of Chemistry, University of Graz
Formic acid (HCOOH), ≥98%, Sigma-Aldrich

Hydrogen bromide, 33% solution in acetic acid (33% HBr/AcOH), for synthesis, Merck
Iron (III) chloride ($FeCl_3$), reagent grade, Institute of Chemistry, University of Graz
Lactic acid, ≥98%, Institute of Chemistry, University of Graz
Palladium hydroxide on carbon ($Pd(OH)_2/C$), 20 wt%, Sigma-Aldrich
p- Toluenesulfonic acid (TsOH), Merck
Silica gel 60 (0.015-0.040 mm), Merck
Silylation reagent, BSA+TMCS for GC, with 5% trimethylchlorsilane, Fluka
Sodium bromate ($NaBrO_3$), ≥ 99%, Sigma-Aldrich
Sodium chloride (NaCl), (techn.), Institute of Chemistry, University of Graz
Sodium hydride (NaH), 95%, Aldrich Chemistry
Sodium hydrosulfite ($Na_2S_2O_4$), (techn.), Institute of Chemistry, University of Graz
Sodium sulfate (Na_2SO_4), ≥ 99%, Institute of Chemistry, University of Graz
Sucrose, powdered sugar, Wiener Zucker
Trehalose dihydrate, ≥99%, Sigma-Aldrich
Trichloroacetic acid (Cl_3CCOOH), 99.5%, Institute of Chemistry, University of Graz
Trichloroacetonitrile, 98%, Aldrich Chemistry
Triethylamine (Et_3N), 99%, Institute of Chemistry, University of Graz
Triethylsilane (Et_3SiH), 99%, Aldrich Chemistry
Trifluoromethanesulfonic acid (triflic acid, TfOH), 98%, Sigma-Aldrich
Trimethylsilyl trifluoromethanesulfonate (TMSOTf), 99%, Aldrich Chemistry

3.2 Instruments

3.2.1 Sample Preparation

Balance, Sartorius BP3100P, max. 3100 g, d=0.05 g
Analytical balance, AND GR-200, max. 210 g, d=0.1 mg
Autovortex, SA6, Stuart Scientific, Staffordshire, United Kingdom
Rotavapor, IKA® RV10 digital with water bath HB 10 digital and membrane vacuum pump ABM MZ 2C, Schwabach, Germany
Water content determination: Metrohm Coulometer 831 KF, Metrohm Stirrer 728

3.2.2 HPLC-TOF-MS

TOF-MS: Agilent 6230 TOF LC/MS G6230B
Agilent 1260 Infinity Series
HiP Degasser G4225A
Bin Pump G1312B

ALS Autosampler G1329B
TCC Columnthermostat G1316A
DAD Detector G4212B
Software: MassHunter Workstation Rev.B.05.01SP2

3.2.3 HT-GC-MS

Agilent Technologies 7890A GC system
Agilent Technologies 5975C inert XL MSD with Triple-Axis Detector
Agilent Technologies 7693 Autosampler
Software: MS ChemStation Data Analysis G1701EA E.02.02.1431

3.2.4 NMR- spectroscopy

Bruker Avance III spectrometer, 300 MHz
Broadband observe probe head

3.2.5 Autoclave

Parr Instruments Company Series 4570 High pressure/high temperature reactor (max. 350 bar at 500°C) with 500 mL reaction vessel and 4848B reaction control system

Büchi Glas Uster (CH) Art.Nr 49.07052.0001, Fabr. Nr. 4204 with 2.1 L reaction vessel

3.3 Synthesis of Steryl Glucosides

3.3.1 Introduction of Disaccharide Protecting Groups

3.3.1.1 1,3,4,6-Tetra-O-benzyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside (2a)

NaH (8.29 g, 346 mmol) was suspended under vigorous stirring in dry DMF under nitrogen atmosphere in a 1000mL three-necked flask. Sucrose (**1a**) (10.76 g, 31.4 mmol) in 100mL dry DMF was added drop wise, followed by the addition of benzyl bromide (34 mL, 286 mmol, 9 eq) at 0°C. Afterwards the solution was stirred at rt for 4½ h and monitored using TLC (n-hexane/ethyl acetate 2/1) during which the solution changed colour to yellow and the formation of a white precipitate occurred. The reaction was stopped by adding 30 mL of MeOH and the mixture was evaporated to dryness. The remaining residue was extracted with 200 mL water and 100 mL chloroform and the aqueous layer was extracted with chloroform two more times (2x100 mL). The combined organic layers were washed with a saturated solution of NaCl and water (2x100 mL) and dried over Na_2SO_4. After filtration and evaporation of the solvent, a dark brown oil (35.11 g) was obtained which was dissolved in 100 mL diethyl ether, filtered over celite®/charcoal (1 cm of celite and 1 spatula of charcoal) and evaporated under reduced pressure resulting in a brown oil.
Crude yield: 33.47 g, 31.5 mmol, >99% of theory
R_f: 0.67 (n-hexane/ethyl acetate 2/1)

3.3.1.2 1,3,4,6-Tetra-O-benzoyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranoside (2b)

A 5.0426 g (14.7 mmol, 1eq.) sample of dry **1a** was treated with 50 mL of dry pyridine in a 250 mL two-necked flask equipped with a drying tube and a dropping funnel and cooled on an ice bath. Dry benzoyl chloride (14.8 mL, 129 mmol, 8.7 eq.) was added drop wise while stirring for over 1 hour during which the formation of a white precipitate was observed (salt). After the addition of BzCl, the solution was allowed to attain ambient temperature and stirred for another 2½ h until TLC confirmed complete protection of the sugar. The solution was poured into 52 mL of ice-water, which resulted in the formation of a colourless syrup which was dissolved in $CHCl_3$ (25 mL). The two layers were separated, the aqueous phase was extracted again with chloroform (2x20 mL) and the combined organic layers were washed with water (2x50 mL) and dried overnight (Na_2SO_4). After filtration, the solvent was removed under vacuum resulting in a white to yellow syrupy residue.
Yield: 17.3256 g, 14.7 mmol, >99% of theory
R_f: 0.72 (acetone/toluene 1/9)

3.3.1.3 1,3,4,6-Tetra-O-acetyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (2c)

To a stirring solution of **1a** (5.4384 g, 15.9 mmol) in pyridine (36 mL), dry acetic anhydride (20 mL, 212 mmol, 13 eq) was slowly added at 0°C. The solution was allowed to attain rt upon which it changed its colour from colourless to slightly pink. The reaction was stirred over-night and poured on 49 g of ice. The resulting white precipitate was dissolved in chloroform (20 mL) and the two phases were separated. The aqueous phase was extracted two more times with $CHCl_3$ (2x20 mL), the combined organic layers were dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a yellow oil. Upon standing, the product crystallized out.

Yield: 10.7232 g, 15.8 mmol, >99% of theory

R_f: 0.22 (ethyl acetate/n-hexane 1/1)

MP: 49-50°C (Lit.: 69-70°C [46])

3.3.1.4 2,3,4,6-Tetra-O-benzyl-α-D-glucopyranosyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside (2d)

NaH (1.0123 g, 42 mmol) and trehalose (**1b**) (0.9893 g, 2.9 mmol) were stirred in dry DMF at 0°C. Benzyl bromide (4.7 mL, 40 mmol, 14 eq.) was added drop-wise and after complete addition, the ice bath was removed. After 4 h, the solution showed a bright yellow colour and the formation of a white salt. The reaction was quenched with 3 mL of MeOH and the solvents were removed under vacuum. The obtained oily, brown residue was extracted with water and DCM (25 mL each). The organic phase was washed with water (2x10 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The obtained brown oil was dissolved in Et_2O (13 mL) and filtered over celite®/charcoal (1 cm of celite and 1 spatula of charcoal) followed by removal of the solvent to afford a brown oil (3.9221 g). Due to contaminations (visible in TLC and TOF-MS), the oil was dissolved in diethyl ether (22 mL) again, stirred for several minutes, filtered over celite®/charcoal and dried under vacuum to give a yellow oil.

Yield: 3.908 g (3.7 mmol), 127% of theory

R_f: 0.72 (acetone/toluene 1/9)

3.3.1.5 2,3,4,6-Tetra-O-benzoyl-α-D-glucopyranosyl 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranoside (2e)

To a solution of **1b** (0.9811 g, 2.9 mmol) in dry pyridine (10 mL) was slowly added dry benzoyl chloride (3.5 mL, 30 mmol, 11 eq.) at 0°C under continuous stirring. The solution

was allowed to attain ambient temperature, resulting in the formation of a colourless precipitate. After 3 h, another 1.5 mL of benzoyl chloride were added and the solution was stirred over night. The solution was poured into 14 mL of ice-water and the colourless precipitate which separated out was dissolved in chloroform (10 mL), followed by the separation of the two layers. The aqueous phase was extracted with $CHCl_3$ (2x10 mL) and the combined organic layers were washed with water (2x10 mL), dried over Na_2SO_4, filtered and the solvent was evaporated in vacuo, resulting in a colourless oil.
Yield: 3.575 g, 3.0 mmol, 106% of theory
R_f: 0.15 (n-hexane/ethyl acetate 2/1)

3.3.2 Synthesis of 2,3,4,6-Tetra-O-benzyl-D-glucopyranose (3a)

3.3.2.1 Starting from Sucrose

2a (33.47 g, 31.5 mmol) was dissolved in acetone (300 mL) and after slowly adding 17 mL of HCl conc., the solution was stirred under reflux for 1-½ h. After cooling to r.t., the solvent was removed under reduced pressure, affording a green to brown residue which was partitioned between DCM (150 mL) and water (100 mL). The aqueous layer was extracted with DCM two more times (2x50 mL) and the combined DCM layers were washed with water (3x100 mL), dried over Na_2SO_4, filtered and concentrated under vacuum. The obtained brown oil (39.72 g) was dissolved in hot ethyl acetate (70 mL) and addition of hexane in small portions (350 mL) lead to the formation of a colourless solid (28.48 g, wet). After cooling the remaining solution to 0°C, the precipitate was isolated and the mother liquor was left at -25°C for further crystallization. The dried crystals were purified by recrystallization from MeOH (500 mL) resulting in very fine, colourless needles which were isolated and dried over $CaCl_2$. The MeOH mother liquor was stored at -25°C as well and the crystallization and recrystallization steps were repeated several times.
Yield: 9.8118 g, 18.2 mmol, 58% of theory
R_f: 0.32 and 0.38 (n-hexane/ethyl acetate 2/1)
MP: 151-155°C (Lit.: 151-152°C [37])

3.3.2.2 Starting from Trehalose

3.3.2.2.1 Acetone/HCl

2d (1.6075 g, 1.5 mmol) was dissolved in acetone (26 mL) and 810 µL of HCl conc. were added. The solution was stirred for four days under reflux and further acid was added every day (4x400µL).

3.3.2.2.2 Autoclave

A certain amount of starting material (**2d**) and water were brought into the autoclave reactor (sample 1 and 2: Parr; sample 3 and 4: Büchi) and heated to a certain temperature and pressure for a given time. After addition of chloroform, the two phases were separated and the aqueous phase was extracted with chloroform. The combined organic layers were washed with water, dried over Na_2SO_4, filtered and concentrated under reduced pressure.

Table 3: Autoclave reaction procedure: amount of staring material, water as well as reaction conditions used

Nr.	Starting material [g]; [mmol]	Water [mL]	T [°C]	pressure [bar]	t [h]
1	0.9511; 0.9	200	300	80	1
2	0.8103; 0.8	200	200	14	1
3	1.6095; 1.5	500	200	10	4
4	1.4683; 1,4	500	200	30	4

3.3.3 2,3,4,6-Tetra-O-benzoyl-D-glucopyranose (3c)

3.3.3.1 Sucrose as Starting Material

3.3.3.1.1 General Procedure

To a stirred solution of the starting material (**2b**) in the respective solvent, the acid reagent was slowly added resulting in a pH of 2-1. The solution was heated (a reflux condenser was used) and stirred for a given time under permanent monitoring of the reaction progress using TLC (acetone/toluene 1/9 or 1/3) and LC/MS-TOF. When needed, further reagent or solvent were added. The experiments were carried out a few times with varying amounts of acid reagent and reaction time according to Table 4.

Table 4: Experimental conditions used for the hydrolysis of octa-O-benzoylated sucrose

Nr	Starting material [g]; [mmol]	Solvent [mL]	Starting amount of solvent [mL]	Reagent	Starting amount of reagent	pH	T [°C]	t [h]
1	2.8556; 2.4	acetone	26	HCl conc.	2.6 mL	1	reflux	48
2	0.3964; 0.3	acetone	4	33%HBr/HOAc	0.1 mL	1	reflux	72
3	0.4000; 0.3	DMF	1.2	NH_4Cl	0.0141 g	2-1	70	48
4	0.5348; 0.5	DMF	4.6	Amberlyst-15	0.5091 g	2-1	70	48
5	0.4056; 0.4	acetone	4	Cl_3CCOOH	0.324 g	1	50	24
6	0.4514; 0.4	acetone	4.3	HCOOH	2 mL	1	reflux	24
7	0.7010; 0.6	acetone	6.5	pTsOH	0.37 g	1	reflux	24
8	0.8201; 0.7	acetone	10	H_2SO_4 98%	0.5 mL	1	reflux	24

3.3.3.1.2 Acetonitrile/Triflic Acid

To a stirred solution of the starting material **2b** (0.5463 g, 0.465 mmol) in dry acetonitrile (5 mL), triflic acid (41 µL) was added under nitrogen protection at 0°C resulting in a pH of 1. After addition of the acid, the solution was allowed to attain room temperature and further stirred for 1½ h. Another 41 µL of acid were added, the reaction was heated to 60°C and stirred over-night (under N_2 atmosphere).

3.3.3.2 Trehalose as Starting Material

3.3.3.2.1 General Procedure:

To a stirring solution of the starting material (**2e**) in the respective solvent, the acid reagent was slowly added resulting in a pH of 2-1. The solution was heated (a reflux condenser was used) and stirred for a given time under permanent monitoring of the reaction progress using TLC (acetone/toluene 1/9 or 1/3) and LC/MS-TOF. When needed, further reagent or solvent were added.

Table 5: Experimental conditions used for the hydrolysis of octa-O-benzoylated trehalose

Nr.	Starting material [g]; [mmol]	Solvent [mL]	Starting amount of solvent [mL]	Reagent	Starting amount of reagent [mL]	pH	T [°C]	t [h]
1	0.5303; 0.5	acetone	5	HCl conc.	0.24	1	reflux	24
2	0.5563; 0.5	acetonitrile	5	TfOH	0.041	1	90	48
3	0.8518; 0.7	acetone	10	H_2SO_4 conc.	0.5	1	reflux	24

3.3.3.2.2 Autoclave

The starting material **2e** (1.2102 g, 1.0 mmol) and 500 mL water were brought into the autoclave reactor (Büchi) and heated at 200°C at 30 bar for 4 h. After the addition of chloroform, the two phases were separated and the aqueous phase was extracted with chloroform. The combined organic layers were washed with water, dried over Na_2SO_4, filtered and concentrated under reduced pressure.

3.3.4 2,3,4,6-Tetra-O-acetyl-D-glucopyranose (3d)

3.3.4.1 General Procedure:

The starting material **2c** stirred in the respective solvent was treated with an acid reagent and the solution was heated to reflux and stirred for a given time with permanent monitoring of the reaction progress with TLC and LC/MS-TOF. When needed, further reagent or solvent were added.

Table 6: Experimental conditions used for the hydrolysis of octa-O-acylated sucrose

Nr.	Starting material [g]; [mmol]	Solvent [mL]	Starting amount of solvent [mL]	Reagent	Starting amount of reagent	pH	T [°C]	t [h]
1	0.8358, 1.23	acetone	14	HCl conc.	0.7 mL	1	reflux	1
2	0.6228, 0.92	acetone	10	lactic acid	1 mL	4-3	reflux	24

3.3.5 2,3,4,6- Tetra-O-benzyl-α-D-glucopyranosyl trichloroacetimidate (4)

3a (7.82 g, 14.5 mmol) was dissolved in 65 mL dry DCM and 6.5 mL trichloroacetonitrile and NaH (32.3 mg, 1.3 mmol) were added and the solution was stirred at room temperature for 4h. After filtration over silica gel and evaporation of the solvent, a dark brown oil (11.05 g) was obtained which was purified by re-dissolving in petrol ether/diethyl ether 3/2 (80 mL) and filtration over silica gel. Concentration under reduced pressure afforded a colourless oil which crystallized upon standing within a month. The crystals were dried over $CaCl_2$.
Yield: 9.7353 g, 14.2 mmol, 98% of theory
R_f: 0.63 (α-anomer), 0.57 (β-anomer) (n-hexane/ethyl acetate 2/1)
MP: 69-71°C (Lit.: 77°C [38], 72-73°C [39])

3.3.6 Cholesteryl 2,3,4,6- tetra-O-benzyl-D-glucopyranoside (5)

To a stirred solution of **4** (9.2853 g, 13.6 mmol) and dry cholesterol (4.4032 g, 11.4 mmol) in dry DCM (60 mL) a 4Å molecular sieve (20.9 g) was added followed by a 0.02 M TMSOTf-solution ((217 µL TMSOTf in 60 mL DCM) which resulted in a colour change of the colourless solution to yellow. After 1-1½ h the reaction was quenched with Et_3N (5 mL) and filtered to

remove the molecular sieve. The organic layer was concentrated under vacuum to give a light brown oil which crystallized over-night upon standing (8.49 g). The solid was purified by stirring in MeOH/dioxane 10/1 resulting in the formation of a very fine colourless precipitate which was isolated via centrifugation and dried over $CaCl_2$.
Yield: 6.0358 g, 6.6 mmol, 58% of theory
R_f: 0.81, 0.86 (n-hexane/ethyl acetate 2/1)
MP: 50°C (Lit.: 69-70°C [46])

3.3.7 Removal of Benzyl Ether Protecting Groups

3.3.7.1 $NaBrO_3$ and $Na_2S_2O_4$

To the starting material **5** (313.2 mg, 3.4 mmol) and ethyl acetate (12 mL) $NaBrO_3$ was added as an aqueous solution (159.3 mg in 3.4 mL H_2O) resulting in a bright yellow solution. The mixture was stirred effectively while $Na_2S_2O_4$ (177.3 mg) in 6.8 mL water was added in small portions leading to a decolouring of the reaction mixture. After 1 h additional salt solutions were added ($NaBrO_3$ 279.6 mg in 6 mL H_2O and $Na_2S_2O_4$ 500.2 mg in 19 mL water) and the solution was stirred over-night. After dilution with ethyl acetate and separation of the two phases, the aqueous phase was extracted twice with ethyl acetate (2x10 mL) and the combined organic layers were washed, dried (Na_2SO_4) and concentrated under reduced pressure. The obtained solid was not further purified as analysis using LC/MS-TOF revealed the sole presence of side-products.

