STUDY OF THE MECHANISM OF SWEET ALMOND β-GLUCOSIDASE

AND

SYNTHESIS OF A DISACCHARIDE BUILDING BLOCK FOR SIDE-CHAIN-BRANCHED (1, 3; 1, 6) β-D-GLUCANS

AN ABSTRACT

SUBMITTED ON THE 27TH DAY OF MAY, 2014

TO THE DEPARTMENT OF CHEMISTRY

OF THE SCHOOL OF SCIENCE AND ENGINEERING OF

TULANE UNIVERSITY

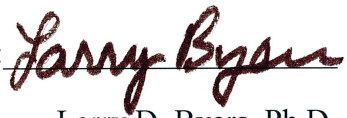
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

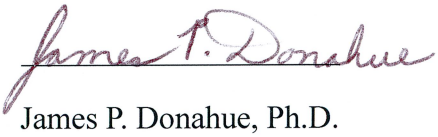
Miaomiao Xie

APPROVED:



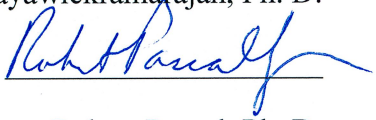
Larry D. Byers, Ph.D.

Chairman



James P. Donahue, Ph.D.

Janarthanan Jayawickramarajah, Ph. D.



Robert Pascal, Ph. D.

ABSTRACT

β-Glucosidase from sweet almond is a family 1 retaining glycohydrolase, catalyzing the highly efficient hydrolysis of a variety of glycosides. Retaining glycosidases operate through a two-step mechanism. The first step is the glycosylation of the enzyme and the second step is the deglycosylation of the glycosylated enzyme intermediate. However, the detailed mechanisms of these two steps remain unclear. In this study, several alkyl glucosides, with leaving groups of varying pKas, were used to study the mechanism of β-glucosidase from sweet almond. The derived β_{eq} value for the equilibrium constant K for glycoside hydrolysis was found to be -0.27, meaning the effective charge on the glycosidic oxygen in the substrate is positive 0.27. According to the negative kinetic Brønsted coefficient (β_{lg} = -0.97) obtained for hydrolysis of aryl glucosides, the effective charge on oxygen of substrate in the glycosylation transition step is minus 0.7. This means there is 76% breaking of the carbon-oxygen bond in the transition state. The negative kinetic Brønsted coefficient (β_{lg} = -0.97) was obtained via hydrolysis of aryl glucosides with pKas greater than 7.1. As the pKa of the leaving group drops below 7.1, the slope of the structure-reactivity correlation changes ($\beta_{lg} \approx 0$). This implies that the rate-limiting step is changed to the breakdown of the glucosyl-enzyme intermediate. The glucoside for 2, 4-dinitrophenol (pKa=4.3) is a good substrate. With this substrate the

rate-limiting step (in kcat) is deglycosylation. The value of the α-secondary deuterium kinetic isotope effect on kcat of 2, 4-dinitrophenol glucoside was found to be 1.12 (± 0.02). The large isotope effect indicates that hydrolysis of the glucosyl-enzyme intermediate occurs via an S_N1-like mechanism; this implies that a relatively large amount of carbo-cation character exists on the anomeric carbon in the deglycosylation step. The measured value of ^2H KIE differs significantly from the kinetic isotope effect (^2H KIE; k_H/k_D=1.01) for the glycosylation step obtained using phenyl β-glucopyranoside (Dahlquist, 1969), where the transition state is more S_N2-like.

β- (1, 3)-Glucans are polysaccharides of D-glucose monomers linked by β-glycosidic bonds. In addition to linear β- (1, 3)-linkages, glucans can also be branched with side chains of varying length via β- (1, 6)-glycosidic bonds along the backbone. β-Glucans are biological response modifiers (BRMs) and have the ability to activate the immune system. They have attracted great attention in recent years as non-specific immune stimulants with low toxicity. One difficulty in this field is the lack of structurally well-defined and pure glucans. This has led to contradictory results from physiological studies and limits their value. Therefore, a synthetic strategy for the production of structurally well-defined glucans is still an area of intense interest. In this work a disaccharide building block for branched oligosaccharides has been synthesized. This disaccharide building block containing the 4-acetoxy-2, 2-dimethylbutanoate (ADMB) group makes it a good substrate for coupling reactions. The sensitive protecting p-methoxybenzylidene groups can be removed without affecting the benzylidene functionality. Through the outlined methodology, the branched oligosaccharides can be synthesized in an expedient and synthetically simple manner.

STUDY OF THE MECHANISM OF SWEET ALMOND β-GLUCOSIDASE

AND

SYNTHESIS OF A DISACCHARIDE BUILDING BLOCK FOR SIDE-CHAIN-BRANCHED (1, 3; 1, 6) β-D-GLUCANS

A DISSERTATION

SUBMITTED ON THE 27TH DAY OF MAY, 2014

TO THE DEPARTMENT OF CHEMISTRY

OF THE SCHOOL OF SCIENCE AND ENGINEERING OF

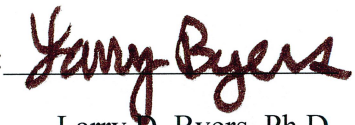
TULANE UNIVERSITY

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

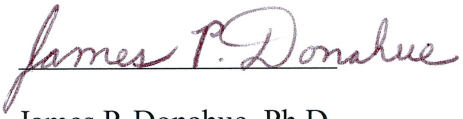
BY

Miaomiao Xie

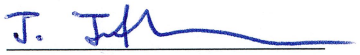
APPROVED: 

Larry D. Byers, Ph.D.

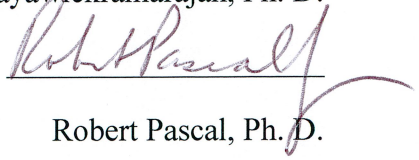
Chairman



James P. Donahue, Ph.D.



Janarthanan Jayawickramarajah, Ph. D.



Robert Pascal, Ph. D.

ACKNOWLEDGEMENT

I wish to thank Dr. Larry Byers for his time, effort, and patience. I want to thank Dr. Harry Ensley for his education and instruction on my synthesis. I also want to thank my committee members Dr. Janarthanan Jayawickramarajah, Dr. Robert Pascal and Dr. James P. Donahue. Finally, I want to thank all members of the Tulane Chemistry Department are and all my friends and family.

TABLE OF CONTENTS

Part II Synthesis of a Disaccharide Building Block for Side-Chain-Branched (1, 3; 1, 6) β-D-Glucans

LIST OF SCHEMES

LIST OF FIGURES

LIST OF TABLES

APPENDICES

Abbreviations:

2, 4-DNPG (2, 4-dinitrophenyl β-D-glucopyranoside)

BSP (1-benzenesulfunyl piperidine)

DABCO (1, 4-diazabicyclo [2.2.2] octane)

DBU (1, 8-diazabicyclo [5.4.0] undec-7-ene)

DEAD (diethyl azodicarboxylate)

DMF (dimethylformamide)

DNP (2, 4-dinitrophenol)

MES (2-(N-morpholino)ethanesulfonic acid)

PIPES (piperazine-N, N'-bis(2-ethanesulfonic acid)

PMP (*p*-methoxybenzylidene)

pNPG (*p*-nitrophenyl β-D-glucopyranoside)

pNP (*p*-nitrophenoxide)

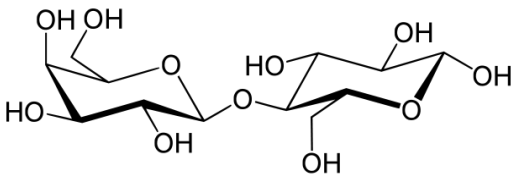
pNPOH (*p*-nitrophenol)

Part I Study of the Mechanism of Sweet Almond β-Glucosidase

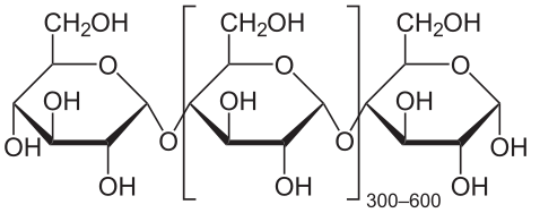
INTRODUCTION

Glycohydrolases

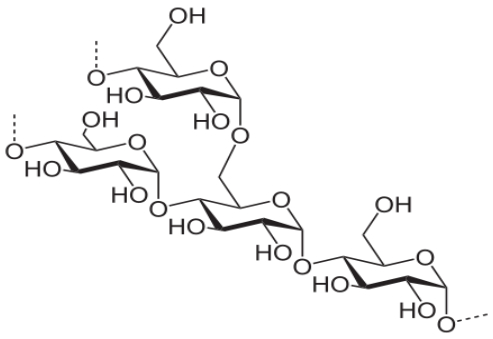
Glycohydrolases (GH), enzymes which catalyze the hydrolysis of a glycosidic bond, are extremely common enzymes with a broad distribution in nature. The importance of understanding glycohydrolase activity is apparent given the involvement of carbohydrates and their conjugates in a myriad of biological processes. Glycohydrolases also have numerous substrates and a great variety of functions for both humans and the rest of the natural world. For example, the deficiency of lactase, the enzyme that degrades the milk sugar lactose, leading to lactose intolerance in some populations; amylases, enzyme that catalyzes the breakdown of starch (amylose and amylopectin) into sugars, are widely used in the fermentation industry; lysozyme can damage bacterial cell walls by catalyzing hydrolysis of 1, 4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan (Figure 1).