3.3.7.2 Cyclohexene

A 241.6 mg (0.3 mmol) sample of **5** was treated with 60 mL of EtOH/cyclohexene 2/1 (vol/vol) and under nitrogen protection and effective stirring, one spatula of $Pd(OH)_2$ was added followed by heating of the mixture to reflux conditions. The solution was stirred over night, the catalyst was removed by filtration and centrifugation and the organic layer was evaporated under vacuum affording a white to grey powder which was re- dissolved in pyridine, filtered over celite and concentrated again to give a colourless solid.
Yield: 124 mg, 0.2 mmol, 85% of theory
R_f: 0.07 (petrol ether/acetone 1/1)

3.3.7.3 Et_3SiH

The starting material **5** (82.4 mg, 0.3 mmol) was suspended in 30 mL of EtOH. One spatula of of $Pd(OH)_2/C$ was added under nitrogen atmosphere and vigorous stirring. After the addition of 300 µL (2 mmol) Et_3SiH, the mixture was heated to reflux. After 2 h, additional Et_3SiH (150 µL, 1 mmol) was put into the solution and it was stirred for 4 days with constantly adding further reactant (in total 2.1 mL, 13 mmol of Et_3SiH). After complete deprotection, the solution was filtered over celite and centrifuged to remove the catalyst. Evaporation of the solvent in vacuum resulting in a fine greyish- white powder.
Yield: 175 mg, 0.3 mmol, >99% of theory
R_f: 0.07 (petrol ether/acetone 1/1)

3.3.7.4 Formic acid

The starting material (**5**) (285 mg; 0.3 mmol) was stirred into a 10% formic acid solution in EtOH (20 mL) and under inert atmosphere, one spatula of $Pd(OH)_2/C$ was added. The mixture was heated to reflux and stirred over-night.

3.3.7.5 $FeCl_3$

To a stirring solution of **5** (195.9 mg, 0.2 mmol) in dry DCM (10 mL) $FeCl_3$ was added. The formation of a white precipitate could be observed and after 30 min the reaction was quenched with 20 mL of water.

4 Results and Discussion

A new synthesis route for steryl glucosides (see Figure 15) which was developed by the working group before, was further investigated. Sucrose is used as starting material and the pyranosyl moiety of the disaccharide is employed to form the glycosylic part of the desired product during a five-step reaction sequence. In the first step, the hydroxy groups of the sugar are protected using benzyl ethers to avoid their participation in further reaction steps. Next, the protected disaccharide is hydrolyzed under acidic conditions to cleave the glycosidic bond between the pyranosyl and the fructosyl part of sucrose. The fructosyl moiety can be regarded as a special protection group for the anomeric OH-group of the glucose moiety which can be removed in an easy and quick way. The separated pyranosyl molecule with the free OH-group at C1 and four benzylated OH-groups is further transformed to its trichloroacetimidate derivative, which is a standard procedure for the formation of glycosidic bonds as already described in Chapter 2. Under alkaline conditions and prolonged reaction times, the α-anomer is preferably formed due to stereroelectronic effects. In the following step of the reaction sequence, the carbohydrate derivative is linked to cholesterol to form a steryl glucoside which is still protected at its hydroxy groups. In the last step, the hydroxy groups of the molecule are deprotected by cleaving the ether bond via hydration using $Pd(OH)_2$ as a catalyst and cyclohexene as a hydride donor.

In addition to verify the reliability of the new synthesis method, other sugar protection groups were introduced and tested for their suitability for this method. Two standard protection groups, namely benzoyl and acetyl esters, were introduced in the first step as their removal is fast and simple by using sodium in methanol.
Also, another disaccharide, namely trehalose, was used as starting material instead of sucrose. Due to the α(1→1)α glycosidic linkage of trehalose, two molecules of the pyranosyl compound could be obtained from one educt molecule during reaction step 2 which would increase the carbon efficiency of the reaction and avoid the separation of the glucose and the fructose moiety.

All reactions procedures of the presented sequence as well as further experiments using different protection groups and trehalose described in Chapter 3 are further discussed below. Every reaction was monitored using TLC and LC-MS-TOF and reaction times were adapted where necessary.

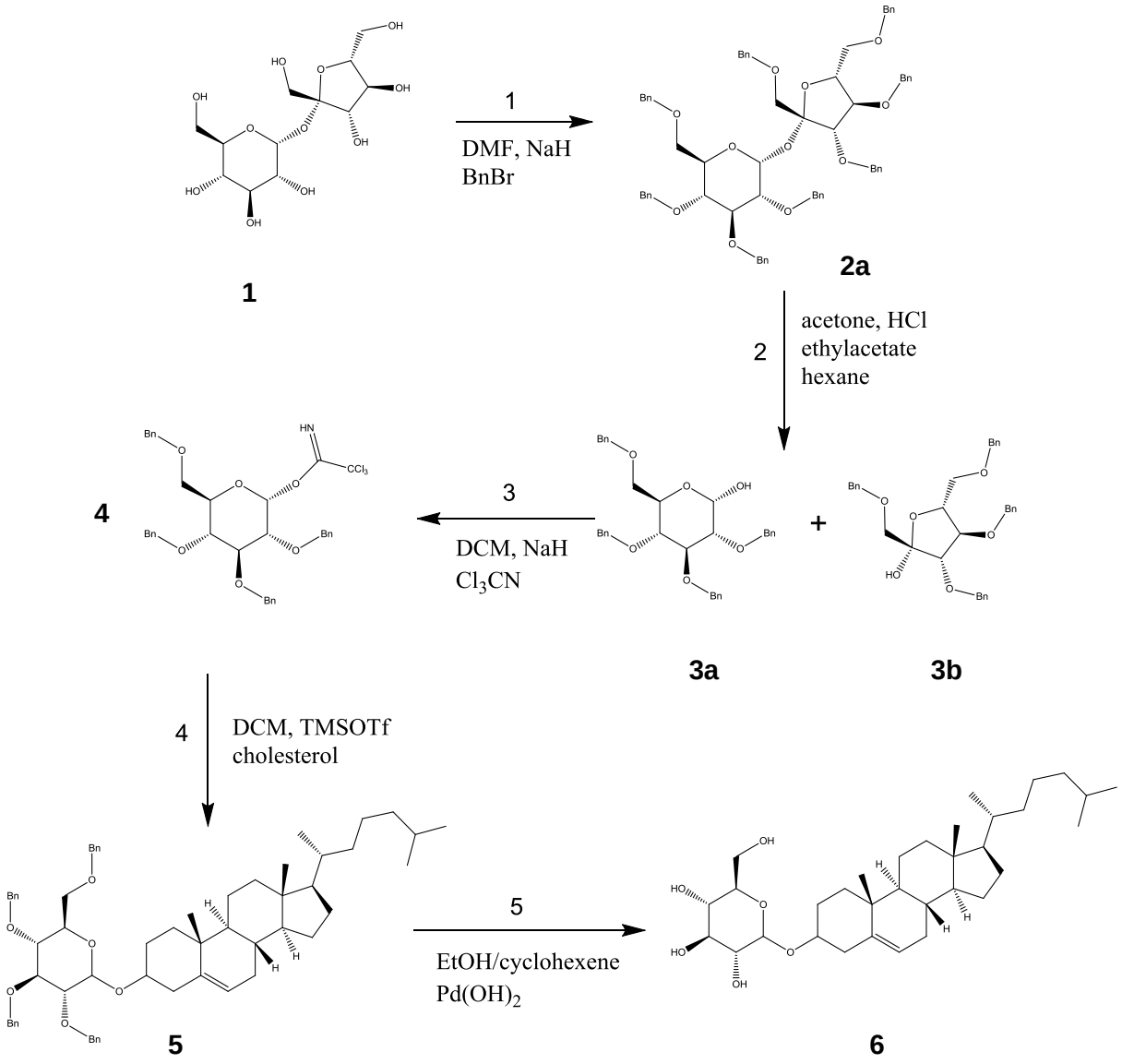


Figure 15: New synthesis route for cholesteryl glucoside starting from sucrose via a five-step reaction sequence

4.1 Step 1 – Protection of the Disaccharide

The protection of the hydroxy groups of sucrose (see Figure 16) or trehalose is carried out in the presence of a base, leading to the formation of alkoxides which can further react as strong nucleophiles attacking the alkyl halides. Absolutely crucial for a successful and complete protection of the sugar is the absence of water in the reaction medium to minimize the formation of unwanted side products. Therefore, the use of dry solvents as well as the storage of the sugar over a drying agent (e.g. desiccator with $CaCl_2$) is important.

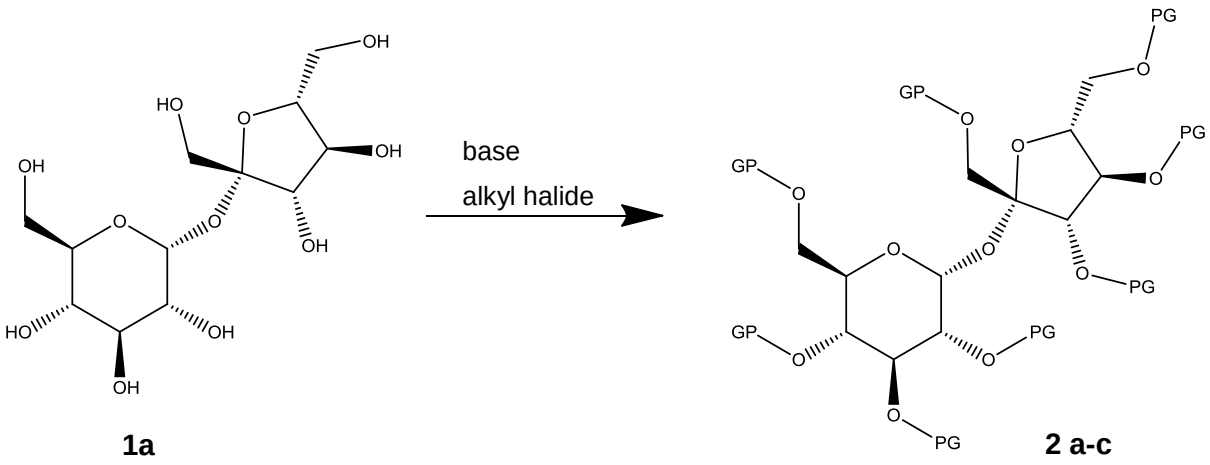


Figure 16: Reaction scheme for the protection of sucrose under basic conditions using alkyl halides

4.1.1 1,3,4,6-Tetra-*O*-benzyl-β-D-fructofuranosyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranoside (2a) [40]

The complete protection of the sugar with benzyl groups requires the presence of a strong base. In the present reaction, sodium hydride was used to deprotonate the hydroxy groups of the sugar, leading to the formation of alkoxides which can further react with benzyl bromide. Bromide ions are released as leaving groups and the formation of sodium bromide can be observed during the reaction as white precipitate. Crucial for a successful and complete protection of the sugar is the absence of water in the reaction medium which is also important due to safety issues. Sodium hydride reacts violently with water, leading to the formation of sodium hydroxide and hydrogen gas. Thus, the used solvent was stored over a molecular sieve (4 Å) with a colour indicator and its water content was measured coulometrically prior to use. Furthermore, introducing the base into the solvent and the following addition of the sucrose suspension was carried out portion-wise in the presence of a molecular sieve and under nitrogen protection.

Reaction control using TLC revealed the presence of several side- products (see Figure 17), which probably represent incomplete protected sugar molecules (spots below the reaction product) with a higher polarity or dibenzyl ether (spot above the reaction product). The obtained dark brown oil (raw product) was stirred in diethyl ether for several minutes and filtered over celite®/charcoal as the only cleaning procedure. The proposed vacuum distillation of Kessler et al. [40] to remove dibenzyl ether which is formed during the reaction was not performed but instead the raw material was used in the following reaction step.

A big advantage of this experimental setup is the use of sucrose as powdered sugar which was bought in the supermarket and represents a cheap and readily available starting material. In addition, time consuming and costly clean up procedures like column chromatography can be avoided as the raw oily material is used for the next reaction step.

The formed dibenzyl- ether and other side products do not hinder the subsequent hydrolysis reaction and can be removed in the second step of the reaction sequence.

Crude yield: 33.47 g, 31.5 mmol, >99% of theory

R_f: 0.67 (n-Hexane/ethyl acetate 2/1)

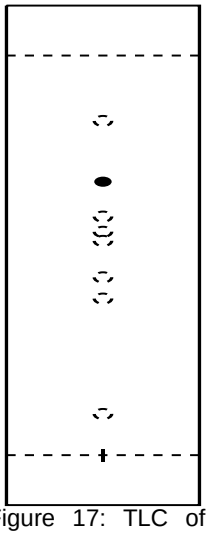


Figure 17: TLC of the reaction product (black dot) and impurities (white dots)

LC/MS-TOF

Positive APCI ionization mode: calculated for $[C_{68}H_{70}O_{11}+NH_4]^+$: 1080.52564; found: 1080.528763 $^m/_z$; Δm: -2.89 ppm

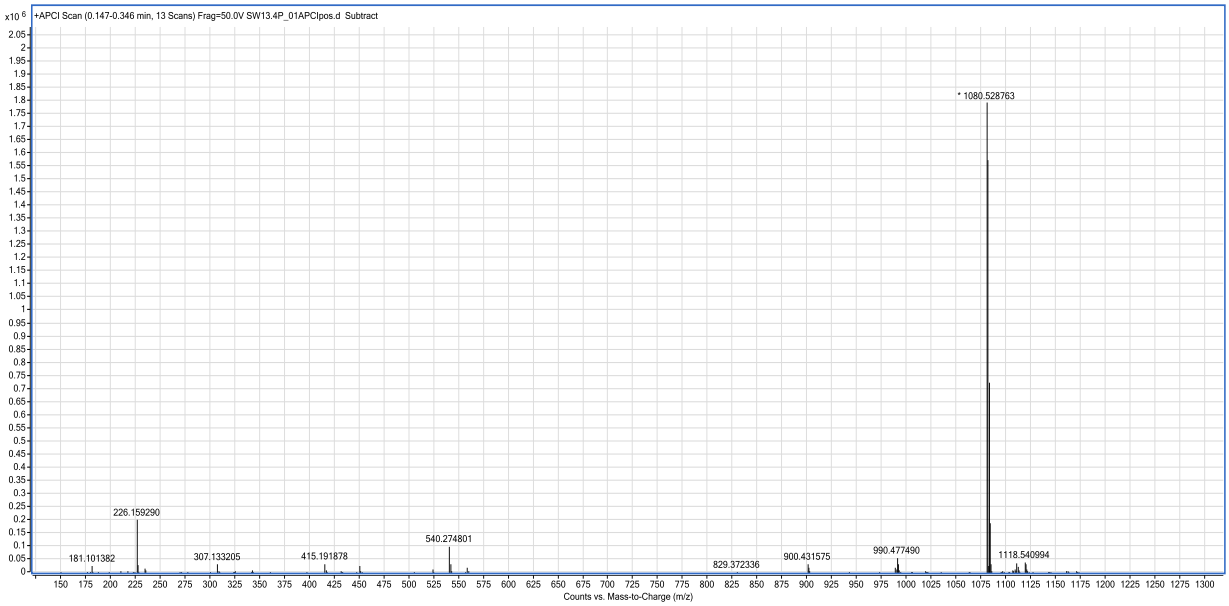


Figure 18: TOF-mass spectrum using positive APCI ionization of 1,3,4,6-tetra-O-benzyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside

NMR

Due to the above described impurities, the NMR is not in complete accordance to the literature ([41])

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.40-7.13 (56H, m) 5.75 (1H,d) 4.92-4.07 (28H, m) 3.95 (1H, t) 3.78-3.40 (10H, m) 3.38 (1H, dd) 2.96, 2.91, 2.37, 2.28, 2.21, 1.62 (all s, impurities)

Solvent peaks: 7.26 ($CHCl_3$); 1.21, 3.48 (Et_2O); 8.02, 2.96, 2.88 (DMF) [42]

^{13}C NMR ($CDCl_3$, 300 MHz) δ 138.91, 138.62, 138.47, 138.35, 138.22, 138.20, 138.05, 128.41, 128.33, 128.30, 128.26, 128.07, 128.00, 127.96, 127.90, 127.79, 127.76, 127.73, 127.67, 127.64, 127.60, 127.53, 127.47, (aromatic C) 77.45, 77.03, 76.60 (solvent)

Solvent peaks: 77.36 ($CHCl_3$); 15.20, 65.91 (Et_2O); 162.62, 36.50 (DMF) [42]

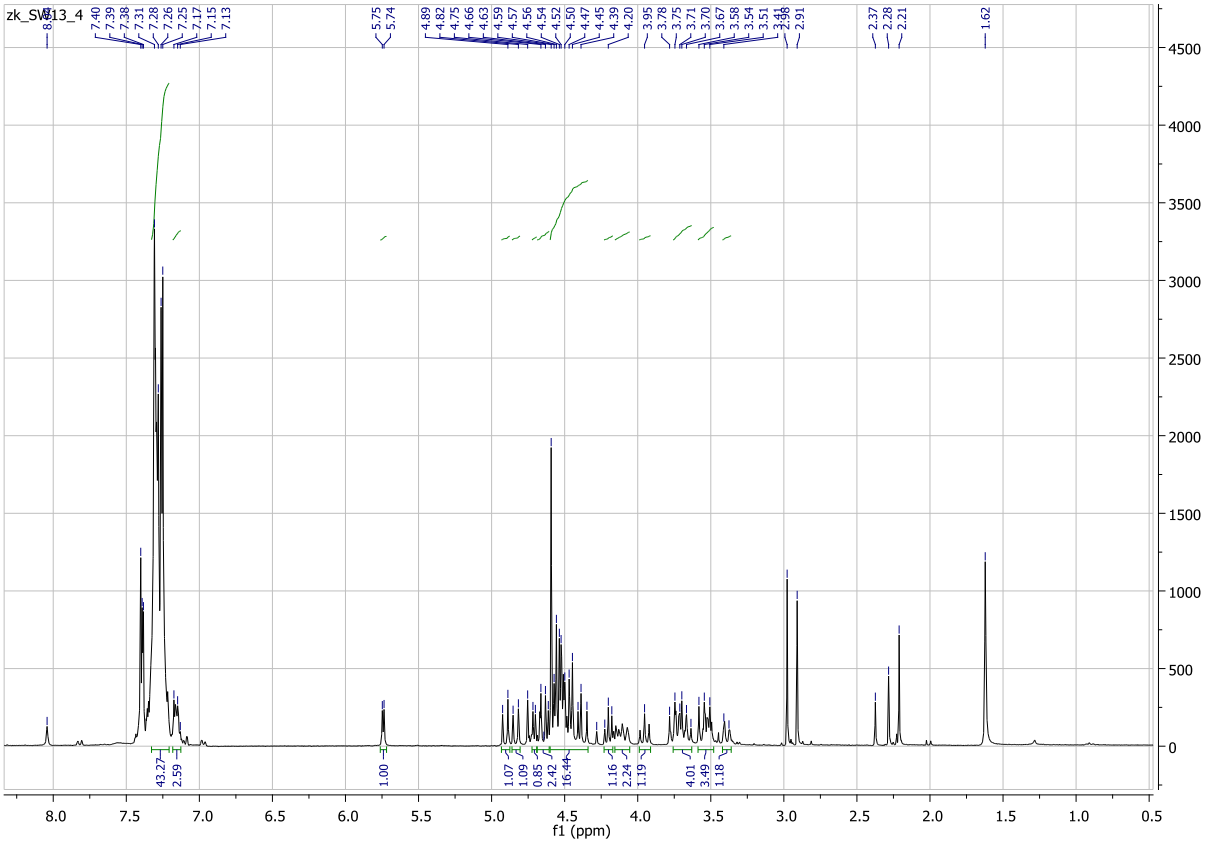


Figure 19: ^{1}H NMR spectrum of 1,3,4,6-tetra-O-benzyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside

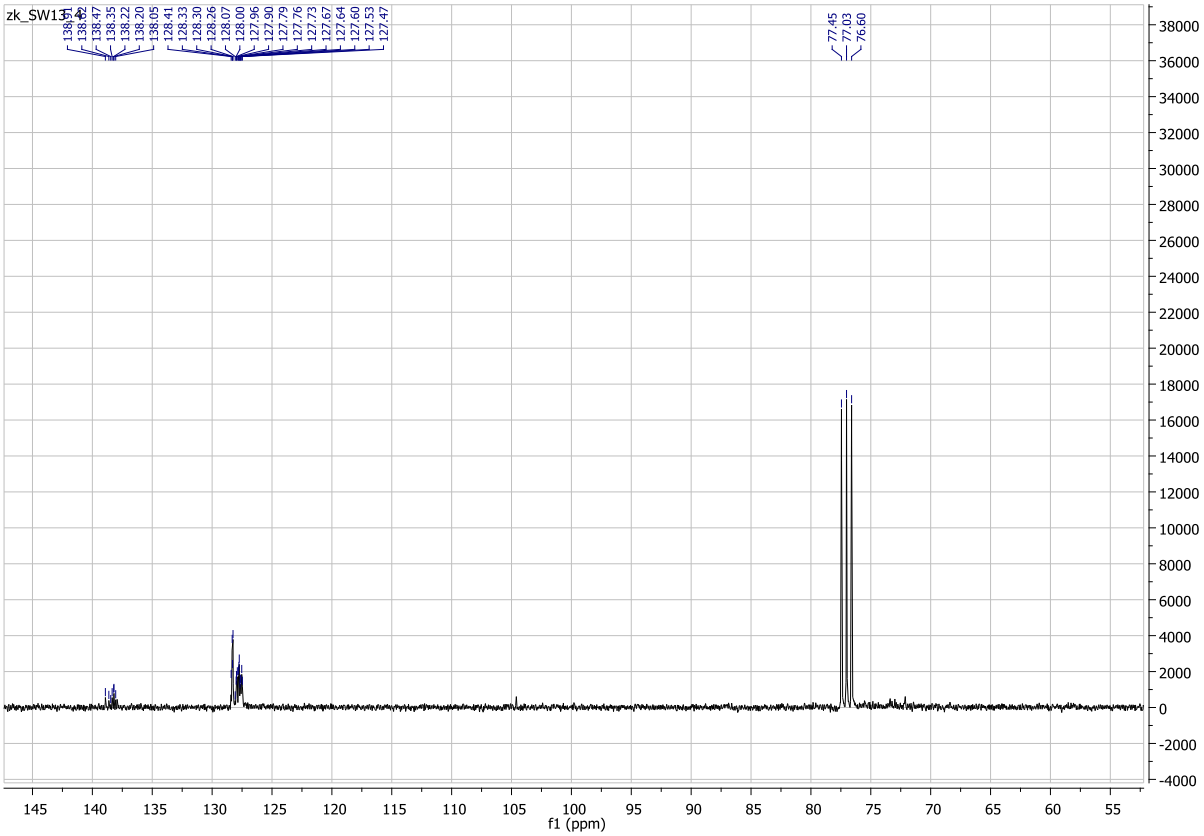


Figure 20: ^{13}C NMR spectrum of 1,3,4,6-tetra-O-benzyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside

4.1.2 1,3,4,6-Tetra-*O*-benzoyl-β-D-fructofuranosyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranoside (2b) [43], [44]

The reaction was carried out in pyridine which does not only act as a solvent but also as a base leading to the desired formation of alkoxides. Again, water free conditions are important to minimize side reactions. That's why the solvent was freshly distilled prior to use and a drying funnel was used to seal the reaction flask. The formed salt (pyridinium chloride) is dissolved by adding of water during the work up procedure. The white, sticky syrup which precipitates during this step, represents the desired product which can be dissolved completely in chloroform after stirring the mixture for several minutes. Another important point is the complete removal of the solvent by using a rotary evaporator, which requires good vacuum conditions and high temperatures (80°C) as the remaining syrup has a high viscosity, making it hard for present traces of solvent to evaporate.

Yield: 17.33 g, 14.7 mmol, >99% of theory

R_f: 0.72 (acetone/toluene 1/9)

LC/MS-TOF

Positive APCI ionization mode: calculated for $[C_{68}H_{54}O_{19} + NH_4]^+$: 1192.35976; found: 1192.358712 $^m/_z$; Δm: 0.88 ppm (see appendix)

NMR

The recorded NMR- spectrum is in good consistency with the literature ([44]):

^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.22-7.11(45H, m) 6.27-6.20 (2H, m) 6.01-5.96 (2H, m) 5.77 (1H, t) 5.44-5.39 (1H, dd) 4.77-4.57 (6H, m) 4.44-4.39 (1H, dd) 4.39-4.31 (1H, dd) (spectrum is presented in the appendix)

Solvent peaks: 7.26 ($CHCl_3$); 8.62, 7.68, 7.29 (pyridine) [42]

^{13}C NMR ($CDCl_3$) δ 166.00, 165.99, 165.78, 165.52, 165.50, 165.37, 165.05, (Ph*C*OO-) 130.18, 129.92, 129.77, 129.73, 129.63, 129.51, 129.26, 129.15, 128.81, 128.75, 128.70, 128.52, 128.37, 128.29, 128.24, (aromatic C) 123.89, 104.87, 90.97, 79.07, 71.37, 70.02, 69.24, 68.96, 64.94, 64.01, 62.22 (C1-C6, C1'-C6') 77.46, 77.03, 76.61 (solvent) (spectrum is presented in the appendix)

Solvent peaks: 77.36 ($CHCl_3$); 149.90, 123.75, 135.96 (pyridine) [42]

4.1.3 1,3,4,6-Tetra-*O*-acetyl-β-D-fructofuranosyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (2c) [45], [46]

As already described above, water- free conditions are important during the introduction of protection groups as well as the addition of the reagent at 0°C due to the generation of heat during the reaction. The reaction was stirred over night, as monitoring of the reaction using TLC and LC/MS-TOF indicated incomplete protection after the proposed reaction time in [45]. The obtained syrup crystallized completely over night, resulting in a colourless solid which was not further purified (explains the low melting point compared to the literature).
Yield: 10.72, 15.8mmol, >99% of theory
R_f: 0.22 (ethyl acetate/n-hexane 1/1)
MP: 50°C (Lit.: 69-70°C [46])

LC/MS-TOF

Positive APCI ionization mode: calculated for $[C_{28}H_{38}O_{19}+NH_4]^+$: 696.23455; found: 696.235053 $^m/_z$;Δm: -0.72 ppm (see appendix)

NMR

The recorded NMR-spectrum reveals the presence of traces of pyridine and shows satisfying but not complete accordance to the literature ([45], [46]).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.69 (1H, d) 5.49-5.36 (4H, m) 5.09 (1H, t) 4.90-4.86 (1H, dd) 4.35-4.13 (10H, m) 2.19 (3H, s) 2.13-2.11 (19H, m) 2.06 (3H, s) 2.03 (3H, s) (spectrum is presented in the appendix)
Solvent peaks: 7.26 ($CHCl_3$); 8.62, 7.68, 7.29 (pyridine) [42]
^{13}C NMR ($CDCl_3$, 300 MHz) δ 170.74, 170.52, 170.14, 170.12, 170.06, 169.91, 169.68, 169.53, (*C*OCH_3) 149.11, 149.00, 123.98, 104.01, 103.97, 89.89, 75.63, 74.93, 70.24, 69.61, 68.15, 63.64, 62.83, 61.72 (C1-C6, C1'-C6') 77.44, 77.02, 76.60 (solvent) (spectrum is presented in the appendix)
Solvent peaks: 77.36 ($CHCl_3$); 149.90, 123.75, 135.96 (pyridine) [42]

4.1.4 2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranoside (2d) [40]

During the addition of sodium hydride to the solvent, the base reacted quite strongly and the solution thickened a little although the used DMF was tested on its water content beforehand and stored over a molecular sieve. After adding the sugar and the reagent and stirring for a few hours, the solution showed a bright yellow colour and again the formation of a colourless precipitate. After the work up procedure, the obtained brown oil was dissolved in diethyl ether and filtered twice to reduce the presence of unwanted side products. Again, neither column chromatography nor distillation was carried out to completely purify the raw product before reaction step 2. The high yield can be explained by side products (like dibenzylether) as the obtained oil was not further purified.
Crude yield: 3.91 g, 3.7 mmol, 127% of theory
R_f: 0.72 (acetone/toluene 1/9)

LC/MS-TOF
Positive APCI ionization mode: calculated for $[C_{68}H_{70}O_{11}+NH_4]^+$: 1080.52564; found: 1080.526029 $^m/_z$; Δm: -0.36 ppm (see appendix)

NMR
The recorded NMR-spectrum reveals the presence of impurities but can be compared to predicted spectra (obtained from SciFinder [47], [48]) as no applicable spectra in the literature were available.
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.45-7.13 (39H, m) 5.26 (1H, dd) 5.03-4.36 (9H, m) 4.20-4.17 (1H, m) 4.05 (1H, t) 3.74-3.53 (3H, m) 3.42-3.88 (1H, dd) 2.98, 2.91 (all s, impurities) (spectrum is presented in the appendix)
Solvent peaks: 7.26 ($CHCl_3$); 1.21, 3.48 (Et_2O); 8.02, 2.96, 2.88 (DMF) [42]
^{13}C NMR ($CDCl_3$, 300 MHz) δ 138.90, 138.35, 138.28, 138.23, 137.84, 128.43, 128.37, 128.35, 128.01, 127.99, 127.90, 127.81, 127.69, 127.66, 127.51, 127.50, 127.41, (aromatic C) 94.46, 81.81, 79.38, 75.52, 75.09, 72.70, 72.13 (C1-C6, C1'-C6', *C*H_2Ph) 77.47, 77.04, 76.62 (solvent) (spectrum is presented in the appendix)
Solvent peaks: 77.36 ($CHCl_3$); 15.20, 65.91 (Et_2O); 162.62, 36.50 (DMF) [42]

4.1.5 2,3,4,6-Tetra-*O*-benzoyl-α-D-glucopyranosyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranoside (2e) [43], [44]

The benzoylation of trehalose was again carried out using pyridine as a solvent and as a base. The reaction mixture was cooled in an ice bath during the addition of benzoyl chloride

due to the exothermic salt formation. The mixture was stirred overnight, as reaction monitoring using TLC and LC-MS-TOF indicated incomplete protection after stirring for several hours. Again, the product crystallized out after pouring the mixture on ice and dissolution in chloroform allowed further work-up procedures. A colourless oil was obtained which was not further purified.

Yield: 3.58 g, 3 mmol, 106% of theory

R_f: 0.15 (n-hexane/ethyl acetate 2/1)

LC/MS-TOF

Positive APCI ionization mode: calculated for $[C_{68}H_{54}O_{19}+NH_4]^+$: 1192.35976; found: 1192.359802 $^m/_z$; Δm: -0.04 ppm

NMR

The spectrum was recorded with good accordance to the literature ([49]).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.15-7.28 (87H, m) 6.33 (2H, t) 5.78 (2H, t) 5.96 (2H, t) 5.56-5.51 (2H, dd) 4.36-4.30 (2H, m) 4.06-3.88 (4H, dq) (spectrum is presented in the appendix)

Solvent peaks: 7.26 ($CHCl_3$); 8.62, 7.68, 7.29 (pyridine) [42]

4.2 Step 2 – Hydrolysis of the Protected Disaccharide

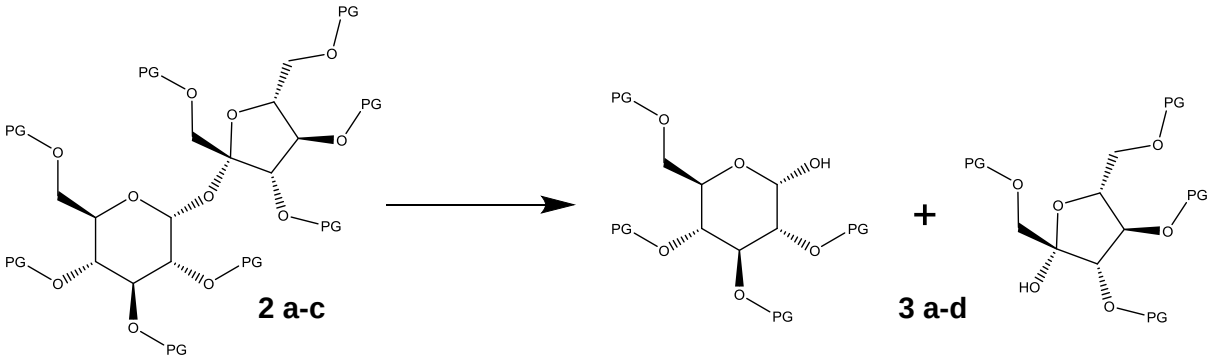


Figure 21: reaction scheme for the hydrolysis reaction of the fully protected disaccharide sucrose

During the second step of the reaction sequence, the disaccharide is hydrolyzed at its anomeric center under acidic conditions. When sucrose is used as a disaccharide, the fructose moiety can be considered as a kind of highly acid labile protection group for the anomeric hydroxy group and due to the different chemical properties, it can be separated from the pyranoide moiety which is further used in the third step. Kessler et al. describe the hydrolysis of benzylated sucrose under mild conditions by adding hydrochloric acid to a solution of the starting material in an organic solvent (e.g. ethanol, acetone) and stirring for

20 to 60 minutes at 50 to 60°C. Due to short reaction times and low temperatures, a cleavage of the benzyl protection groups can be avoided which is a big problem for other described synthesis methods. [40] Furthermore, purification of **3a** using column chromatography is not necessary as it can be obtained simply by precipitating from hexane whereas the fructose moiety (**3b**) stays in solution due to its lower polarity. A yield of 70% [40] respectively 51% [50] can be achieved. Losses can be explained by incomplete precipitation from hexane, losses during purification of the solid by recrystallization or incomplete hydrolysis. To increase the yield and to avoid the loss of one monosaccharide (fructosyl-moiety), trehalose was used as a disaccharide source in step 1, because two molecules of protected glucopyranose with a free hydroxy group at C1 could be obtained by cleaving the glycosidic bond of one molecule of trehalose.

Also, different kinds of protection groups were introduced: benzyl-, benzoyl- and acetyl-groups were used to test their effect on the hydrolysis reaction. Although different hydrolysis conditions were tested, only benzyl protection groups in combination with sucrose showed the wanted hydrolysis reaction under the tested conditions in acceptable yields. Acetyl protection groups are too acid labile and are cleaved off before the anomeric center is hydrolysed. Benzoyl protection groups are stable under strong acidic conditions but apparently hinder the hydrolysis at the anomeric carbon. A possible explanation is the armed/disarmed concept of carbohydrates which is due to the different electronic properties of ethers vs. ester protection groups. Ethers are passive protection groups, which do not influence the build-up of a positive charge at C1, whereas esters are electron- withdrawing groups and disfavour the development of positive charges. [19] Stick and Williams stress these electronic influences with the example of tetra-O-acetyl-α-D-glucopyranosyl bromide, which can be stored in cold and dry conditions for a few months, in contrast, tetra-O-benzyl-α-D-glucopyranosyl-bromide is unstable and in general not isolated but used in situ for further reactions due to the high reactivity of the anomeric centre. [19] During the acidic hydrolysis of sucrose, protons enter the molecule leading to the build-up of a positive charge and subsequently to the formation of either fructofuranosyl oxonium ions or glucopyranosyl oxonium ions ([51]–[53]) which is apparently prohibited in the present reaction if benzoyl ester protection groups are present.

Trehalose cannot be cleaved under the tested conditions, neither with benzyl nor with benzoyl groups due to the high stability of the α(1→1)α glycosidic linkage. The glycosidic oxygen has a very low bond energy (-4.2 $kJmol^{-1}$) compared to other disaccharides like sucrose (+113 $kJmol^{-1}$). [54], [55].

4.2.1 2,3,4,6-Tetra-O-benzyl-D-glucopyranose (3a) [40]

The use of benzyl protection groups and HCl as acid to hydrolyse benzylated sucrose is described in patent DE19534366C2. The reaction is very simple and fast according to the TLC and LC-MS-TOF monitoring. The reaction mixture was allowed to stir for prolonged reaction times (90 min instead of 60 min). The reaction mixture showed a dark red colour, the below drawn TLC shows several spots which are due to the formation of side products (loss of PG) and the impurities of the used starting material from step 1. One of the drawbacks of the reaction is the loss of the fructofuranosyl-part of sucrose which lowers the carbon efficiency of the reaction. The separation of the fructofuranosyl- and the pyranosyl- moiety is to a great extend responsible for the diminishment of yield. Furthermore, losses of some protection groups during the acidic hydrolysis as well as several filtration steps reduce the outcome of the desired end-product.

The separation of the fructosyl-moiety (**3b**) is only possible as its physico-chemical properties differ from the glucosyl moiety. Its lower polarity prevents crystallization when a solution of both compounds in ethylacetate is treated with hexane whereas the majority of the pyranoside precipitates immediately after the addition of hexane. Storing the remaining solution at freezing conditions helps the remaining pyranoside molecules to precipitate. Also during recrystallization of the product, the majority of the end product precipitated immediately after cooling the solution to 0°C. Only small amounts were further obtained after storing the mother solution in the freezer. The obtained crystals were extremely fine, colourless needles with a satisfactory purity. Over all, the hydrolysis using HCl is simple and avoids time consuming work up procedures (column chromatography) and due to the mild conditions, the loss of protection groups can be avoided to a great extend. The fructose moiety can be separated simply due to its lower polarity although the separation is mainly responsible for the loss of yield. However, the use of cheap starting materials and the ease of the hydrolysis compensates for the losses.