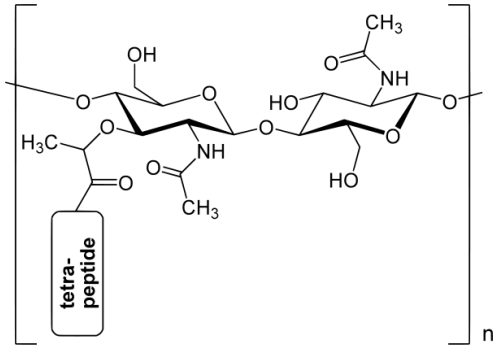
Lactose



Amylose



Amylopectin



Peptidoglycan

Figure 1. Substrates of various glycohydrolases.

Due to the natural variety of carbohydrates, glycohydrolases also have a large diversity. Glycohydrolases are classified into EC 3.2.1 by the IUB Enzyme Nomenclature system (1984). The three digits indicate enzymes hydrolyzing O-glycosyl linkages. They can also be classified into over one hundred families on the basis of their amino acid sequence similarities. Henrissat [1] originally classified over 50 families of glycohydrolases based on their amino acid sequences. Because there is a direct relationship between primary sequence and folding similarities, this method can be used

to predict the mechanism, the active sites and possible substrates of new enzymes. However, the stereospecificity of the enzyme-catalyzed glycoside bond cleavage allows us to also classify glycohydrolases according to the anomeric configuration of the product, namely, "retaining" and "inverting". Classical glycohydrolases have two carboxylic acid-containing side-chains in the active site. For the inverting GHs, a single displacement mechanism is employed in which general acid and general base activity by the active site carboxylic acid/carboxylate residues leads to protonation of the glycosidic oxygen and deprotonation of water molecular, thus leaving group departure is accompanied by concomitant nucleophilic attack by water (Figure 2). Retaining GHs process via a double displacement mechanism in which an enzyme-glycosyl intermediate is formed with the active site carboxylate residue acting as a nucleophile. The second residue, a carboxylic acid, is thought to function as a general acid catalyst in the glycosylation step and a general base in the deglycosylation step (Figure 3).

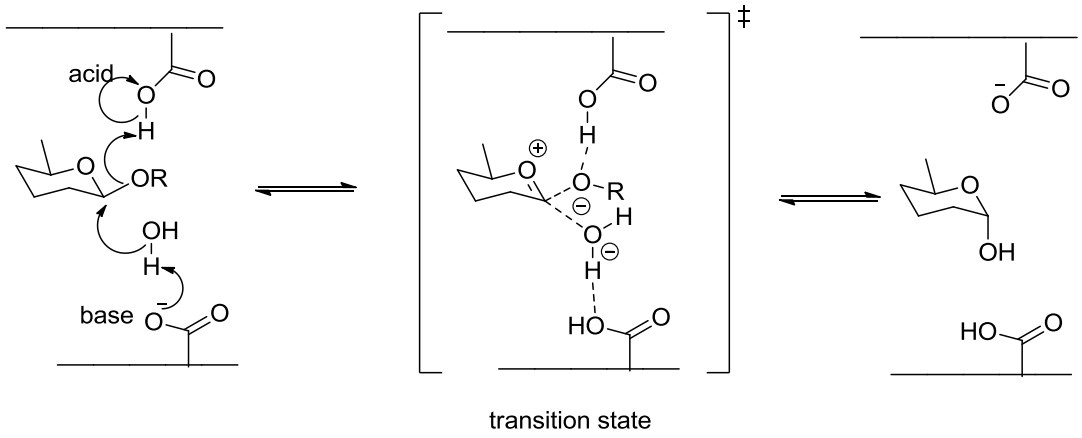


Figure 2. General mechanism of inverting glycohydrolases

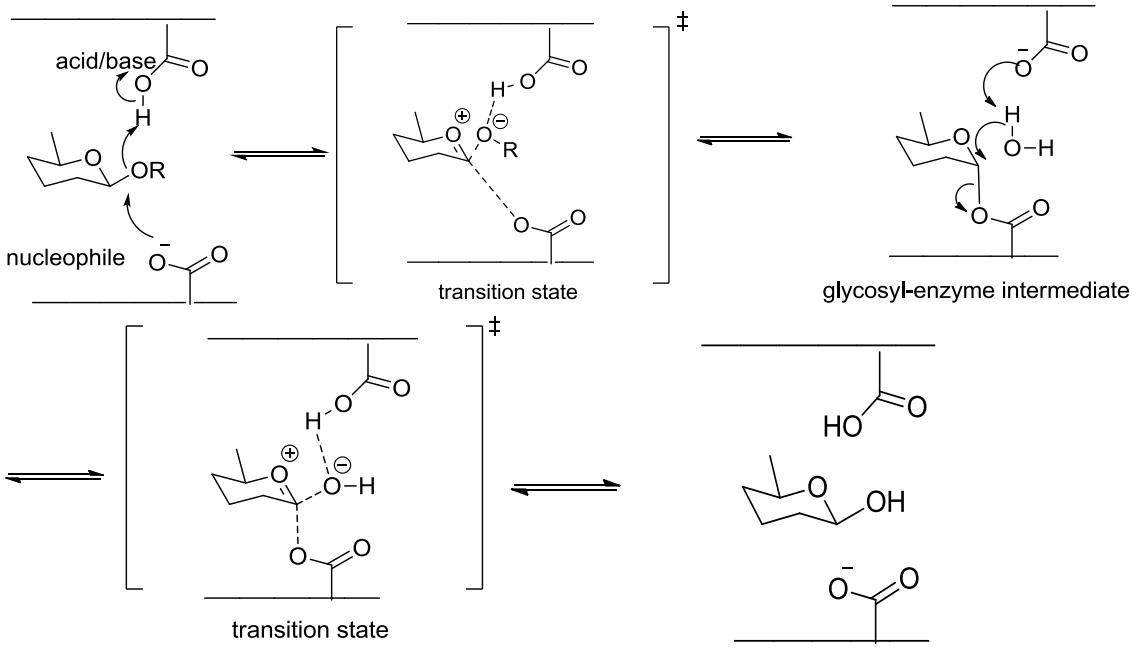


Figure 3. General mechanism of retaining glycohydrolases

β-Glucosidases: function and application

β-Glucosidases (E.C. 3.2.1.21) are glycohydrolases that hydrolyze the non-reducing terminal β-D-glucosyl residues and release β-D-glucose. These enzymes are found in many domains of living organisms, archaea, eubacteria, and eukaryotes and they play a variety of functions.

Although the definition of β-glucosidases is straightforward, the abundance of non-reducing terminal β-D-glucosyl residues in nature has led to the assignment of many E.C. numbers for enzymes that hydrolyze glycosidic bonds. For example, β-glucocerebrosidases (3.2.1.45), cholesteryl-β-glucosidase (3.2.1.104), strictosidine-β-glucosidase (3.2.1.105), amygdalin β-glucosidase (3.2.1.117), prunasin β-glucosidase (3.2.1.118), vicianin β-glucosidase (3.2.1.119), raucaffricine β-glucosidase (3.2.1.125), and coniferin β-glucosidase (3.2.1.126). Some substrates of β-glucosidases are showed in Figure 4.

Since the E.C. numbers tell us relatively little about the mechanism, structure, or relationship to other glycoside hydrolases, the family classification system is necessary. β-glucosidases mainly fall into glycoside hydrolase families GH1, GH3, GH5, GH9, and GH30 [1, 2].