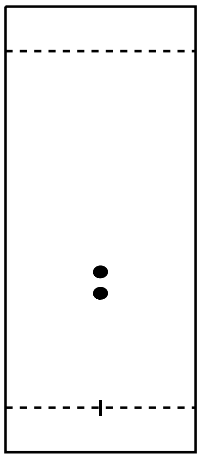


Figure 22: TLC oft he obtained reaction product

Yield: 9.81 g, 18.2 mmol, 58% of theory

R_f: 0.32 and 0.38 (α- and β-anomer) (n-hexane/ethyl acetate 2/1)

MP: 151-155°C (Lit.: 151-152°C [37])

LC/MS-TOF

Positive APCI ionization mode: calculated for $[C_{34}H_{36}O_6+NH_4]^+$:558.28501; found: 558.285759 $^m/_z$; Δm: -1.34 ppm

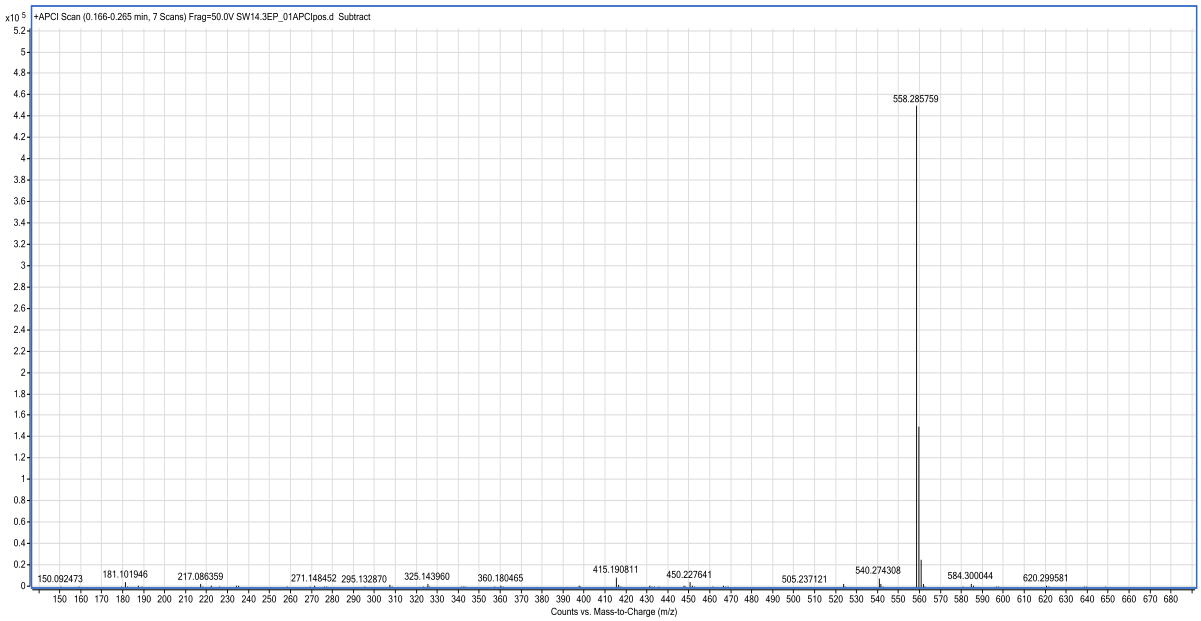


Figure 23: TOF-mass spectrum using positive APCI ionization (above) and IR (below) of 2,3,4,6-tetra-O-benzyl-D-glucopyranose

NMR

The spectrum was recorded with good accordance to the literature [41], [56]

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.29-7.07 (20H, m) 5.18 (1H, m) 4.91-4.41 (8H, m) 4.01-3.89 (2H, m) 3.68-3.32 (5H, m) 2.97 (OH)

Solvent peaks: 7.26 ($CDCl_3$); [42]

^{13}C NMR ($CDCl_3$) δ 138.67, 138.19, 137.86, 137.85, 128.53, 128.43, 128.39, 128.18, 128.08, 128.02, 127.99, 127.95, 127.89, 127.82, 127.76, 127.73, 127.66 (aromatic C), 91.35 (C1), 81.75 (C2), 79.99 (C3), 77.67 (C4), 77.04, 76.62 (solvent), 75.74, 75.10, 74.77, 73.53, 73.30 (O*C*H_2Ph), 70.31 (C5), 68.52 (C6)

Solvent peaks: 77.36 ($CHCl_3$); [42]

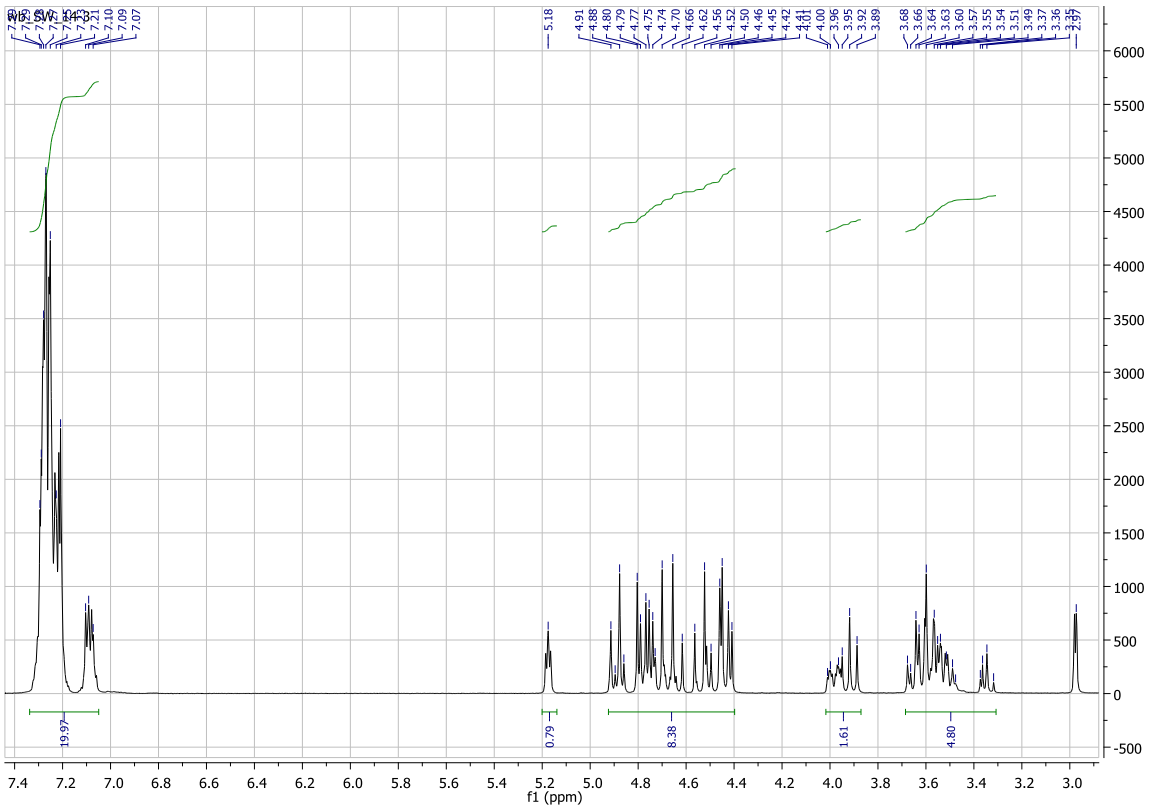


Figure 24: ^{1}H NMR spectrum of 2,3,4,6-tetra-O-benzyl-D-glucopyranose

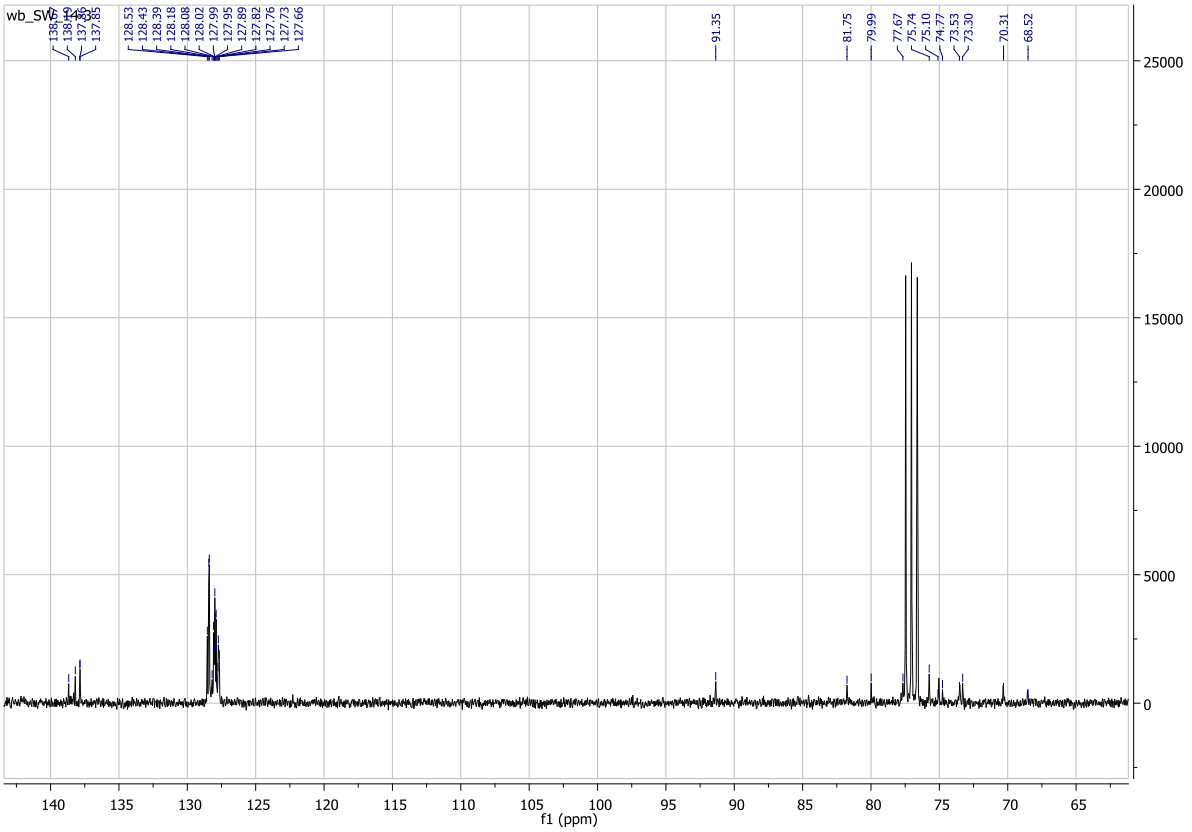


Figure 25: ^{13}C NMR spectrum of 2,3,4,6-tetra-O-benzyl-D-glucopyranose

As already discussed previously, benzylated trehalose was used as starting material for the hydrolysis reaction as well. Due to the high bonding energy of the α(1→1)α bond, the hydrolysis of step 2 could not be performed under the tested conditions. Not only different acidic conditions were used, but also reactions in subcritical water at high pressure and temperatures were performed as described in [49] or [50] for the hydrolysis of sucrose or oligosaccharides.

4.2.2 2,3,4,6-Tetra-O-benzoyl-D-glucopyranose (3c)

The hydrolysis of the benzoylated sucrose resp. trehalose could not be achieved with the tested methods. As the procedure of benzylated sucrose, using HCl, did not result in the desired reaction, harsher conditions using different kinds of acids and other reagents were tested in combination with prolonged reaction times. Table 4 in Chapter 3 gives an overview of the various methods including the used solvent, acidic reagent, obtained pH of the reaction mixture as well as reaction temperature and time using octa-*O*-benzoylated sucrose as starting material. The reactions were constantly monitored using TLC and LC-MS-TOF but under the given conditions, no reaction could be observed. Neither the desired hydrolysis of the anomeric bond, nor unwanted side reactions like cleaving the ester bond of the protection groups took place. Also varying amounts of acid reagent or reaction times could not lead to a successful reaction. Only the use of triflic acid in acetonitrile was able to hydrolyse the starting material to a certain extent, but only a conversion of appr. 15% (determined with LC-MS-TOF) compared to the starting material was achieved after stirring the solution for 5 days which makes the use of triflic acid inefficient for the hydrolysis reaction. In addition, triflic acid is costly and its use represents great safety issues. The suggested explanation of this resistance to hydrolysis of the glycosidic linkage is already explained at the beginning of Chapter 4.2 and Chapter 2.3.

Also, benzoylated trehalose and its behaviour under acidic conditions were investigated. As expected, the combination of benzoyl ester protection groups and the strong α(1→1)α linkage of trehalose prevents the molecule to undergo hydrolysis under the tested conditions. Also, experiments using an autoclave (as already described in Chapter 4.2.1) could not achieve a successful conversion of the starting material.

4.2.3 2,3,4,6-Tetra-O-acetyl-D-glucopyranose (3d)

Acetyl ester protection groups show a different behaviour to benzoyl protection groups. Mild acidic conditions do not lead to the desired hydrolysis reaction, rather as conditions get harsher, a loss of protection groups can be observed. Starting with lactic acid and a pH of 4 to 3, the pH was further decreased as no observable effects were present at this pH value. Acetic acid was used to reach a pH of 3-2. Still when no reaction was observable, trichloroacetic acid was added to a pH of 2, which did not alter the composition of the reaction mixture considerably. A further reduction of the pH of the mixture lead to the loss of protection groups which can be clearly seen in the mass spectrum in Figure 26. As acetyl protecting groups show a lower stability than benzoyl ester groups, the loss of PG at low pH values can be expected. The resistance to the hydrolysis of the glycosidic bond at higher pH values can be explained with electronic effects of ester protecting groups on carbohydrates and has already been discussed in former chapters.

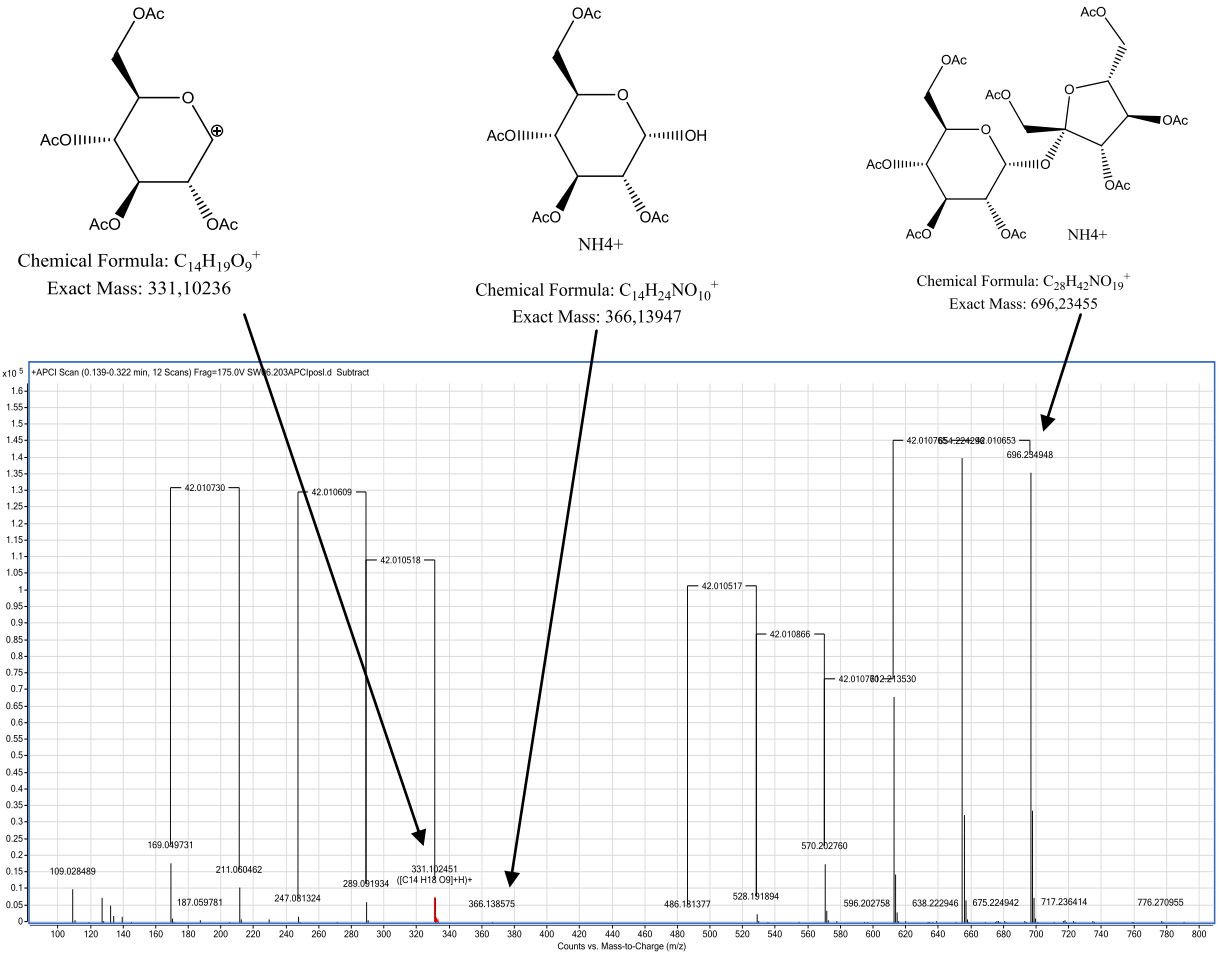


Figure 26: TOF-mass spectrum using positive APCI ionization: monitoring of the loss of PG during the hydrolysis reaction of octa-acetyl-sucrose under acidic conditions; detected ions are formed $[M+NH_4]^+$ or $[M+H-H_2O]^+$ adducts

4.3 Step 3 – Synthesis of 2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate (4) [38]

The synthesis of the imidate derivative was carried out using a base in order to promote the formation of an alkoxide and the addition of the formed anion to an electron-poor nitrile. Due to the choice of the base (sodium hydride) and prolonged reaction times (4 h), the formation of the α-anomer of the resulting protected glucopyranosyl trichloroacetimidate was favoured (for further explanations see also Chapter 2.3). Using TLC to monitor the reaction, the initial formation of both anomers can be observed but base-catalysed anomerization of the β-anomer leads to the formation of the thermodynamically more stable α-anomer.

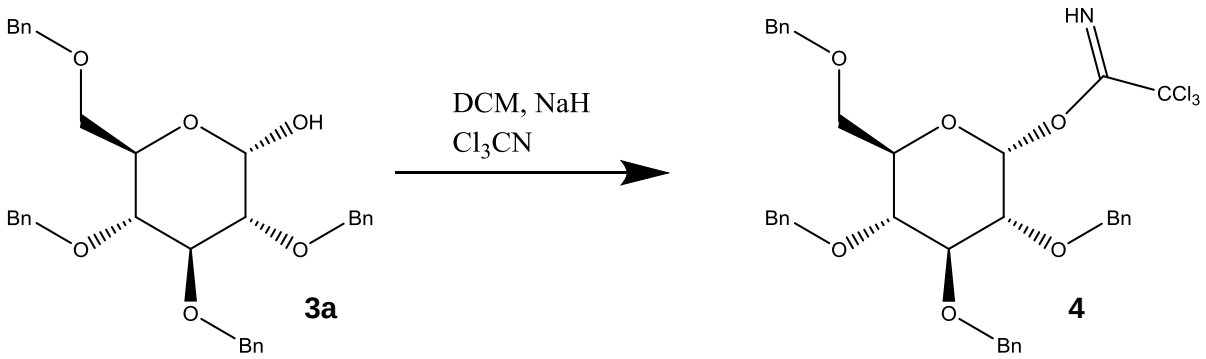


Figure 27: Reaction scheme for the addition of trichloroacetonitrile to 2,3,4,6-tetra-O-benzyl-D-glucopyranose

Schmidt et al. [38] describe the precipitation of the oily product within a few hours, but the obtained reaction product became not solid only when stored at 4°C for four weeks. Due to this long dead time, future experiments should aim at using the oily product for the following reaction step 4 of the synthesis sequence.

Yield: 9.74 g, 14.2 mmol, 98% of theory
R_f: 0.63 (α-anomer), 0.57 (β-anomer) (n-hexane/ethyl acetate 2/1)
MP: 69-71°C (Literature: 72-73°C resp. 77°C[38])

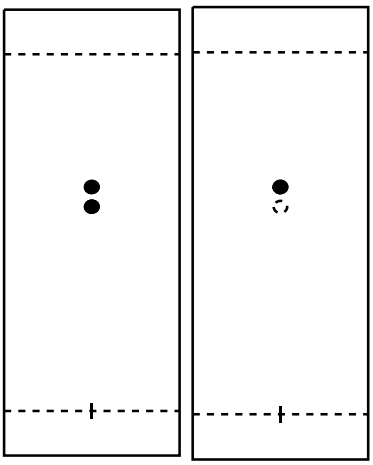


Figure 28: TLC of the reaction after 30 min. (left) and after 3 h (right)

NMR

The recorded NMR-spectrum confirms the presence of the α-anomer when compared to the literature ([59]).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.61 (1H, s) 7.36-7.30 (22H, m) 7.29-7.17 (2H, m) 6.56 (1H, d) 5.01-4.47 (10H, m) 4.11-4.00 (2H, m) 3.85-3.67 (5H, m)

Solvent peak: 7.26 ($CDCl_3$) [42]

^{13}C NMR ($CDCl_3$) δ 161.32 (C7), 138.61, 138.05, 137.95, 137.84, 128.42, 128.39, 128.36, 128.13, 128.08, 128.01, 127.90, 127.87, 127.83, 127.77, 127.73, 127.72, 127.64, 127.57 (aromatic C), 94.37 (C1), 91.28 (C8), 81.38 (C3), 79.36 (C2), 77.45, 77.03, 76.79 (solvent, C4), 76.61, 75.65 (O*C*H_2Ph), 75.35 (O*C*H_2Ph), 73.49 (O*C*H_2Ph), 73.12 (C5), 72.88 (O*C*H_2Ph), 68.01 (C6)

Solvent peak: 77.17 ($CDCl_3$) [42]

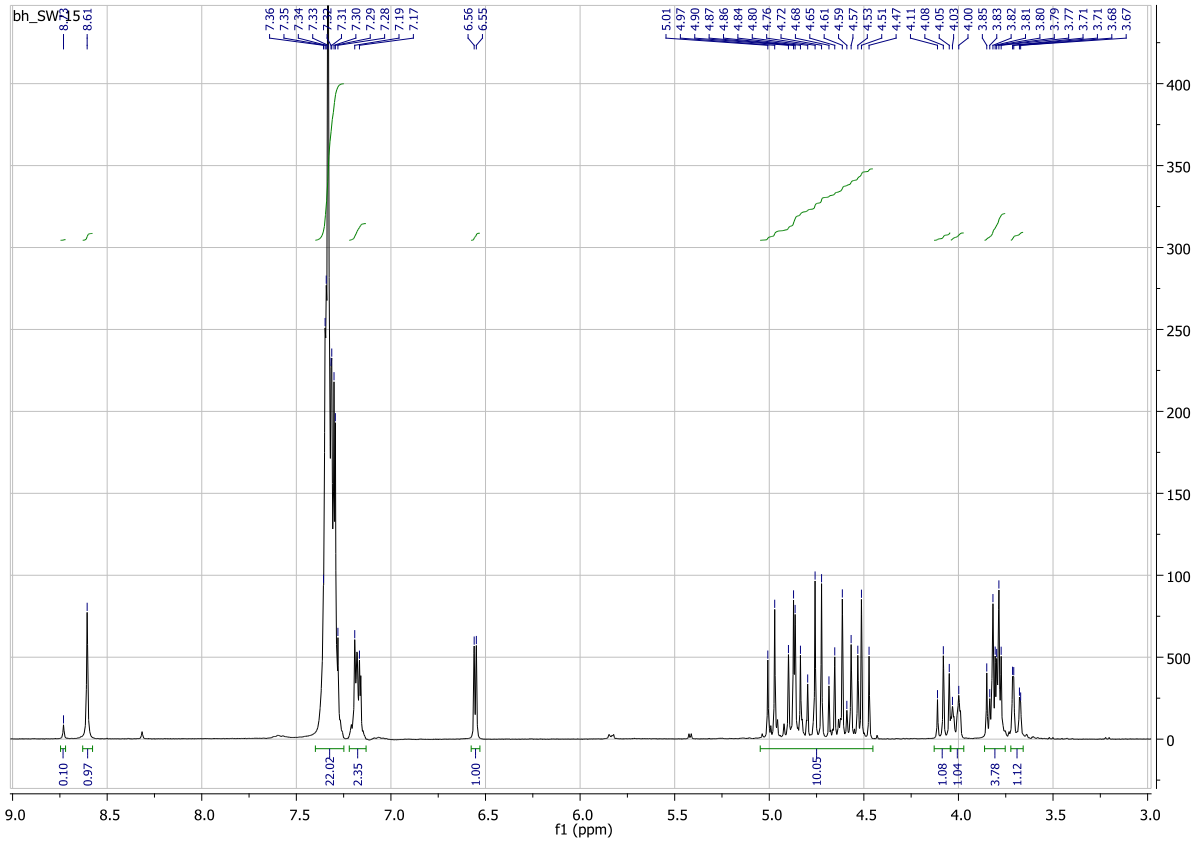


Figure 29: ^{1}H NMR spectrum of 2,3,4,6-tetra-O-benzyl-D-glucopyranose trichloroacetimidate

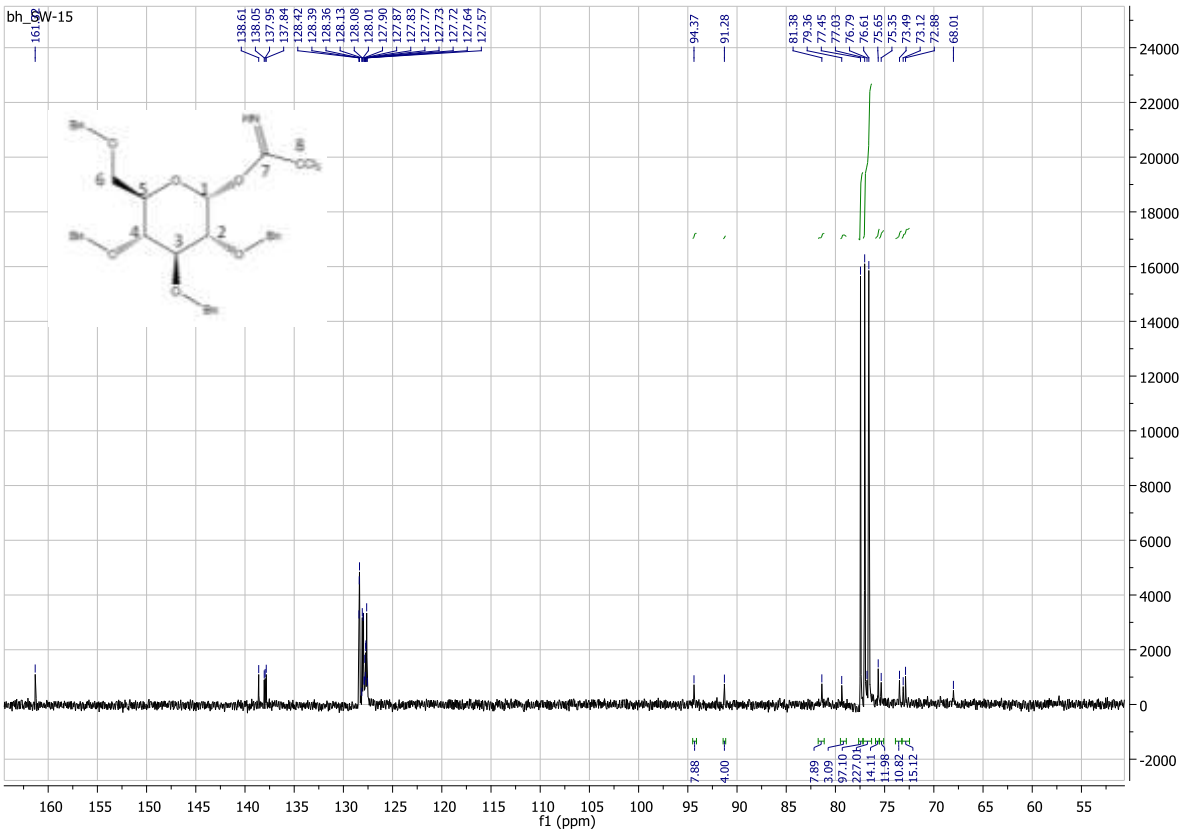


Figure 30: ^{13}C NMR spectrum of 2,3,4,6-tetra-O-benzyl-D-glucopyranose trichloroacetimidate

4.4 Step 4 – Synthesis of Cholesteryl 2,3,4,6- tetra-*O*-benzyl-D-glucopyranoside (5) [50]

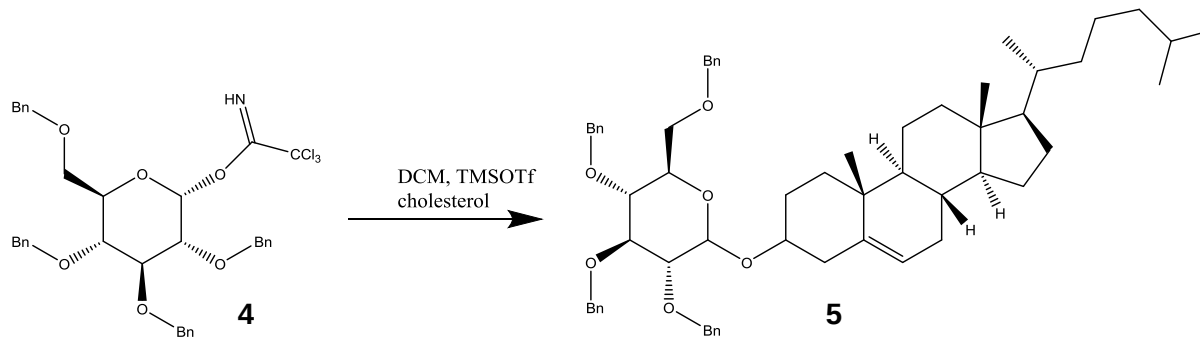


Figure 31: Reaction scheme of the formation of the glycosidic bond between cholesterol and the sugar compound using the trichloroacetimidate method

During the third step of the reaction sequence, the formation of a glycosidic linkage between the glycosyl donor, namely the benzylated glucopyranoside and cholesterol as the glycosyl acceptor, has to be carried out. The formation of glycosidic bonds is not easy to perform, an activation of the donor is necessary, also the determination of the stereoselectivity of the reaction product can cause problems. As already described, the used benzyl ethers are chemically inert and therefore do not have an active influence on the development of charges during the reaction (see Chapter 2). Using the trichloroacetimidate derivative, the formation of the glycosidic linkage can be performed using trimethylsilyl triflate as a promoter. Water free conditions are necessary to prevent any side reactions and therefore the used DCM was dried by distillation over P_2O_5 and stored over a molecular sieve with 4Å. Also, cholesterol was stored under dry conditions and a fresh TMSOTf- solution was prepared right before the start of the reaction. In addition, a drying funnel was used to seal the reaction flask. The monitoring of the reaction using TLC can be seen in Figure 32. The spots on the left side represent the staring material whose intensity decreases as time moves on and the formation of the desired product (black spots in the middle resp. on the right) can be observed. The reaction yield is at 58% which is very low compared to other steps of the sequence. This might be explained by the purification step of the fine precipitate, which was not easy to separate from the matrix. Investigation by using the oily product for the last reaction step of the sequence could be made in future experiments. Also, the end product showed the formation of an α/β-anomeric mixture, which can be seen in TLC as well as in the NMR-spectrum (appr. a ratio of 50/50). As 2,3,4,6- tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate was used as glycosyl donor, the β-anomer of the reaction product should be obtained ([13]) but according to [19], using TMSOTf as a promoter and DCM as a solvent gives preferably the α-D-glycoside of the product. This discrepancy of reaction stereo-control

conditions explains the obtained α/β-ratio of the target molecule. Reaction optimization concerning the stereoselectivity should be performed in future experiments.

Yield: 6.04 g, 6.6 mmol, 58% of theory

R_f: 0.81, 0.86 (n-hexane/ethyl acetate 2/1)

MP: 112-118 °C (no value found in literature)

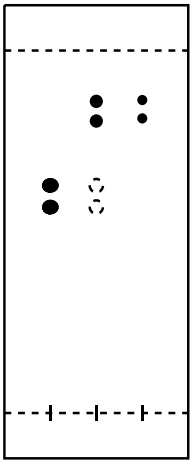


Figure 32: TLC: starting material t=0 (left), reaction product black dots t=45 min. (middle) and end point at t=80 min. of the reaction (right)

LC/MS-TOF

Positive APCI ionization mode: calculated for $[C_{61}H_{80}O_6+NH_4]^+$: 926.62932; found: 926.628875 $^m/_z$; Δm: 0.48 ppm

NMR

The recorded NMR-spectrum shows some impurities but confirms the presence of both anomers when compared to the literature ([60]).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39-7.14 (26H, m) 5.37-5.35 (0.57H, br d) 5.31-529 (0.49H, br d) 5.01-4.53 (11H, m) 4.02 (1H, t) 3.90 (1H, br d) 3.80-3.43 (10H, m) 2.45-2.38 (3H, m) 2.03-1.87 (6H, m) 1.52-0.87 (34H, m) 0.70 (3H, d)

Solvent peak: 7.26 ($CDCl_3$) [42]

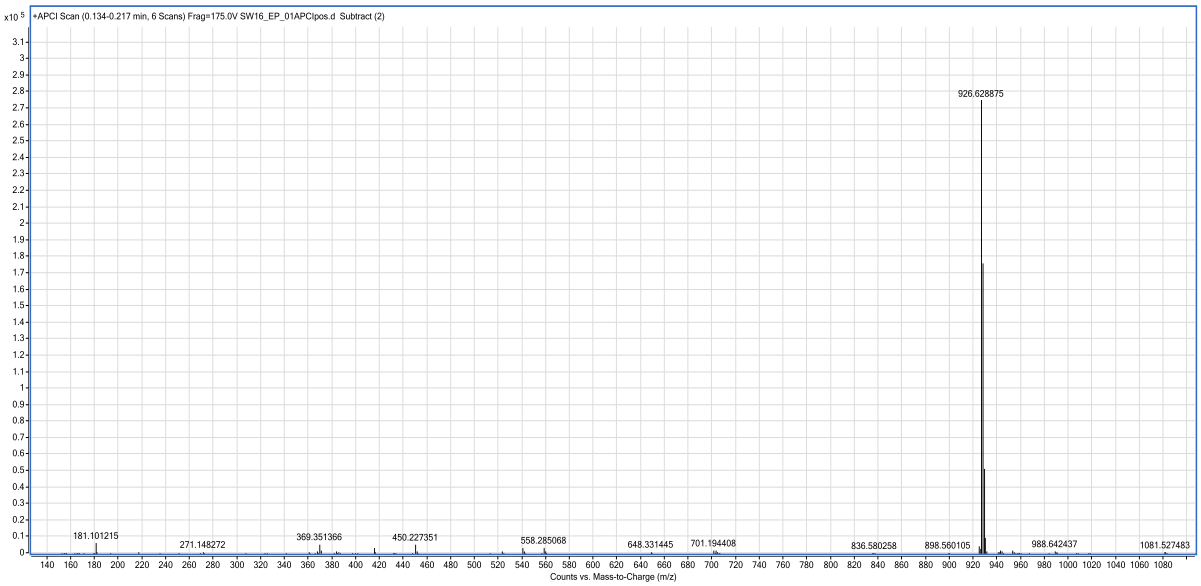


Figure 33: TOF-mass spectrum using positive APCI ionization of cholesteryl 2,3,4,6-tetra-O-benzyl-D-glucopyranoside

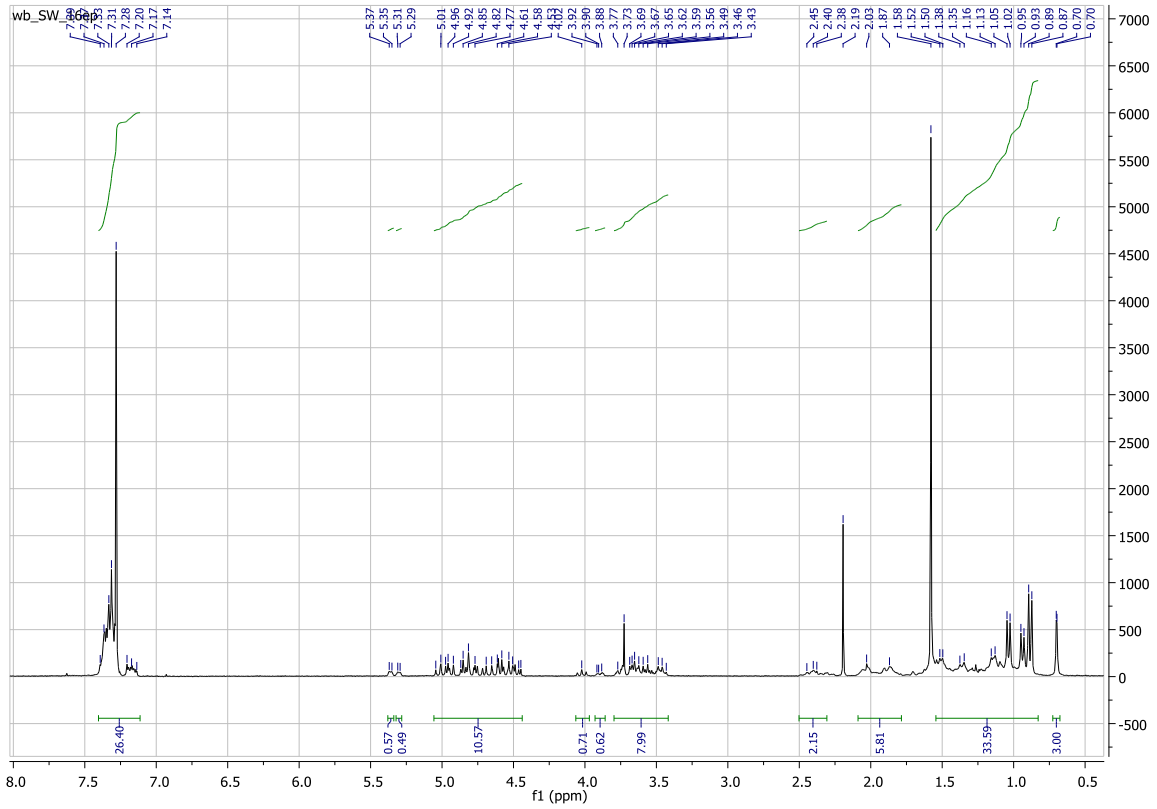


Figure 34: ^{1}H NMR spectrum of cholesteryl 2,3,4,6-tetra-O-benzyl-D-glucopyranoside

4.5 Step 5 – Removal of Sugar Protecting Groups

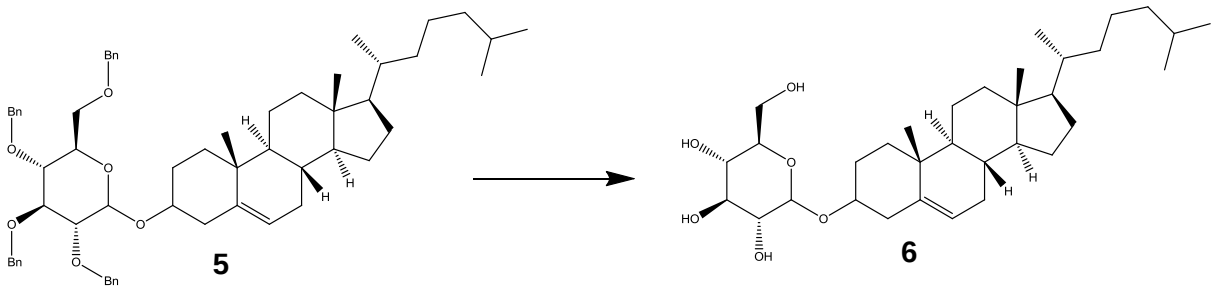


Figure 35: Reaction scheme of the removal of protecting groups of the sugar moiety

Benzyl ethers are widely used as protecting groups in carbohydrate chemistry due to their stability and high tolerance to various chemical conditions. [61], [62] The removal of the protecting groups can be performed using hydrogenation reactions with hydrogen gas and Pd/C or $Pd(OH)_2$/C as catalyst or by catalytic transfer hydrogenation which represents a widely used alternative method to avoid the handling of flammable hydrogen gas. Different

hydrogen donors such as formic acid, cyclohexene, cyclohexadiene in combination with a palladium catalyst can be employed. [62]–[64] Also, other methods using strong Lewis acids, anhydrous $FeCl_3$ or strong basic conditions (e.g. sodium in ammonia) can be used. [61] In order to work out the best method for the given reaction, different approaches were tried. The best results concerning yield and reaction time were obtained by using the $Pd(OH)_2/C$ catalyst in combination with cyclohexene as the hydrogen donor.