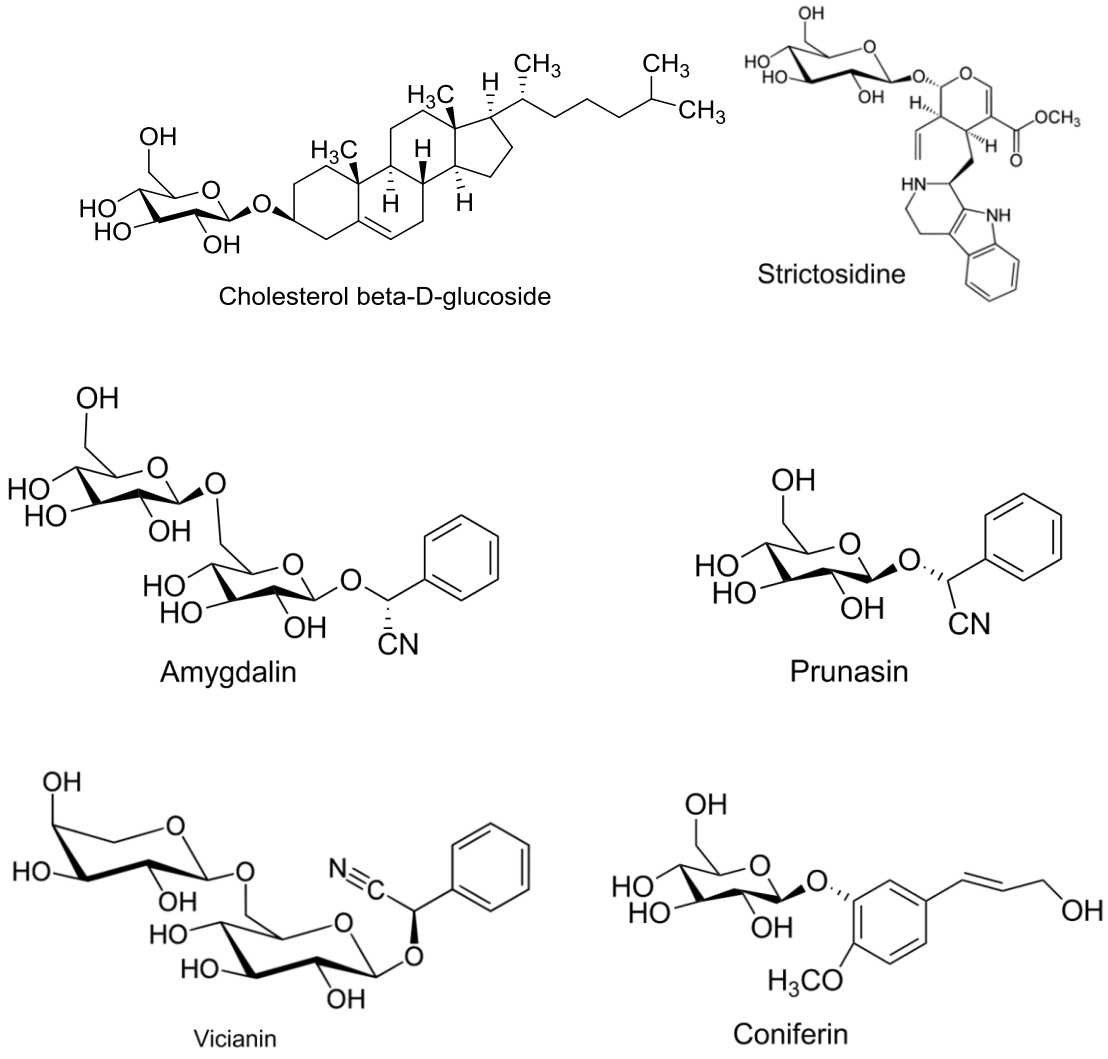


Figure 4. Structures of selected β -glucosidase substrates

One of the best-studied β-glucosidases is glucocerebrosidase (EC.3.2.1.45), commonly known as human acid β-glucosidase. This enzyme's function is to hydrolyze glucosylceramide, an intermediate in glycolipid metabolism, generating glucose and ceramide. Gaucher's disease is caused by a hereditary deficiency of this enzyme. The

disease is the most common of the sphingolipidoses and is the most frequently inherited disorder in Ashkenazi Jews. The carrier frequency is approximately 1 in 15 [3]. In Gaucher's disease, glucosylceramides accumulate in the lysosomes of certain tissues leading to their engorgement. The disorder has classically been divided into three types, of which Type 1 Gaucher's disease is by far the most common. The treatment of type 1 Gaucher's disease include β-glucocerebrosidase enzyme replacement therapy (ERT), substrate reduction therapy (SRT) and chaperon-mediated therapy (CMT) [4]. Due to the application of ERT for treatment of Gaucher's disease, an intimate knowledge of the structure and application of enzyme is necessary. The structure of the enzyme has been determined by x-ray crystallography [5] and produced in recombinant mammalian and insect cells, and gene-activated human cells [6].

In plants, β-glucosidases play an important role in the plant chemical defense against pathogens [7]. Many plants defensive compounds are stored in an inactive glucosylated form in order to stabilize them and increase the solubility of these compounds. When cell disruption happens, the defense compounds are activated by hydrolysis of the glucosidic linkage catalyzed by β-glucosidases. Numerous plants have been found to contain glycosides that can release toxic compounds, such as cyanide and hydroxamic acids [8, 9]. For example, cyanogenic glucosides are amino acid derived phytoanticipins found in many different plant species. The cyanogenic glucosides can be hydrolyzed to yield toxic HCN when tissue disruption happens. HCN can protect plants by blocking cellular respiration of the invader via inactivation of the mitochondrial cytochrome oxidase [7]. In plants, β-glucosidases also serve to activate a number of plant hormone groups. Plant hormones are signal molecules produced in plants. Plant

hormones regulate cellular processes in targeted cells and may determine such activities as the formation of flowers, stems, shedding of leaves and ripening of fruits. Plant hormones that are activated by β-glucosidases include auxins [10], cytokinins [11] and abscisic acid [12].

Another application of β-glucosidases of increasing interest is the bioconversion of plant biomass to produce glucose [13], or breaking through plant cell walls to establish pathogenic or symbiotic relationships [14]. β-Glucosidases are important enzymes in cellulolytic microorganisms, hydrolyzing cellulose into glucose molecules [13]. β-Glucosidases play a key role in the conversion of cellulose based waste materials into glucose for use in fermentation to alcohol or as potential fuels. Consequently, this has been an area of increasing study and interest focusing on the identification and production of β-glucosidases with high glucose tolerance. The applicable β-glucosidases have been isolated from bacteria and fungi. [14, 15]

β-Glucosidase from sweet almond

Since β-glucosidases have a great plurality of functions and applications, a better understanding of how β-glucosidases works is highly desirable. To fully take advantage of the properties of β-glucosidases, a detailed study of the mechanism is necessary. β-Glucosidase from sweet almond is a subject of study with a number of advantages. The enzyme is commercially available and the assay using aryl glucosides is

well known and efficient. The almond β-glucosidases was first isolated and studied in 1837 [16]. β-Glucosidase from sweet almond is a family 1 [17] retaining [18] glycohydrolase, catalyzing the highly efficient hydrolysis of a variety of glycosides [19]. Although one of the earliest enzymes investigated [16], the details of its mechanism of action have only been partially revealed. One of the reasons for this is that β-glucosidase from sweet almond has not been crystallized. Another reason is that β-glucosidase from sweet almond has several isozymes with different molecular weights. By now, four isozymes of almond β-glucosidase have been characterized and identified. Isozyme A has molecular weight of 135kDa, isozyme B has molecular weight of 190-200kDa, isozyme AB has molecular weight of 155kDa and the new isozyme has molecular weight of 58kDa [20]. The enzyme used in this study is isozyme A.

Enzyme kinetics and kinetic isotope effects

Carefully examining enzyme kinetics can lead to a better understanding of the function and mechanism of an enzyme, how the enzyme activity is controlled and how strongly a substrate or inhibitor can bind. pH profiles, the pH dependence of an enzyme-catalyzed reaction, can provide a powerful approach to identifying enzyme active sites residues which play a key role in substrate binding. When properly applied, pH profiles may yield a great deal of information concerning the mechanism of the enzyme-catalyzed reaction. Kinetic isotope effects have proved to be powerful and sensitive probes to investigate the mechanism and transition state structures of enzyme-catalyzed reactions.

Through measurement of the relative rate constants of hydrolysis of substrates containing various isotopic substitutions, primary and secondary isotope effects have been used to interpret the conformational and bonding changes in the transition state of enzyme-catalyzed reactions. Solvent kinetic isotope effects (SKIEs) are determined by comparing the rate constants of a reaction measured in H_2O and measured in D_2O. The ratio of the rate constants (k^{H2O}/k^{D2O}) larger than one is a normal SKIE and the ratio less than one is known as an inverse SKIE. Since many enzyme reactions occur in aqueous solution, the study of SKIE in such reactions is of great importance.

The Mechanism of β-Glucosidase from sweet almond

The catalytic mechanism of β-glucosidase from sweet almond involves a glycosyl-enzyme intermediate. The generally accepted mechanism of β-glucosidase from sweet almond is shown in Figure 5.

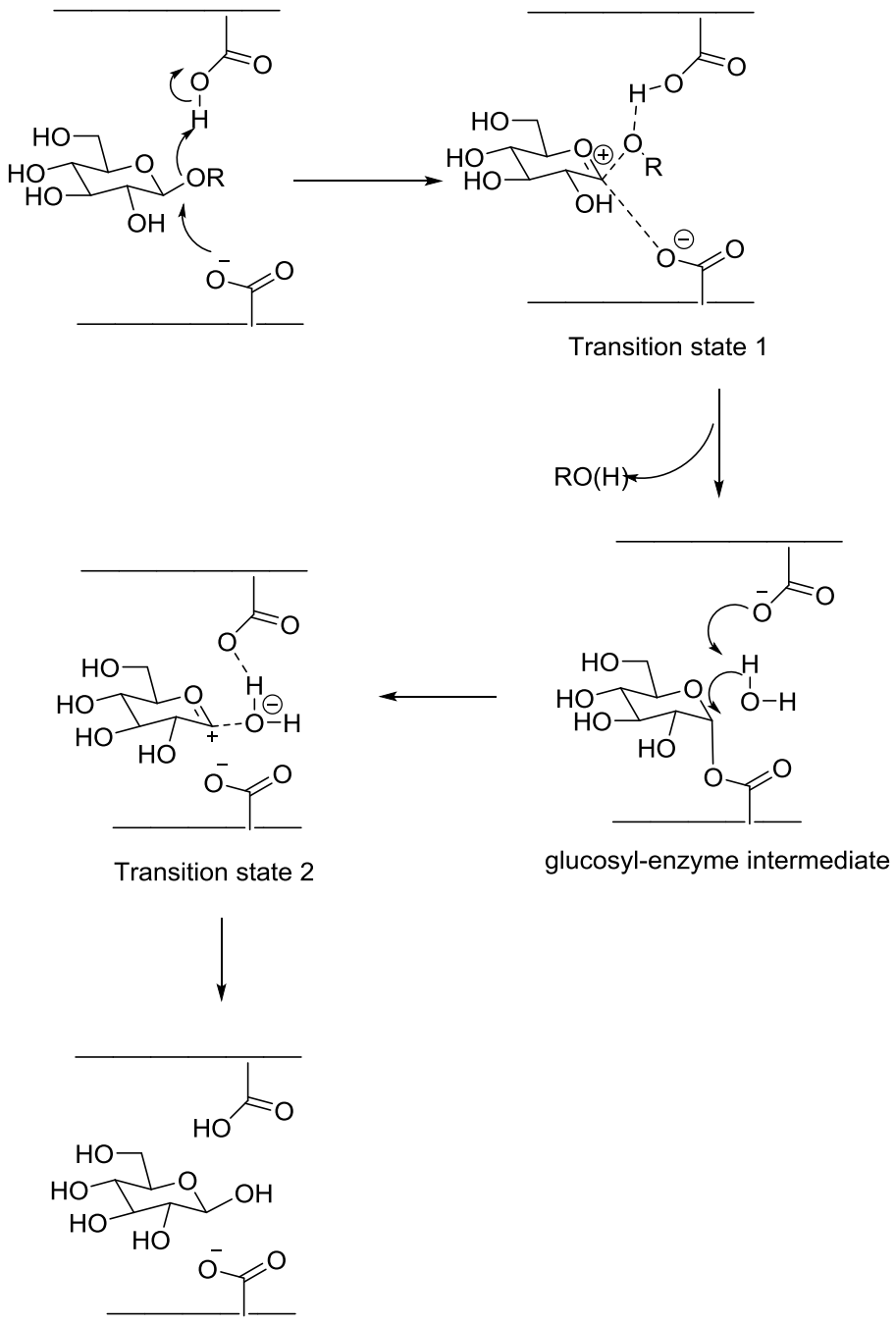
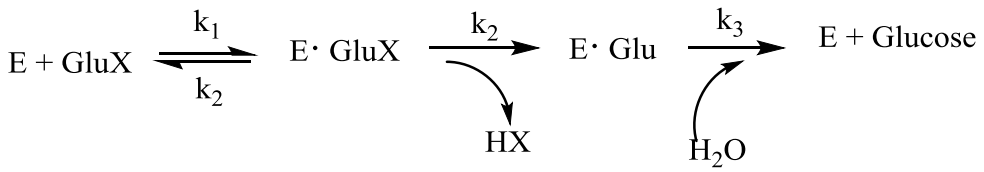


Figure 5. Generally accepted mechanism for hydrolysis by β-glucosidase

The minimal kinetic mechanism for β-glucosidases is:



Then, $k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$ $K_m = \frac{k_3}{k_2 + k_3}\frac{k_{-1} + k_2}{k_1}$ $\frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2}$ eq. 1

The Michaelis constant, K_m, indicates the substrate concentration that produces 1/2 V_{max} for the catalyzed reaction. V_{max} is the maximal velocity of the eznyme. The value of K_m is independent of the amount of enzyme used but does depend on assay conditions. Factors such as pH, the presence of inhibitors or activators, temperature, ionic strength etc. will impact K_m. K_m also provides a measure of the affinity of the enzyme for the substrate when $k_2 \ll k_{-1}, k_3$. The catalytic constant kcat, also called turnover number, indicates how many moles of substrate per unit time will be converted to product by one mole of the enzyme under a particular condition when all substrates are saturating. kcat is equal to V_{max} divied by total enzyme concentration. The apparent second-order rate constant of the reaction, $kcat/K_m$, shows the enyzme's catalytic efficiency.

In general, there are two extreme mechanisms for the reaction: An S_N1 type reaction with formation of an oxocarbonium ion and an S_N2 with formation of a covalent glucosyl-enzyme intermediate (Figure 6). There are several pieces of evidence that show the transition state 1 is a S_N2 type mechanism. For the β-glucosidase-catalyzed hydrolysis of phenyl β-glucopyranoside, Dahlquist et al. [21] found no secondary deuterium kinetic isotope effect (^{2}H KIE; k_H/k_D=1.01). Further, Berti et al. [22] obtained a large (1.032)

primary ^{13}C KIE at C-1 position for the methyl glucoside hydrolysis. These observations strongly suggest an A_ND_N (S_N2) type mechanism in which no change in hybridization occurs at C-1 in the rate-limiting step.

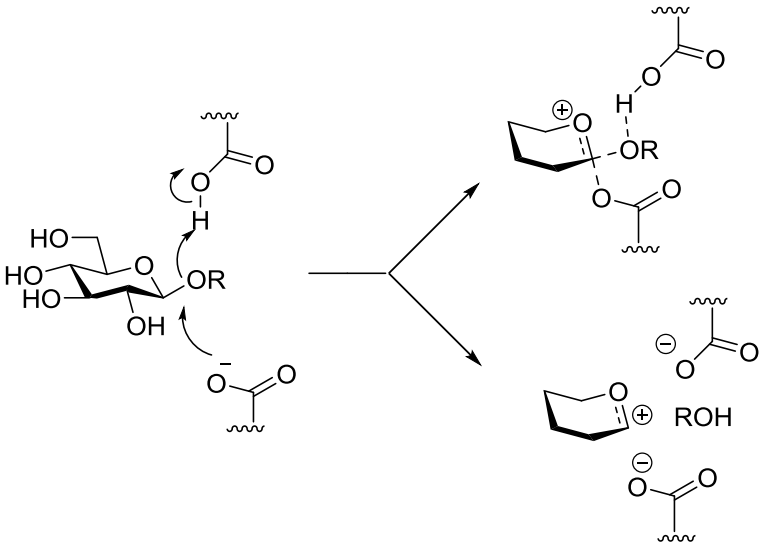


Figure 6. Two possible transition states for the exocyclic cleavage mechanism for the retaining β-glucosidase