4.5.1 $Pd(OH)_2/C$ with Cyclohexene

The reaction was carried out according to [65], cyclohexene was used in great excess in a mixture with the used solvent. Using LC/TOF-MS and TLC to monitor the reaction (see Figure 37), the cleavage of the ether bonds could be tracked precisely. After stirring for 24 h, the removal of the protecting groups was complete. An important remark is the insolubility of the starting material in the used solvent mixture (in the literature, the reaction is carried out with the soluble α-anomer). The starting material (**5**) was suspended in the solvent and after the addition of the catalyst the mixture was heated to reflux, which in turn led to complete dissolution of the starting material. Problems occurred during the filtration when trying to remove the catalyst. Due to its fine particles, the removal was very time-consuming as several centrifugation and filtration steps were needed. This led to losses of the target molecule which had a negative impact on the obtained yield. Nevertheless, due to the fast and complete deprotection, a second try with a greater amount of starting material was performed. As a greater amount of catalyst and an improved filtration technique using a celite® bed was used, the problems of the first experiment could be solved and a yield of >99% was achieved. Using high temperature GC/MS (see Figure 36) as well as LC/MS-TOF (see Figure 38), the high purity of the product could be verified. Also, the presence of the double bond of the cholesterol moiety could be proven by LC/MS-TOF which remained stable under the used reaction conditions.
Yield: 124 mg, 0.2 mmol, 85% of theory (first experiment)
Big: 3.7427 g, 6.8 mmol, > 99% of theory (second experiment)
MP: 215-217°C (Literature: α-anomer:206-208°C, β-anomer: 258-260°C [67])

LC/MS-TOF

Negative APCI ionization mode: calculated for $[C_{33}H_{56}O_6+HCOO]^-$: 593.40589; found: 593.405519 $^m/_z$; Δm: 0.63 ppm

NMR

Due to the presence of both anomers of **6** the NMR- spectra can't easily be interpretated but characteristic peaks of both anomers are present when compared to the literature [30], [65].

^{1}H NMR (d_6 DMSO, 300 MHz) δ 5.36-5.26 (1H, m) 4.92-4.70 (3H, m) 4.50-4.40 (2H, m) 4.20 (1H, d) 3.67-3.57 (1H, m) 3.48-3.39 (2H, m) 3.18-2.98 (3H, m) 2.93-2.86 (1H, m) 2.51 (3H, m) 2.40-2.05 (2H, m) 2.00-1.70 (6H, m) 1.60-0.85 (40H, m) 0.65 (3H, s)

Solvent peak: 3.36 (DMSO)

^{13}C NMR (d_6 DMSO) δ121.66, 77.2, 73.90, 70.48, 56.64, 56.04, 50.01, 42.32, 38.76, 37.28, 36.64, 36.13, 35.67, 31.88, 28.25, 27.87, 24.34, 23.68, 23.14, 22.86, 21.08, 19.56, 19.01, 12.14, 39.12 - 40.79 (solvent peaks)

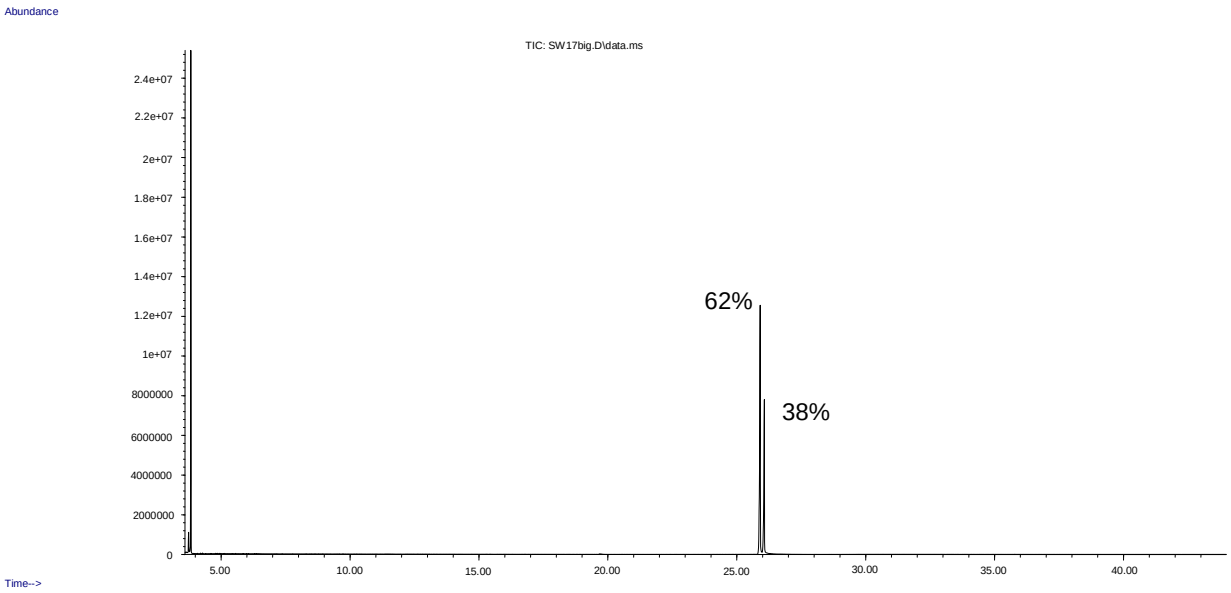


Figure 36: GC/MS of cholesteryl-D-glucopyranoside, two anomers are found with a ratio of 62% (α-anomer) to 38% (β-anomer). The anomeric classification was verified with a cholesteryl-β-D-glucopyranoside reference material from sigma aldrich

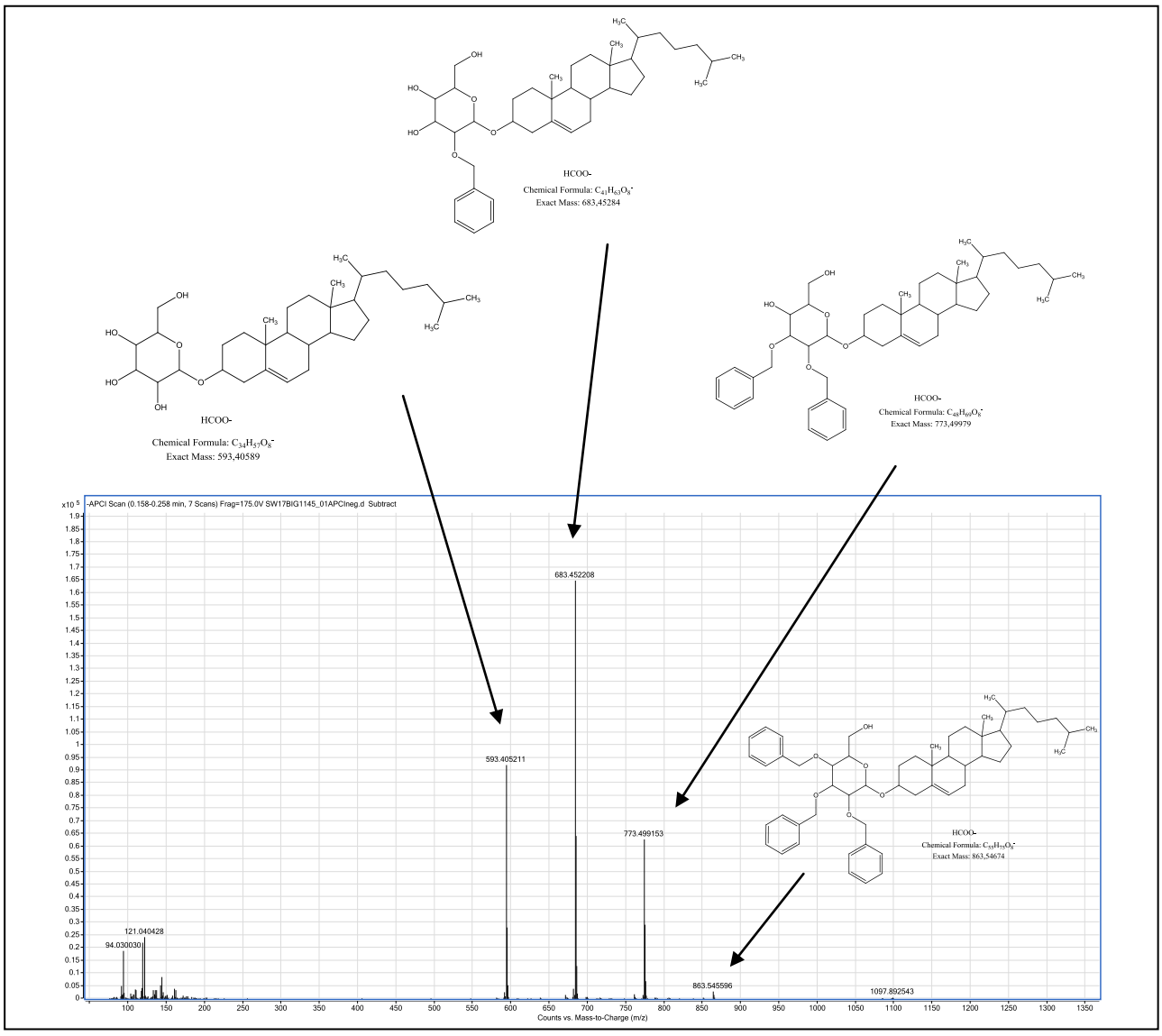


Figure 37: TOF-mass spectrum using negative APCI ionization of the monitoring of the removal of the benzyl ether protecting groups after 45 min. reaction time

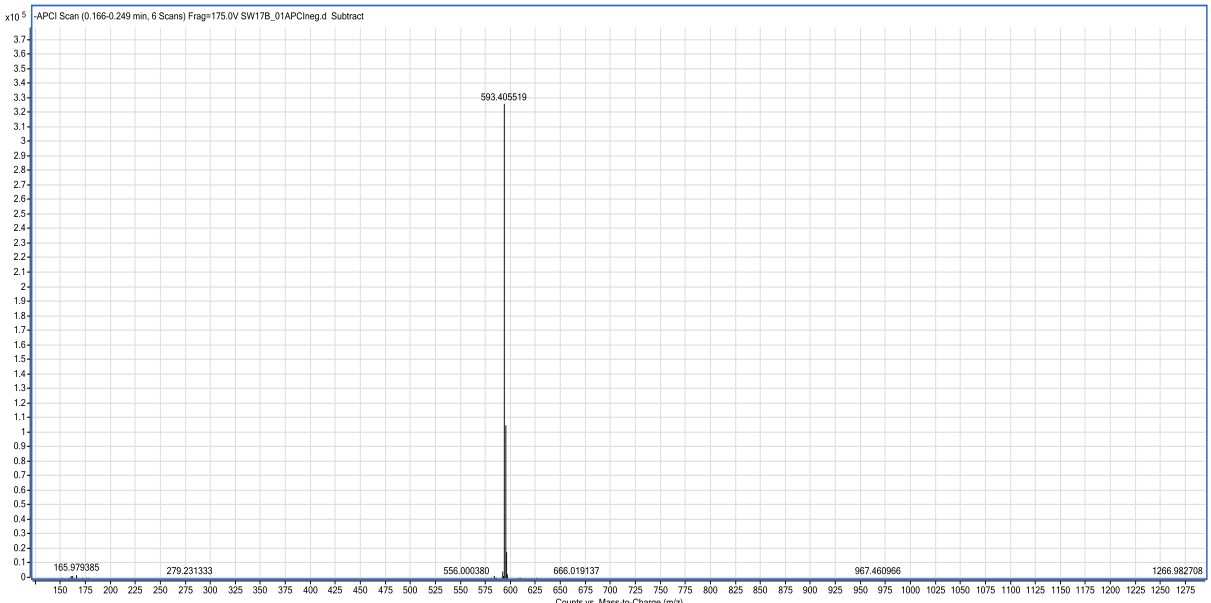


Figure 38: TOF-mass spectrum using negative APCI ionization of cholesteryl-D-glucopyranoside at the end of the reaction

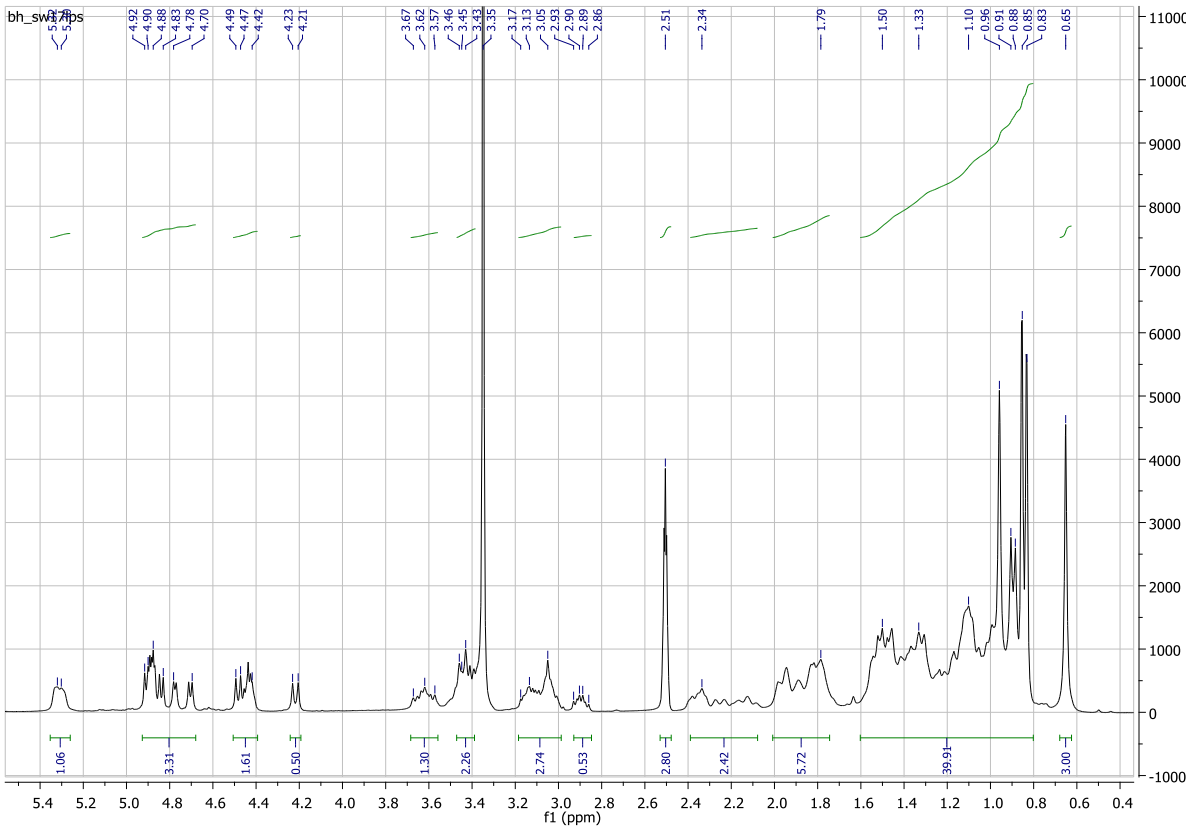


Figure 39: ^{1}H NMR spectrum of cholesteryl-D-glucopyranoside

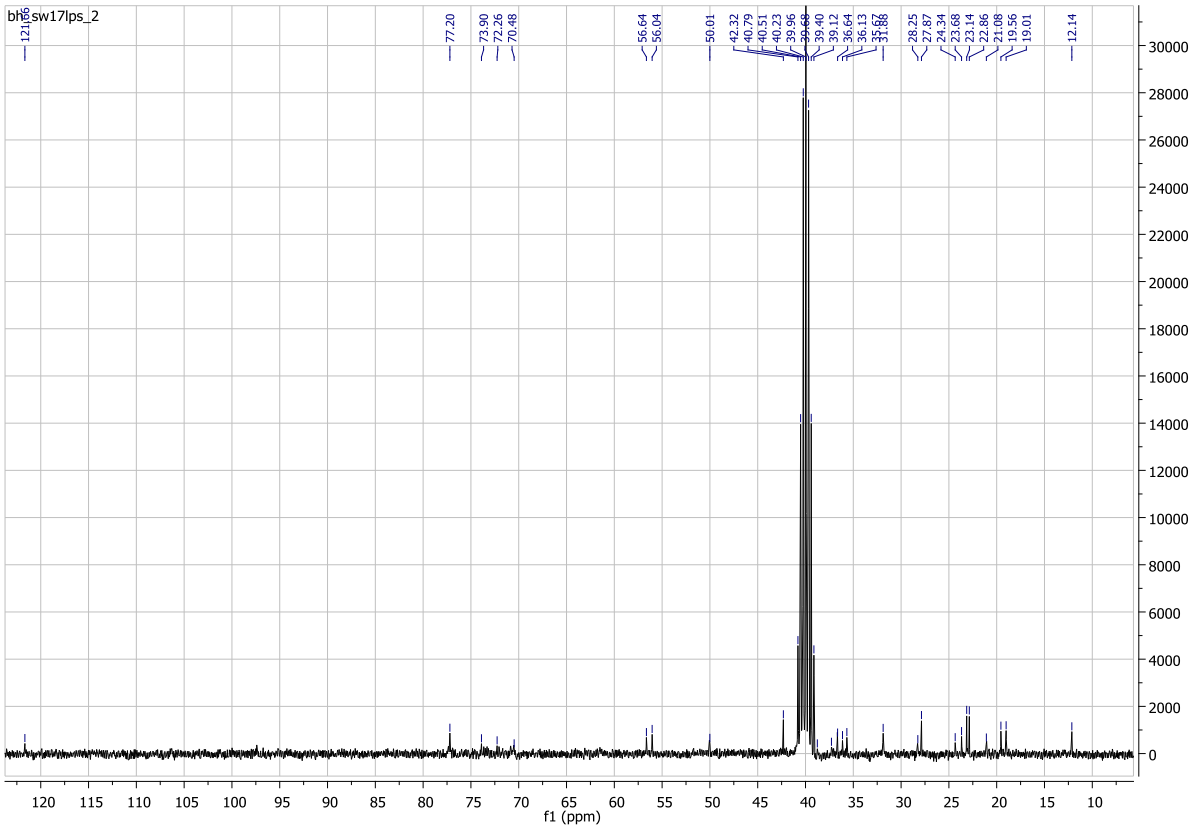


Figure 40: ^{13}C NMR spectrum of cholesteryl-D-glucopyranoside

4.5.2 $Pd(OH)_2/C$ with Et_3SiH

The $Pd(OH)_2/C$ and Et_3SiH reaction system could also prove its suitability for the desired reaction. It is suggested, that an in situ generation of molecular hydrogen takes place upon the addition of silane to the palladium catalyst which in turn serves as a hydrogen donor. [63] The method was able to completely remove the benzyl protecting groups. However, the reaction took four days to complete and a great excess of Et_3SiH had to be added portion-wise throughout the reaction time. The in situ generation of hydrogen could be seen after every addition of Et_3SiH (bubbles) which came along with the loss of effective reactant and might be the reason for prolonged reaction times and the great use of silane. Unlike expected, the double bond of the cholesterol moiety stayed intact although this catalytic hydrogen transfer system is usually able to reduce double bonds.
Yield: 175.1 mg, 0.3 mmol, >99% of theory

4.5.3 $Pd(OH)_2/C$ with Formic Acid

Using only a minor amount of acid, a reaction could not be observed and therefore a higher amount of acid was added carefully (drop- wise). This did not result in the deprotection of the sugar moiety but instead, the glycosidic bond of the compound was cleaved due its lability in acidic conditions.