Studies by Rosenberg and Kirsch [23] revealed an ^{18}O KIE of 1.038 for the hydrolysis of *p*-nitrophenyl glucoside. This result is indicative of almost complete C-O bond cleavage in the transition state. For the same reaction, Dale et al. have found no solvent KIE on the second order rate constant. Furthermore, the hydrolysis of aryl glucosides with pKa>7.1 gave a large negative Brønsted coefficient (β_{lg} = -0.97) [19], suggesting that the bond breaking is very advanced in the rate-determining transition state with little, if any, proton transfer to the leaving group. These pieces of evidence suggest that the enzyme shows relatively little evidence of acid/base catalysis (Figure 7). To

investigate this further, Golden et al. examined the solvent KIE for the reaction with methyl glucoside and obtained a value of $^{DOD}(kcat/Km)^{lim} = 1.05(\pm 0.08)$ [24]. The lack of any significant solvent KIE can be interpreted as suggesting that the rate limiting step may involve little proton transfer to the leaving group oxygen. However, it is hard to image there is no protonation for the departure with such a poor leaving group (pKa=15.5). A more likely explanation is that protonation of the substrate occurs prior to C-O bond cleavage. If we consider the protonation of the leaving group as an equilibrium, with equilibrium constant Keq, occurring prior to the glucosylation step, with a rate constant of k, then the overall rate constant is given by $k_2 = Keq \times k$. It can then be estimated that $^{D_2O}Keq > 1$ and $^{D_2O}k < 1$. Thus, $^{D2O}k_2$ may coincidently be ≈ 1. (Figure 8)

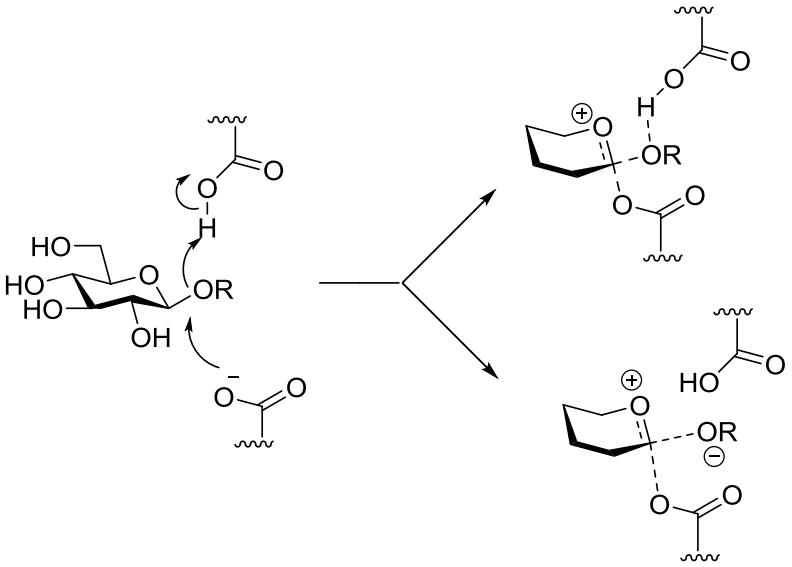


Figure 7. Two possible proton transferring transition states for double displacement mechanism.

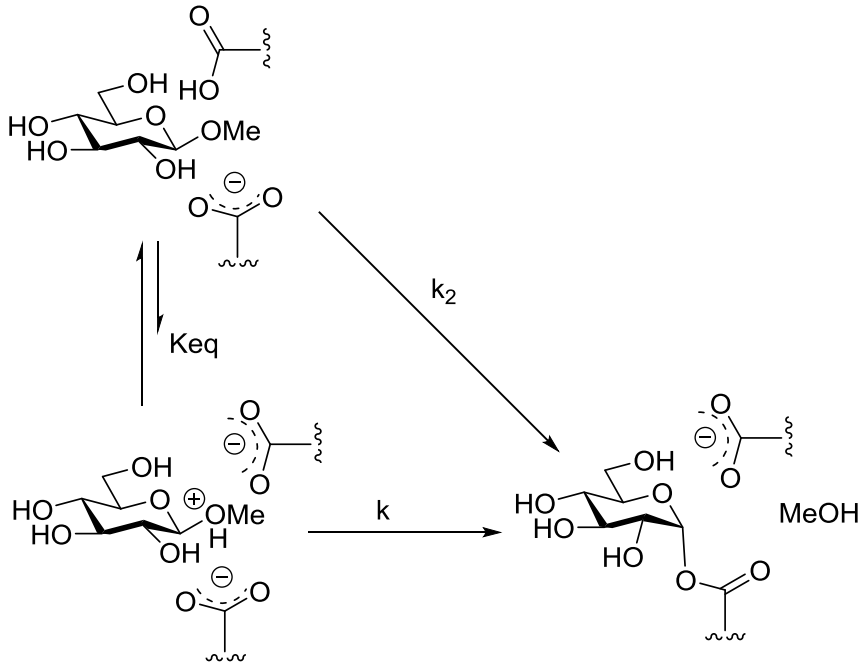


Figure 8. Possible mechanism of β-glucosidase from sweet almond for hydrolysis of methyl glucoside [24].

Research Purpose

The purpose of this work is to further investigate the mechanism of β-glucosidase from sweet almond. Despite extensive research on the enzyme, the details of its mechanism have only been partially revealed.

The lack of a significant SKIE of the β-glucosidase from sweet almond [19, 24] even for the substrates with large pKa, remains an enigma. In this work various alkyl glucosides with varying pKas were used to further study the mechanism of β-glucosidase. It is already known that the Brønsted coefficient for hydrolysis of aryl glucosides with pKa greater than 7.1 is -0.97. However, when the pKa drops beneath this mark, the slope of the structure-reactivity correlation levels off to 0. This means that the rate-limiting step has changed and is now the breakdown of the gluco-enzyme intermediate (Figure 5). The detailed mechanism remains unclear. The pKa of 2, 4-dinitrophenol is 4.3, thus 2, 4-dinitrophenol glucoside serves as an ideal substrate to examine the deglucosylation step. In this work, the secondary KIE of 2, 4-dinitrophenol glucoside will be measured to further understand if the transition state is a concerted A_ND_N mechanism or stepwise (A_N+D_N) mechanism.

MATERIALS AND METHODS

Materials

The almond enzyme was chromatographically purified from Sigma-Aldrich (specific activity = 56 units/mg) and Calzyme Laboratories (specific activity = 12 units/mg). The substrate, para-nitrophenyl-β-D-glucopyranoside (pNPG) was obtained from Sigma-Aldrich. The glucose assay, buffering salts, and the DCl and NaOD were also obtained from Sigma-Aldrich. The D_2O (99.9%) was obtained from Cambridge Isotope Laboratories, Inc., Andover MA. Full experimental details for synthesis of other substrates are given below.

The buffer solutions consisted of 0.01 M buffer (formate, pL 2.9-4.0; acetate, pL 3.5-5.8; MES, pL 5.5-7.0; PIPES, pL 6.1-7.5) and 0.01 M NaCl. The buffers were made up in either deionized water or deuterium oxide. The pH measurements were made with an Accumet pH meter using a micro combination glass electrode. The pD values were estimated by adding 0.41 to the pH meter reading [25] and adjusted with either NaOD or DCl. ^{1}H NMR were recorded on a Varian Inova spectrometer at 400 MHz.

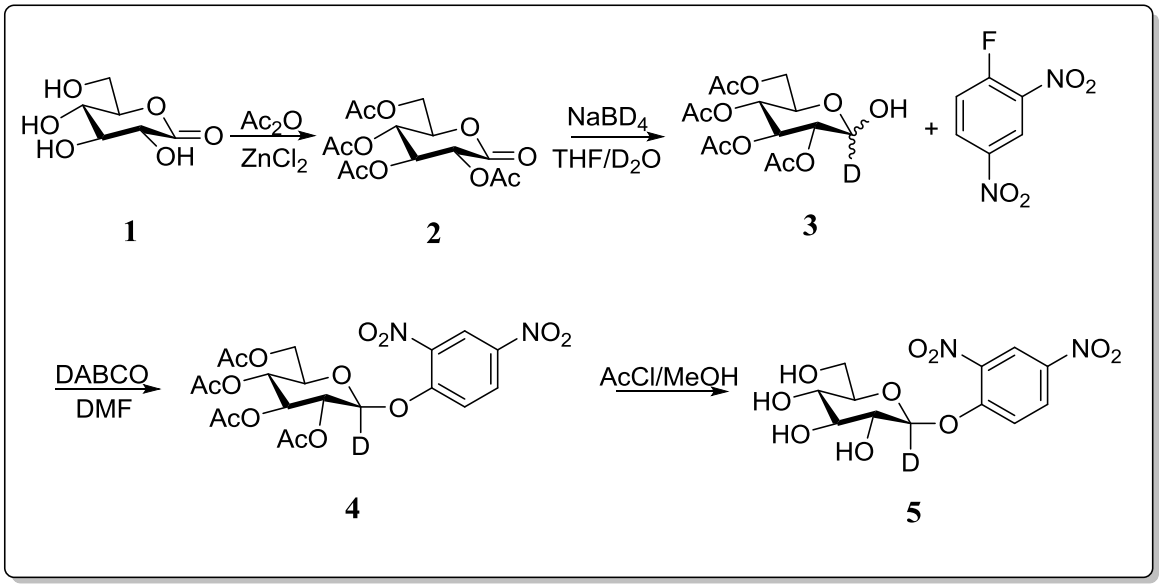
Methods

Synthesis

Pathway 1: Synthesis of 2, 4-dinitrophenyl β-D-glucopyranoside and 2, 4-dinitrophenyl β-D-[1-^{2}H]-glucopyranoside [26]