4.5.4 $NaBrO_3$ and $Na_2S_2O_4$

Adinolfi et al. [61] presented the interesting approach of removing benzyl ether protecting groups by using a redox-system consisting of sodium bromate and sodium dithionite in an ethyl acetate/water mixture. The proposed decomposition of formed HBrO (aqueous solution) liberates $Br^{\cdot}$ which brominates the side chain of the aromatic moieties. Oxidative opening using sodium dithionite results in the removal of the protecting groups. Using this system at the investigated reaction, the formation of the desired product could not be observed but instead, various reaction side-products were obtained.

4.5.5 $FeCl_3$

Anhydrous iron(III)chloride in dry DCM is reported to be highly effective for the cleavage of benzyl ethers at room temperature. [66] Complexation of the compound to the oxygen atom of the ether and subsequent replacement by a proton is proposed as a reaction mechanism. However, due to the Lewis acid character of iron(III) chloride, the cleavage of the glycosidic bond of the protected cholersteryl glucoside was observed.

4.6 Evaluation of the Synthesis Route

The displayed synthesis route represents an easy, fast and competitive method for the synthesis of steryl glucosides which could further be used as a reference and standard material for quantitative analysis purposes. An overall yield of 32% was achieved, which represents an improvement compared to previous results from the working group (Christopher Ludwig, 17% [50]) and exceeds overall yields of other synthesis methods (Koenigs-Knorr: 30%, trichloroacetimidate-method: 21% [50]). The high purity of the synthesised cholesteryl-D-glucopyranoside could be determined using GC/MS and LC/MS-TOF. A mixture of the α- and β-anomer was obtained, whereof the α-anomer contributes with 62% and the β-anomer with 38%.

The big advantage of the present synthesis route is the use of the readily available and cheap starting material sucrose which was bought in the form of powdered sugar in the supermarket. Furthermore, no time consuming and intense clean up procedures like column chromatography during any of the reaction steps is necessary but instead, simple and fast work-up methods can be performed.

Steps two and four represent the bottlenecks of the synthesis route, as they contribute highest to losses during the entire pathway. The optimization attempts of reaction step 2 by using different kinds of protecting groups or another disaccharide as starting material did not yield in better reaction outcomes. But due to patient and accurate working methods, the yield could be increased compared to former reactions. Nevertheless, this step still carries the potential to be further optimized to reach the yield achieved in the literature. Reaction step 4 has not been described in literature before, but different reaction conditions could lead to anomerically pure target molecules as already described in Chapter 4.4. Also, the work-up procedure for this reaction step should be further optimized in order to account for losses in yield.

Benzyl ether has proven to ideally represent protecting groups for the given synthesis pathway. Their introduction is simple and high-yielding and they exhibit excellent stability to all applied reaction conditions. Furthermore, they do not hinder desired reactions, due to their electronically passive properties. In addition, the removal of the protection groups in the last step is easily feasible by using cyclohexene as a hydride donor which avoids the risk of using flammable hydrogen gas. The cleavage of the ether bond is fast and due to the mild reaction conditions, the hydration of the Δ^5 double bond present in the cholesteryl moiety can be prevented.

APCI-TOF-MS was extensively used as an analysis tool as it represents a rapid and direct method to monitor the progress of the described reactions and to identify formed products and by-products. Also, it is an excellent tool for the high sensitive analysis of steryl glucosides.

5 Conclusions and Outlook

Due to the growing demand of biofuels such as FAME, it is of great interest to the biodiesel industry to propose solutions to difficulties and problems related to the use of biodiesel. An important topic is engine performance problems caused by phytosteryl glucosides which occur as precipitates in the fuel matrix. Available and affordable standard and reference materials for those compounds would be a great benefit for the industry in order to facilitate the challenging analysis of steryl glucosides.

The investigated synthesis pathway for cholesteryl glucoside starting from sucrose has proven to be a fast, cheap, high-yielding and therefore competitive preparation method for these natural compounds. The high purity of the products allows for their use as a reference and as a standard material for quantitative analysis purposes.

Sucrose was used as starting material with the fructosyl-part employed as acid labile protecting group for the anomeric hemiacetal OH-group of the glucopyranosyl-moiety. Utilizing trehalose as an alternative starting material could not be employed in the pathway due to the high bonding energy of the glycosidic linkage.

Benzyl ethers have shown to possess the most advantageous properties for protecting groups of the sugar moiety compared to other common protecting groups such as benzoates and acetates. In addition, their removal at the end of the synthesis route was achieved by a straight-forward, fast and high-yielding catalytic transfer hydrogenation using cyclohexene as a hydrogen donor.

The next research undertaking could aim at further optimizing single reaction steps and the synthesis of anomerically pure products. In addition, the pathway could be performed using other sterols, such as sitosterol, stigmasterol and campesterol. Not to mention, synthesis using enzymatic catalysis would be an interesting field for further research.

6 List of Figures and Tables

7 Literature

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8 Appendix

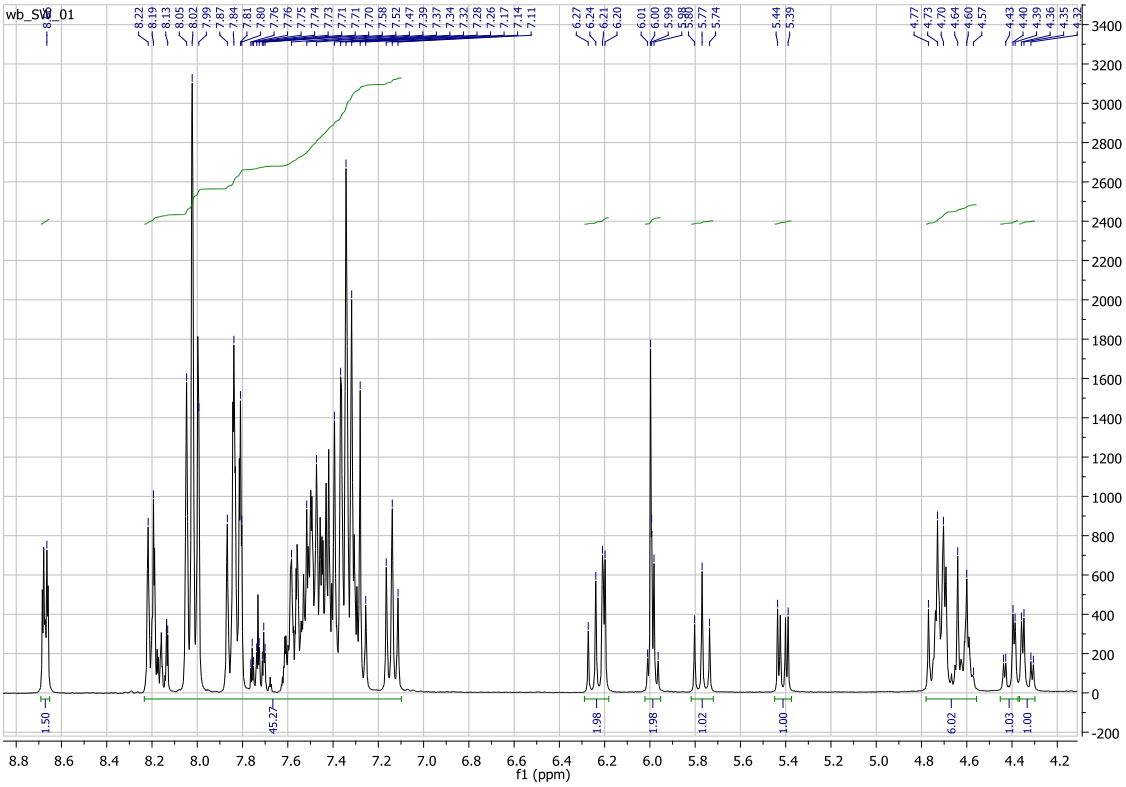

Figure 41: ^{1}H NMR spectrum of 1,3,4,6-tetra-O-benzoyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranoside

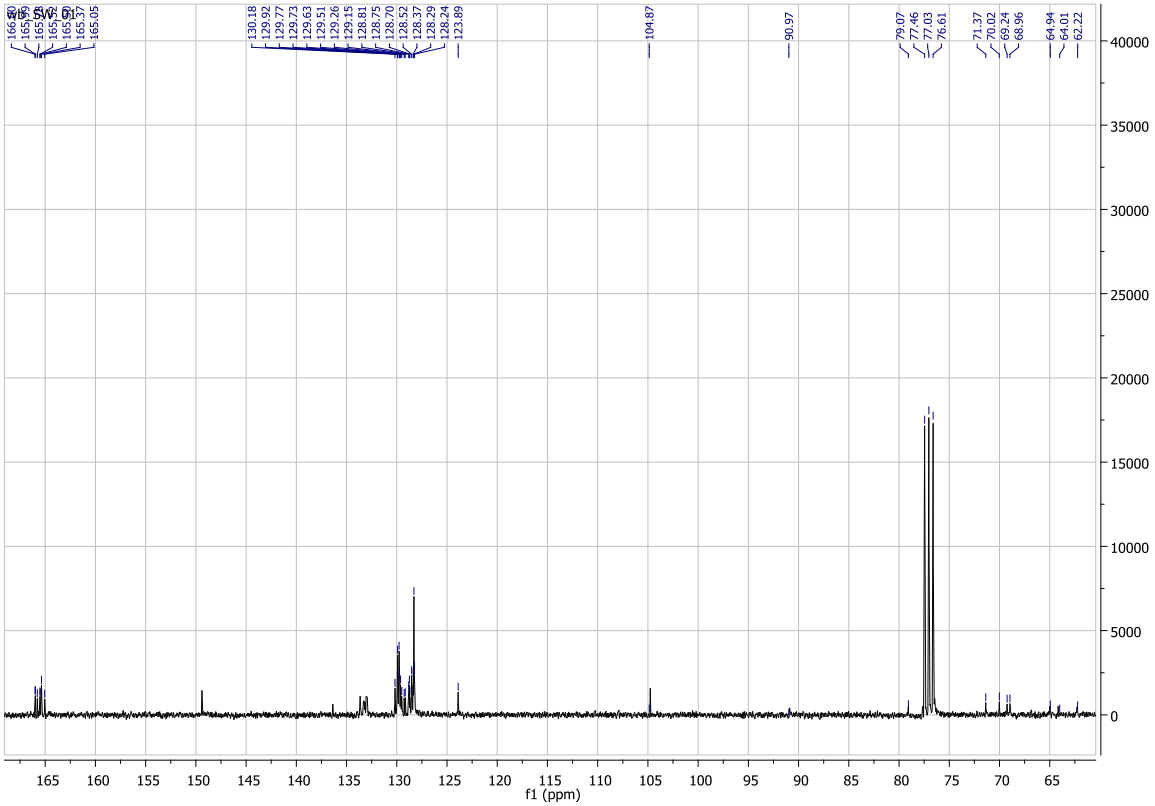

Figure 42: ^{13}C NMR spectrum of 1,3,4,6-tetra-O-benzoyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranoside

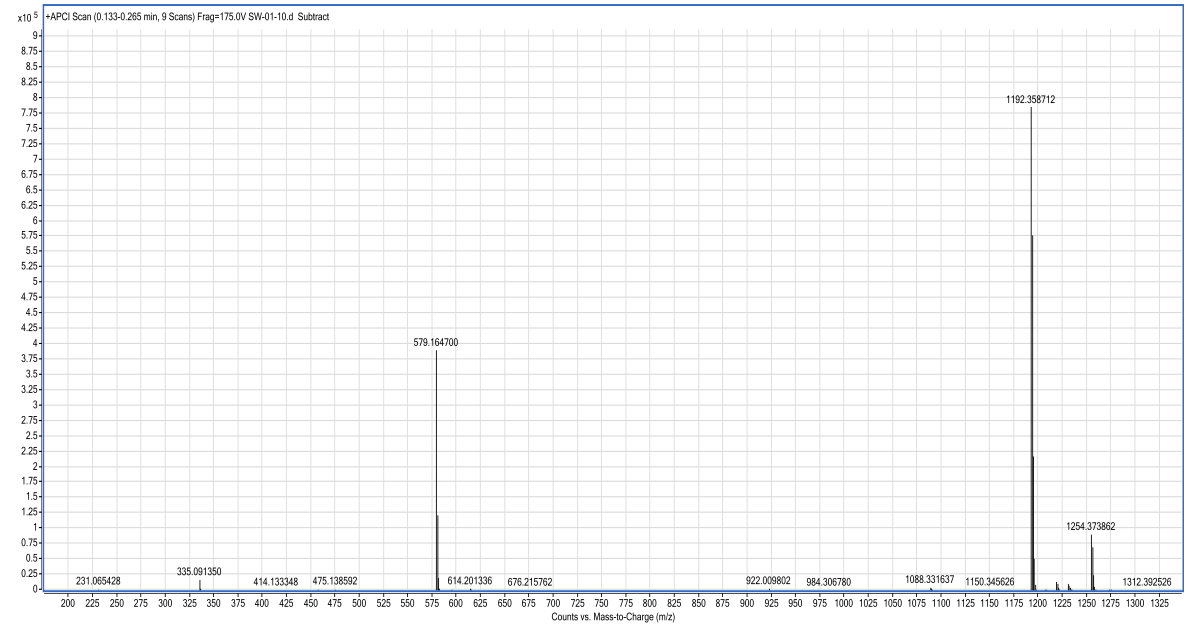

Figure 43: TOF-mass spectrum using positive APCI ionization of 1,3,4,6-tetra-O-benzoyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranoside

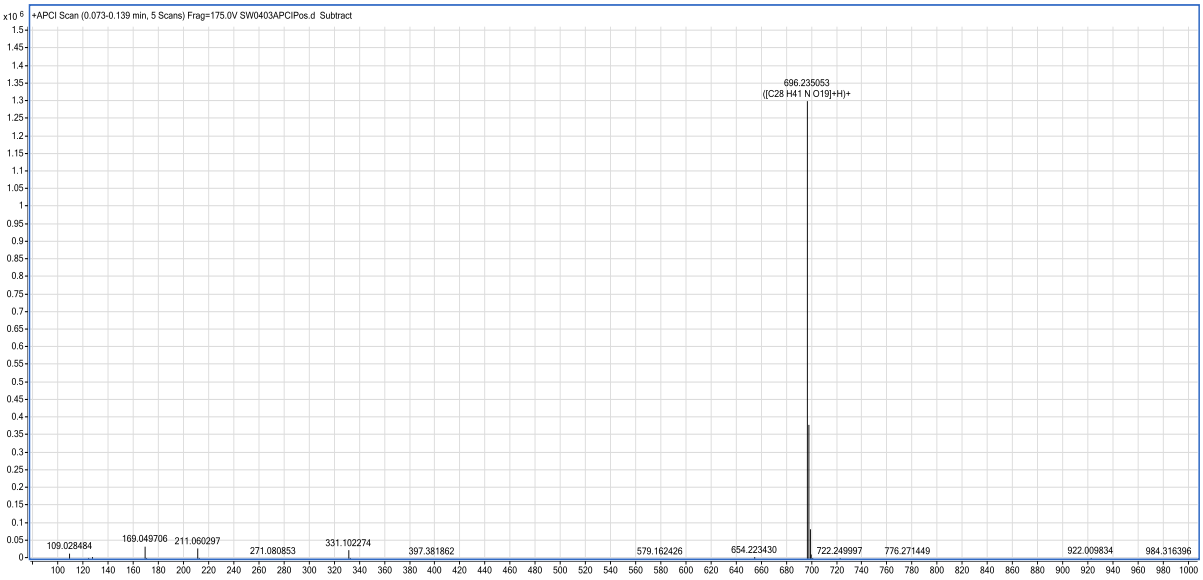

Figure 44: TOF-mass spectrum using positive APCI ionization of 1,3,4,6-tetra-O-acetyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside

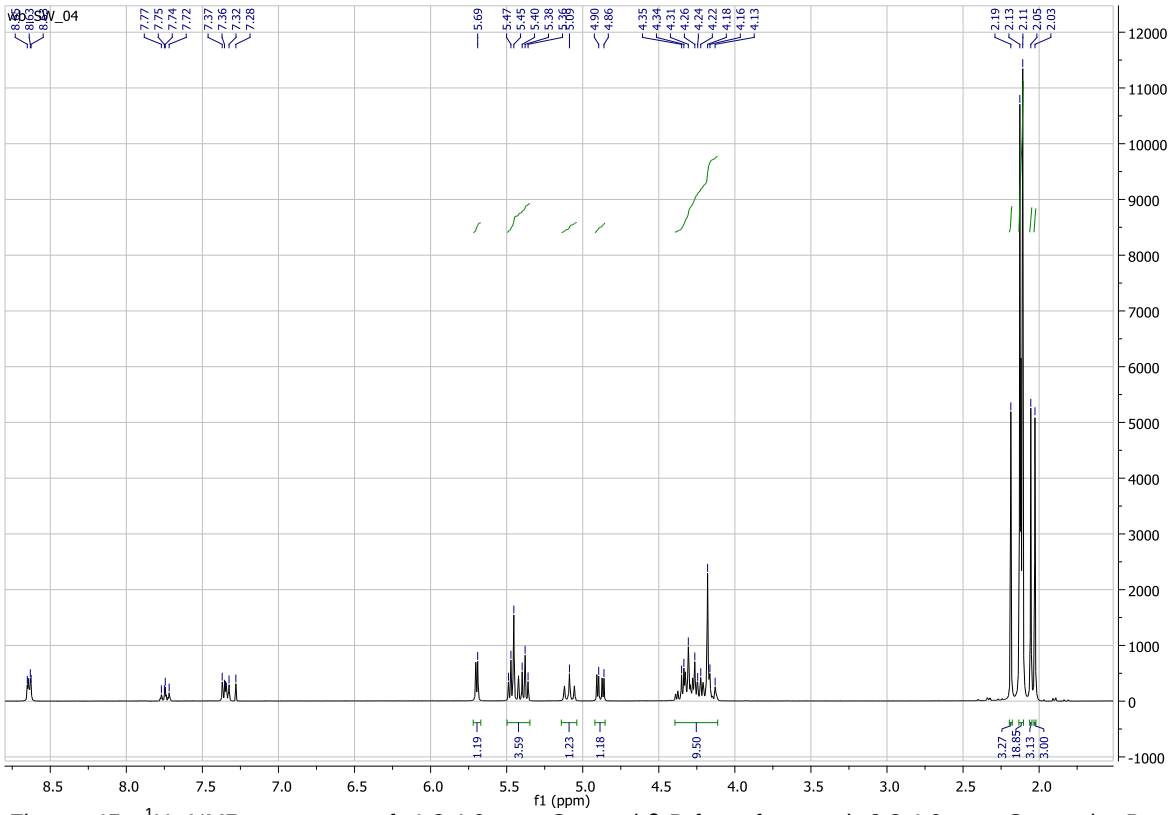

Figure 45: ^{1}H NMR spectrum of 1,3,4,6-tetra-O-acetyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside

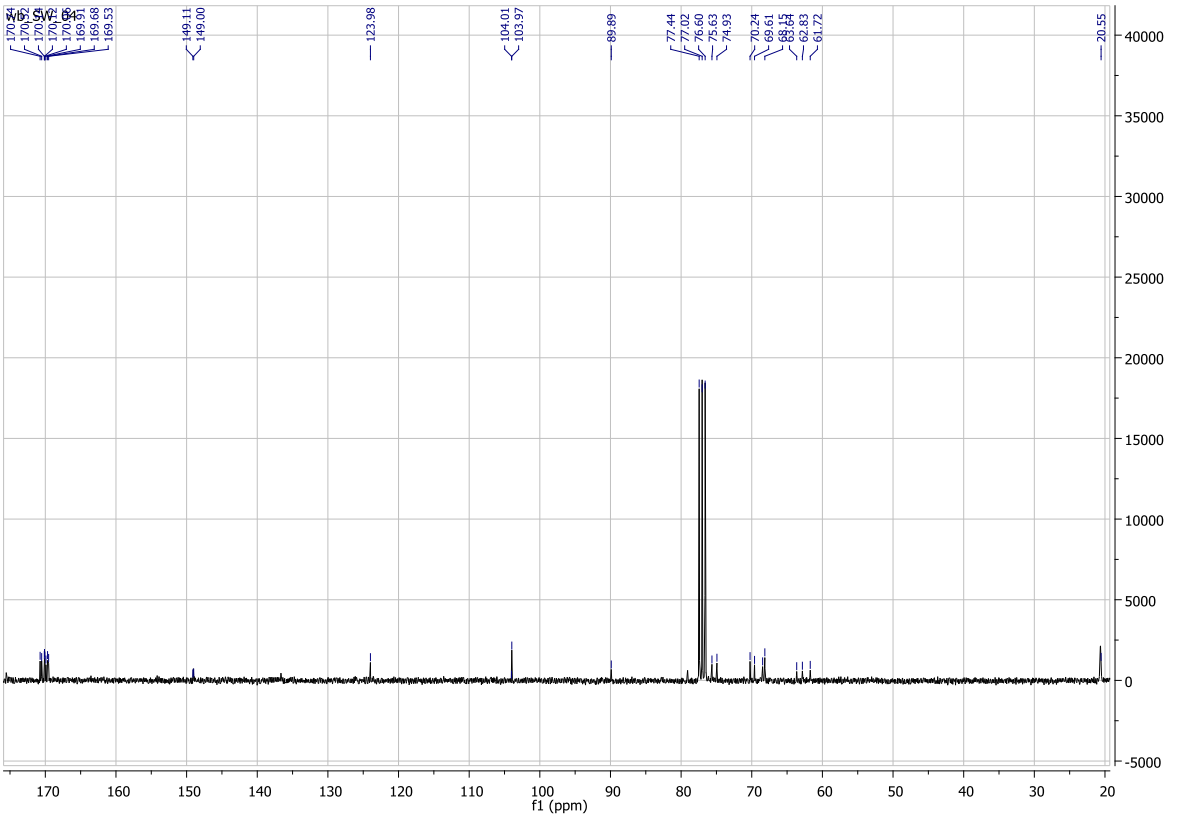

Figure 46: ^{13}C NMR spectrum of 1,3,4,6-tetra-O-acetyl-β-D-fructofuranosyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside

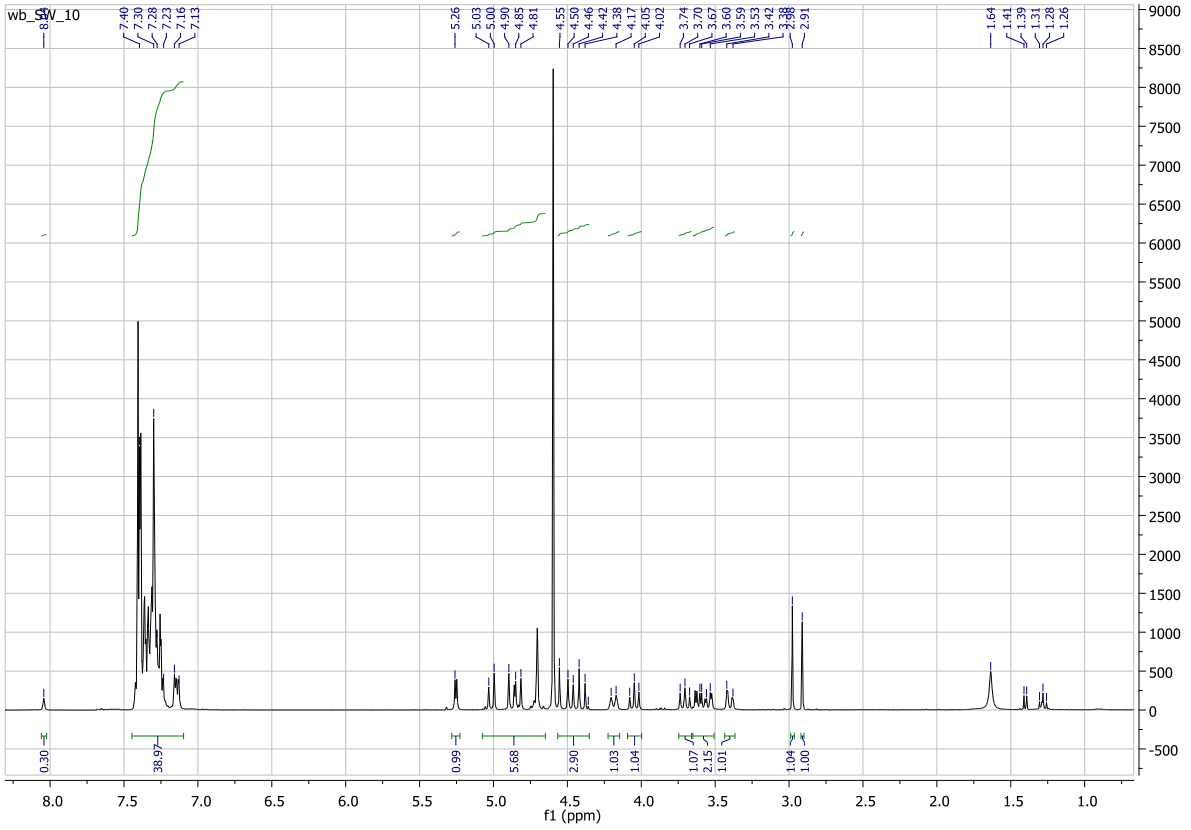

Figure 47: ^{1}H NMR spectrum of 2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside

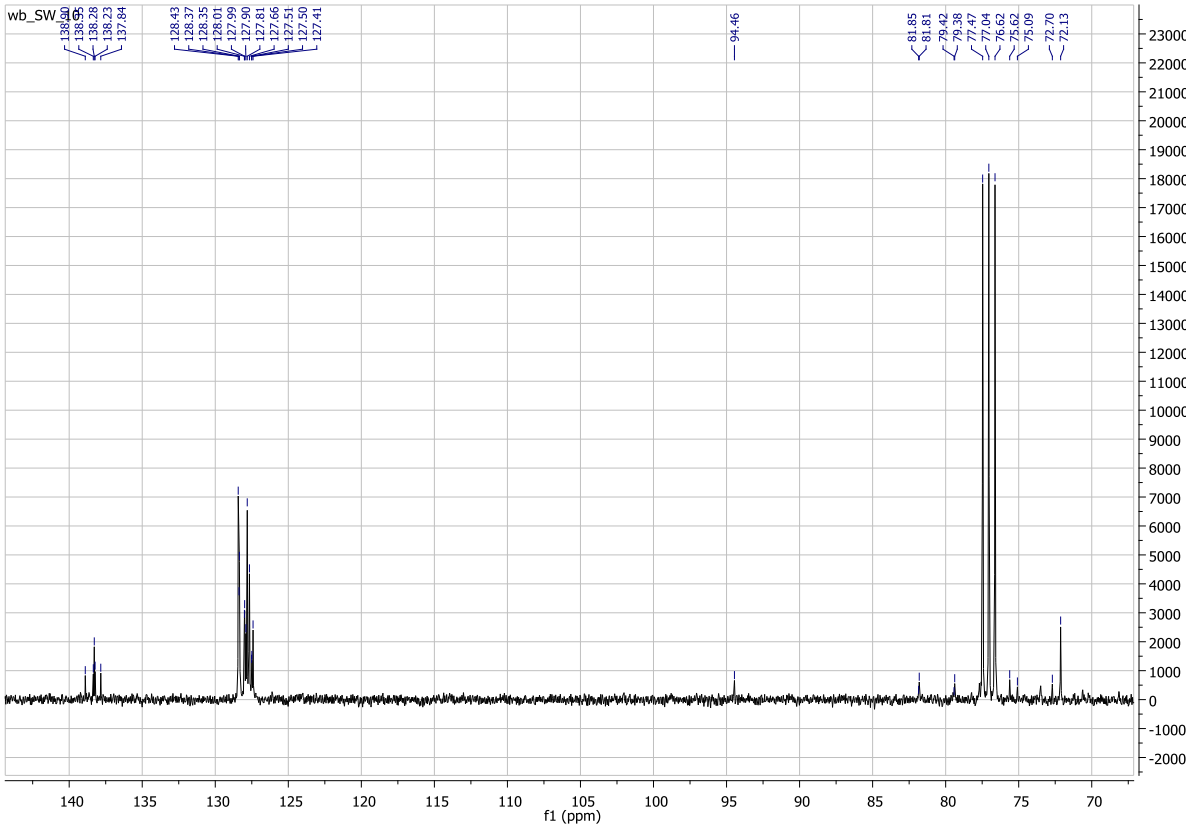

Figure 48: ^{1}H NMR spectrum of 2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside

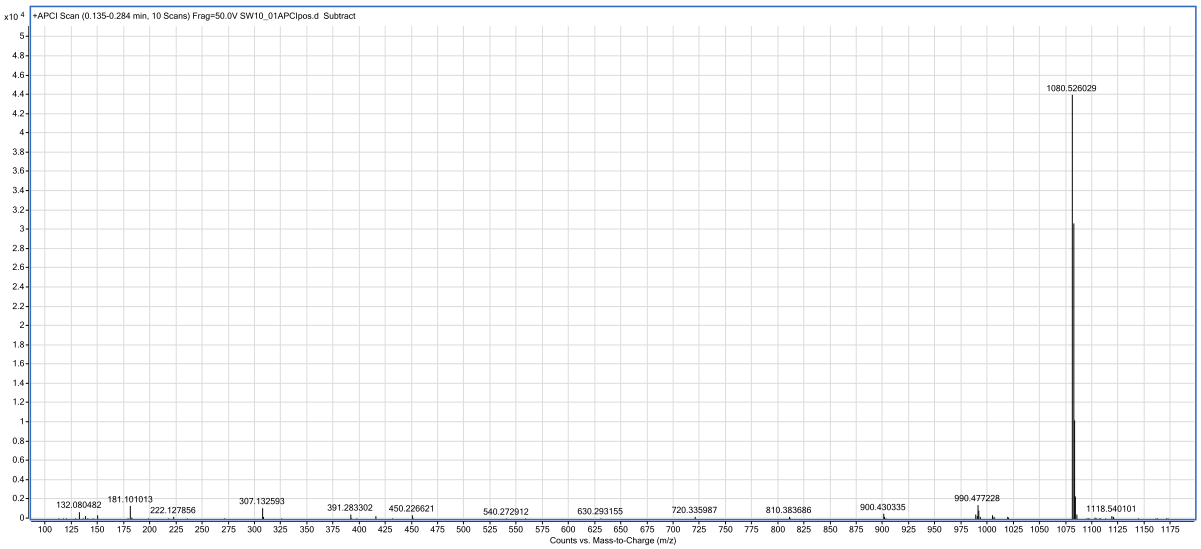

Figure 49: TOF-mass spectrum using positive APCI ionization of 2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside

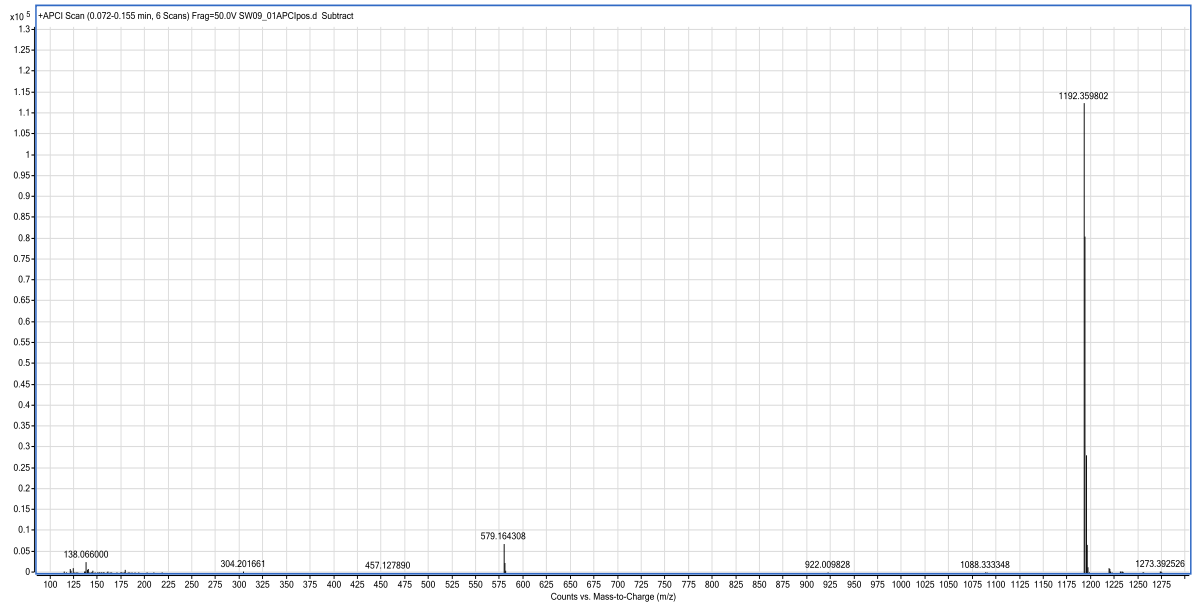

Figure 50: TOF-mass spectrum using positive APCI ionization of 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranosyl 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranoside

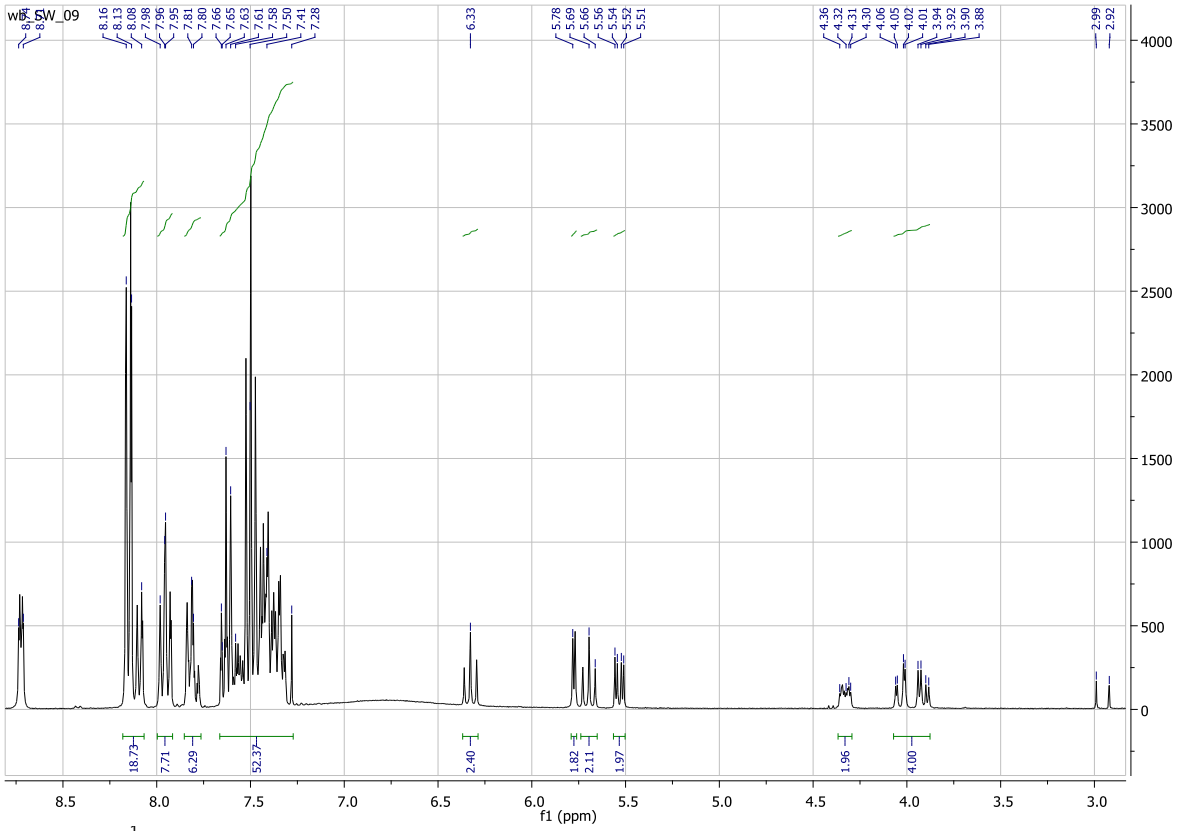

Figure 51: ^{1}H NMR spectrum of 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranosyl 2,3,4,6-tetra-O-benzoyl-α-D-glucopyranoside