A solution of D-glucono-1, 5-lactone **1** (5.0g, 28mmol), acetic anhydride (25mL) and zinc chloride (2.5g) was stirred at room temperature for 1 h. The solution was poured onto ice and was extracted with chloroform (2 x 100mL), and the extract was washed with ice cold water, dried with sodium sulfate and evaporated to give a colorless syrup **2**. This unpurified syrup was dissolved in tetrahydrofuran (60mL) and was cooled to 0^{o}C, and a cold solution of $NaBD_4$ (0.44g, 11mmol) in D_2O (2.0mL) was added dropwise with stirring for 2 h, then the solution was neutralized with Dowex 50-W, filtered and evaporated in *vacuo*. The resulting syrup was washed with methanol. The product **3** was obtained as colorless syrup. This syrup, 1-fluoro-2, 4-dinitrobenzene (4.0g) and DABCO (8.0g) was dissolved in DMF (60mL). The solution was stirred at R.T. for 3h. The mixture was concentrated *in vacuo* to leave an oil which was dissolved in dichloromethane and then washed with saturated $NaHCO_3$ and water. After being dried over Na_2SO_4, the solution was concentrated in *vacuo* to give a pale-yellow solid which

was recrystallized by 95% ethanol to give product **4** (6.8g, 47%). (2, 4-dinitrophenyl β-D-glucopyranoside was made by the same method as was 2, 4-dinitrophenyl β-D-[1-^{2}H]-glucopyranoside, substituting $NaBH_4$ for NaBD4 giving 52% overall yield). The deacetylation was carried out by using methanolic HCl which was prepared by mixing acetyl chloride with dry methanol in the ration of 1:10 (v/v). The product **4** (1.0g, 1.9mmol) was dissolved in methanol-HCl (6 mL) and the reaction was run at 4 ^{o}C for 24 hours and then evaporated to dryness. The residue was washed and recrystallized using methanol and ethyl ether. The final product **5** was obtained as yellow solid (0.4g, 60%).



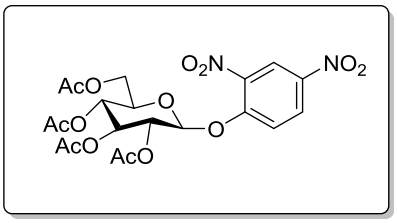
Scheme 1. Synthetic strategy of 2, 4-dinitrophenyl β-D-[1-2H]-glucopyranoside

2, 3, 4, 6-tetra-O-acetyl-2, 4-dinitrophenyl -β-D-[1-^{2}H]-glucopyranoside (4)

^{1}H NMR ($CDCl_3$ 400MHz) δ 8.69 (d, 1H, *J* =2.4Hz), 8.41 (dd, 1H, *J* =9.2Hz and 2.8Hz), 7.45 (d, 1H, *J* =9.6Hz), 5.27-5.32 (m, 2H, H-2, H-3), 5.21(t, 1H, *J* =9.6Hz, H-4), 4.23-4.25 (m, 2H, H-6a, H-6e), 3.93-3.95 (m, 1H, H-5), 2.11 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H).

2, 4-dinitrophenyl *β*-D-[1-^{2}H]-glucopyranoside (5)

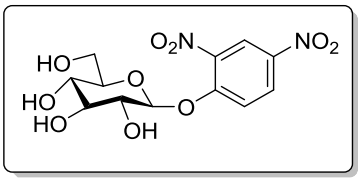
^{1}H NMR (D_2O 400MHz) δ 8.87 (s, 1H), 8.52 (d, 1H, *J* =9.6Hz), 7.59 (d, 1H, *J* =9.2Hz), 3.93 (d, 1H, *J* =10.4Hz), 3.35-3.78 (m, 5H).



6

2, 3, 4, 6-tetra-O-acetyl-2, 4-dinitrophenyl -β-D-glucopyranoside (6)

^{1}H NMR ($CDCl_3$ 400MHz) δ 8.69 (d, 1H, *J* =2.8Hz), 8.41 (dd, 1H, *J* =9.2Hz and 2.8Hz), 7.46 (d, 1H, *J* =9.2Hz), 5.26-5.33 (m, 3H, H-1, H-2, H-3), 5.19 (t, 1H, *J* =9.6Hz, H-4), 4.23-4.25 (m, 2H, H-6a, H-6e), 3.92-3.96 (m, 1H, H-5), 2.11 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H).



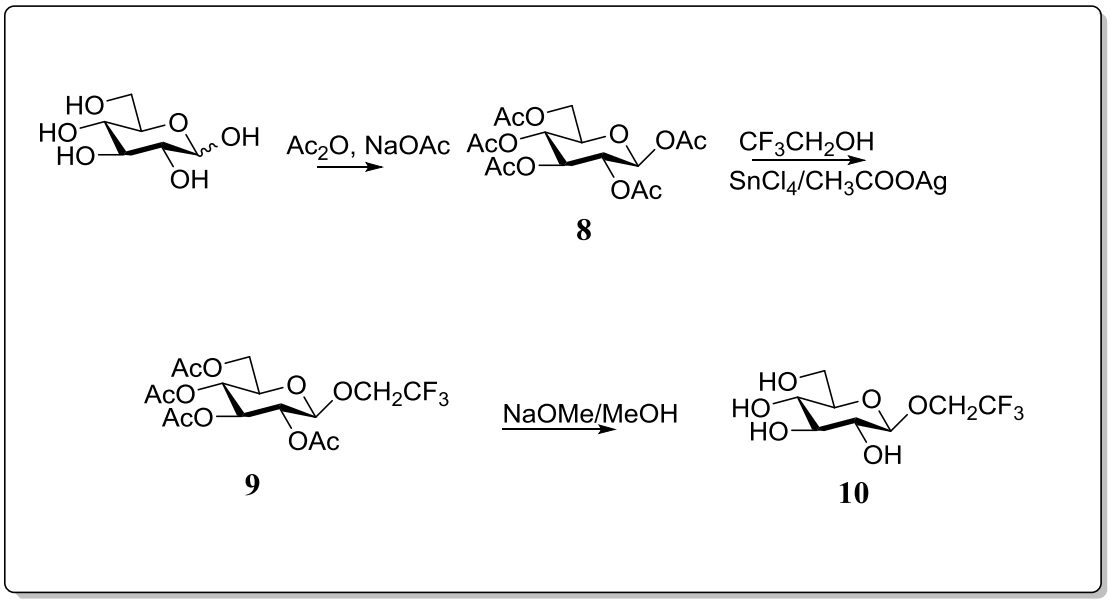
7

2, 4-dinitrophenyl *β*-D-glucopyranoside (7)

^{1}H NMR (D_2O 400MHz) δ 8.85 (s, 1H), 8.50 (d, 1H, *J* =9.6Hz), 7.58 (d, 1H, *J* =9.2Hz), 5.39 (d, 1H, *J* =7.2Hz, H-1), 3.93 (d, 1H, *J* =10.4Hz), 3.33-3.74 (m, 5H).

Pathway 2: Synthesis of trifluoroethyl β-glucopyranoside [27]

Using D-glucose (10.0g, 0.055mol) and anhydrous sodium acetate (5.0g, 0.06mol), followed by addition of acetic anhydride (67mL, 0.71mol). The reaction mixture was heated to 135^{o}C under reflux for 2h. The reaction mixture was poured onto ice and the white solid was filtrated and recrystallized from 95% ethanol to get peracetylated glucopyranose **8** (65% yield). A mixture of peracetylated glucopyranose (5g, 0.13mol) and CF_3CH_2OH (9mL, 2.5eq) was dissolved in dry dichloromethane (15mL), then $SnCl_4$ (2.5mL, 1.5eq) and CH_3COOAg (3.2g, 1.5eq) was added. The reaction mixture was stirred at room temperature for 3 hours, filtered and quenched with saturated $NaHCO_3$. The organic layer was washed and dried. Solvent was removed and the resulting product was purified by column chromatography (EtOAc:Hexane=2:3) to get the product **9** as a white solid (60% yield). To remove the protecting group, **9** (1.0g, 2.3mmol) and MeOH (8mL) were stirred, then catalytic amount of NaOMe was added at R.T. and stirred overnight. The reaction mixture was neutralized with Dowex 50W-X8. After filtration, the filtrate was concentrated in *vacuo* by toluene (3 times). The residue was purified by column chromatography (chloroform: methanol=9:1) yielding a white solid **10** (yield 71%)



Scheme 2. Synthetic strategy of trifluoroethyl β-glucopyranoside

2, 2, 2-trifluoroethyl -2, 3, 4, 6-tetraacetate-β-D-glucopyranoside (9)

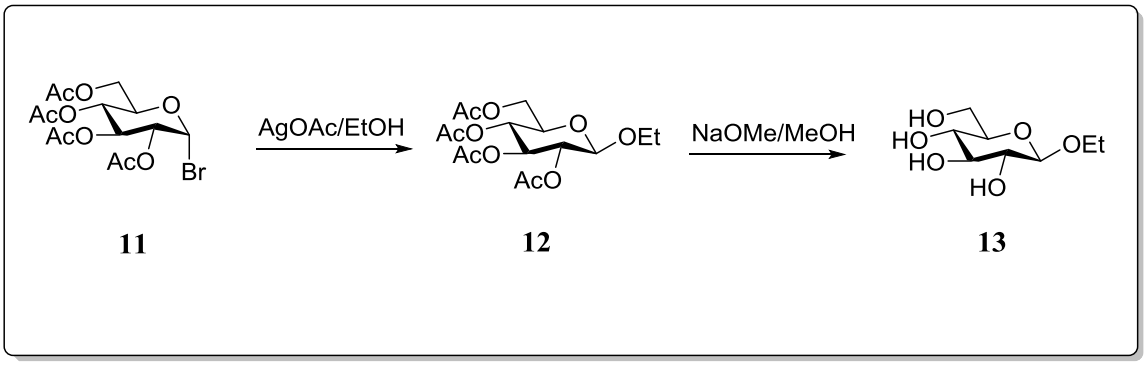
^{1}H NMR ($CDCl_3$ 400MHz) δ 1.995 (s, 3H), 2.012 (s, 3H), 2.034 (s, 3H), 2.080 (s, 3H), 3.709 (ddd, *J* = 2.4Hz, 4.4Hz and 10.0Hz, 1H, H-5), 3.90-3.97 (m, 1H), 4.080-4.149 (m, 2H, H-6e, CH_2CF_3), 4.24 (dd, *J* = 4.4 Hz and 12.8 Hz, 1H, H-6a), 4.627 (d, *J* =7.6 Hz, 1H, H-1), 5.02 (t, *J* = 8.8Hz, 1H, H-2), 5.084 (t, *J* = 9.6Hz, 1H, H-4), 5.206 (t, *J* =9.6Hz, 1H, H-3)

2, 2, 2-trifluoroethyl *β*-D-glucopyranoside (10)

^{1}H NMR (D_2O 400MHz) δ 4.544 (d, *J* = 8 Hz, 1H, H-1), 4.16-4.30 (m, 2H), 3.88 (d, *J* = 12.8Hzm 1H, H-6a), 3.68 (dd, *J* = 6 Hz and 12Hzm 1H, H-4), 3.42-3.48 (m, 2H, H-5&H-6e), 3.35 (t, *J* = 9.2Hz, 1H, H-3), 3.28 (t, *J* = 8.4Hz, 1H, H-2). (NMR spectrum at the end)

Pathway 3: Synthesis of ethyl β-glucopyranoside

A mixture of AgOAc (50mg, 0.3mmol), 4Å molecular sieve (0.5g), EtOH (10 mL) and CH_2Cl_2 (5mL) was stirred at R.T. for 30 minutes, followed by addition of tetra-acetyl-α-bromoglucopyranside **11** (1g, 2.4mmol). The reaction mixture was stirred overnight. After the reaction was completed, the mixture was washed with saturated $NaHCO_3$ and brine, then dried over Na_2SO_4. Solvent was removed in *vacuo* and the resulting product was purified by column chromatography (ethyl acetate: hexane = 2:3) yielding a white solid **12** (yield 0.5g, 58%). Catalytic NaOMe was added to the solution of **2** (0.5g, 1.3mmol) in MeOH (4mL) at R.T. and the reaction mixture was stirred overnight. The reaction mixture was neutralized with Dowex 50W-X8 (0.3g). After filtration, the filtrate was concentrated in *vacuo* by toluene three times. The residue was purified by column chromatography (CH_2Cl_2: methanol = 5:1) yielding compound **13** as a white solid (0.16g 60%).



Scheme 3. Synthetic strategy of ethyl β-glucopyranoside

Ethyl -2, 3, 4, 6-tetraacetate-β-D-glucopyranoside (12)

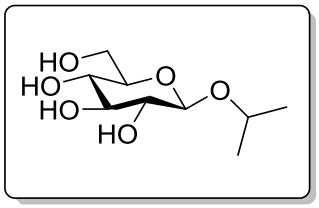
^{1}H NMR ($CDCl_3$ 400MHz) δ 5.18 (t, *J* = 9.2Hz, 1H, H-3), 5.07 (t, *J* = 9.6 Hz, 1H, H-4), 4.96 (dd, *J* = 8 Hz and 9.6 Hz, 1H, H-2), 4.49 (d, *J* = 7.6 Hz, 1H, H-1), 4.24 (dd, *J* = 4.8 Hz and 12.4 Hz, 1H, H-6a), 4.11 (dd, *J* = 2.4 Hz and 12Hz, 1H, H-6e), 3.85-3.93 (m, 1H), 3.67 (ddd, *J* = 2.4Hz, 4.4 Hz and 9.6 Hz, 1H, H-5), 3.52-3.60 (m, 1H), 2.07 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.18 (t, *J* = 7.2 Hz, 3H).

Ethyl- *β*-D-glucopyranoside (13)

^{1}H NMR (D_2O 400MHz) δ 4.43 (d, *J* = 8 Hz, 1H, H-1), 3.91-3.95 (m, 1H), 3.87 (dd, *J* = 2.4 Hz and 12.4 Hz, 1H, H-6a), 3.64-3.70 (m, 2H, CH_2, H-4), 3.40-3.46 (m, 2H, H-5, H-6e), 3.33 (t, *J* = 9.6Hz, 1H, H-3), 3.20 (t, *J* = 9.6Hz, 1H, H-2).

Pathway 4: Synthesis of Isopropyl -β-D-glucopyranoside [28]

Glucose (0.54 g, 3 mmol) was treated with β-glucosidase from sweet almond (0.3g, EC 3.2.1.21 from Sigma, 2.6 units/mg) in propan-2-ol/water (10 mL, 9:1, v/v), the mixture was stirred for 48–72 h at 50 ◦C, then filtered and concentrated under vacuum. The resulting syrup was subjected to flash column chromatography over silica gel (CH_2Cl_2:MeOH = 5:1). The isopropyl-β-D-glucopyranoside was collected as a white solid **14** (0.14g, 28% yield).



14

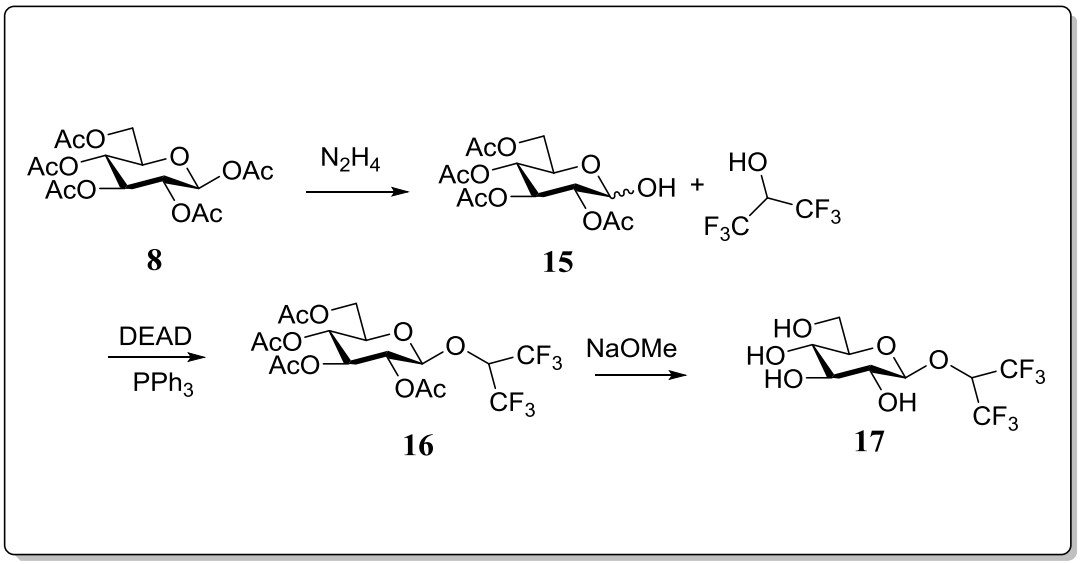
Isopropyl -β-D-glucopyranoside (14)

^{1}H NMR (D_2O 400MHz) δ 1.20 (dd, *J* = 6.4Hz and 14.4 Hz, 6H), 3.18 (t, *J* = 8 Hz, 1H, H-2), 3.32 (t, *J* = 5.6 Hz, 1H, H-3), 3.40-3.46 (m , 2H, H-5, H-6b), 3.67 (dd, *J* = 6 Hz and 12.8Hz, 1H, H-4), 3.88 (dd, *J* = 2 Hz and 12.4 Hz, 1H, H-6a), 4.05-4.11 (m, 1H), 4.51 (d, *J* = 8Hz, 1H, H-1).

Pathway 5: Synthesis of 1, 1, 1, 3, 3, 3-hexafluoro-isopropyl -β-D-glucopyranoside [29]

Hydrazine acetate (0.30 g, 3.2 mmol) was added to a stirred solution of glucose pentaacetate **8** (1.0 g, 2.6 mmol) in DMF (5 mL). After heating to 50^o C for 2 hrs, the reaction mixture was diluted with EtOAc (20mL). The organic solution was washed with saturated aqueous $NaHCO_3$ solution (2 × 20mL), brine (15mL), and dried over Na_2SO_4. Solvent was removed in *vacuo* affording a yellow oil which was purified by flash chromatography over silica gel (EtOAc:hexanes; 1:3) to give an inseparable mixture

of α- and β-**15** (0.6 g, 70%, α/β = 5/1) as a white solid. Triphenylphosphine (0.5g, 2mmol), 1, 1, 1, 3, 3, 3- hexafluoroisopropanol (0.5mL, 5 mmol) and diethyl azodicarboxylate (0.9mL, 40 wt. % in toluene, 2mmol) were added to a stirred solution of the hemiacetalic sugar (0.3g, 1mmol) in dry toluene (10 mL). The mixture was stirred under nitrogen at room temperature for 4 hours. After evaporation under reduced pressure, the residue was chromatographed (hexane: ethyl acetate =3:2)) on silica gel to yield the product as white solid **16** (0.27g, 55%). Protecting groups were removed by stirring **16** (1 g, 2mmol) and a catalytic amount of NaOMe in MeOH (10mL) at R.T. overnight. The reaction mixture was neutralized with Dowex 50W-X8. After filtration, the filtrate was concentrated in *vacuo* using toluene (3 times). The residue was purified by column chromatography (chloroform:methanol=9:1 to 5:1) yielding a white solid **17** (0.4g, yield 68%)



Scheme 4. Synthetic strategy of 1, 1, 1, 3, 3, 3-hexafluoro isopropyl -β-D-glucopyranoside

2, 3, 4, 6-Tetra-*O*-acetate-α/β-D-glucopyranose (15)

(α anomer) ^{1}H NMR ($CDCl_3$ 400MHz) δ 1.88 (s, 3H), 1.90 (s.3H), 2.08(s, 3H), 2.11 (s, 3H), 4.10 (dd, *J* = 3.2 Hz and 7.2 Hz, 1H, H-6e), 4.20-4.27 (m, 2H, H-5, H-6a), 4.89 (dd, *J* = 3.6 Hz and 10.4 Hz, 1H, H-2), 5.07 (t, *J* = 12.0 Hz, 1H, H-4), 5.45 (t, *J* = 3.2 Hz, 1H, H-1), 6.52 (t, *J* = 9.6 Hz, 1H, H-3).

1, 1, 1, 3, 3, 3-hexafluoro isopropyl -2, 3, 4, 6-tetraacetate-β-D-glucopyranoside (16)

^{1}H NMR ($CDCl_3$ 400MHz) δ 1.98 (s, 3H), 2.01 (s, 3H), 2.03 (s, 3H), 2.10 (s, 3H), 3.70-3.76 (m, 1H, H-5), 4.16-4.25 (m, 2H, H-6a, H-6b), 4.40-4.45 (m, 1H), 4.75 (d, *J* = 7.2 Hz, 1H, H-1), 5.08 (t, *J* = 10Hz, 1H, H-2), 5.11 (t, *J* = 9.2 Hz, 1H, H-4), 5.21 (t, *J* = 9.2Hz, 1H, H-3).

1, 1, 1, 3, 3, 3-hexafluoro isopropyl -β-D-glucopyranoside (17)

^{1}H NMR (D_2O 400MHz) δ 3.32-3.48 (m, 4H, H-2, H-3, H-5, H-6e), 3.70 (dd, *J* = 5.2Hz and 12.4Hz, 1H, H-4), 3.88 (dd, *J* = 2Hz and 12.4Hz, 1H, H-6a), 4.71 (d, 8Hz, 1H, H-1), 5.28-5.34 (m, 1H). (NMR spectrum at the end)

Enzyme Kinetics

Determination of the K_m of substrates

The enzyme (0.45mg/mL=12U/mg ≈0.32μM subunits) was prepared in 0.01M MES buffer at pH 6.35. With pNPG as substrate, product concentration was monitored by measuring the absorbance (λ = 400nm for p-nitrophenoxide at pL≥6.3, or at λ=348nm at pL <6.3) on a Hewlett-Packard model 8452A diode array spectrophotometer equipped with circulating water bath. Enzyme was added (the final concentration is about 0.23 unit/mL) into the buffered solution of pNPG (≈0.125mM). Under these conditions ([S] « K_m ≈ 2.5mM) the reactions follow pseudo first order kinetics. The first-order rate constant is equal to V_{max}/K_m. When inhibitors (different alkyl β-glucopyranosides) are in the solution, the rate constant is V_{max}/K_m (1+ [I]/K_i). K_i is the dissociation constant of inhibitor from the EI complex. The apparent K_i is equal to the K_m of alkyl β-glucopyranosides.

To determine the K_m of 2, 4-dinitrophenol glucoside, the initial velocity was measured at different substrate concentrations. According to the Michaelis-Menten equation $v_0 = \frac{V_{max}[S]_0}{K_m + [S]_0}$, the K_m for 2, 4-dinitrophenol glucoside can be determined by measuring v_0 at various substrate concentrations. With final enzyme concentration

approximately 0.2 unit/mL, the initial velocity was measured by recording the absorption at 480nm. The concentration of 2, 4-dinitrophenol is calculated according to ε_{480} (DNP) = 0.76 $mM^{-1}cm^{-1}$ and was varied between 0.5mM and 7.5mM.

Determining the initial velocity and k_{cat}/K_m of alkyl glucosides

Enzyme was added to a buffered solution of 10mM trifluoroethyl β-glucopyranoside, giving a final concentration of about 42μg/mL≈0.5 unit/mL or 142μg/mL≈1.7 unit/mL. The reaction was monitored through removal of aliquots to determine the glucose concentration using the coupled enzyme assay of hexokinase and glucose-6-phosphate dehydrogenase, containing 10mM ATP, 5mM MgSO4, 1mM NAD+, pH = 7.5, and measuring the increase in absorbance at 340 nm due to the formation of NADH. Under these conditions (the concentration of trifluoroethyl β-glucopyranoside [S] «K_m ≈ 121mM at pH =5.0), the kinetics are presudo first-order: $k_{obs} = V_{max}/K_m$. For the first 20 min of the reaction, the production of glucose is linear with time and the velocity under these conditions is equal to the initial velocity: $v_0 = k_{obs}[S]_0$. Thus, dividing this initial velocity by the substrate concentration yields the pseudo first-order rate constant. Dividing the rate constant by the enzyme concentration yields the second-order rate constant k_{cat}/K_m.

pL dependence of trifluoroethyl β-glucopyranoside

The k_{cat}/K_m values were measured at various pH/pD values (25°C). The buffer solutions consisted of 0.01M buffer (formate, pL 2.9-4.0; acetate, pL 3.5-5.8; MES, pL 5.5-7.0; PIPES, pL 6.1-7.5), prepared in deionized water (H_2O) containing 0.01M NaCl. pH value were measured using an Accumet pH meter. pD values were estimated by adding 0.41 to the pH meter reading[11]. The data were fit to the equation:

$$k_{obs} = k^{lim}/[1+10^{(pK1-pH)}+10^{(pH-pK2)}]$$

Determine the reverse rate constant (V/K)r

The reverse reaction for hydrolysis of alkyl glucoside follows the mechanism:

$$E+Glc \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{"E-Glc"}+ROH \xrightarrow{k_2} GlcOR+E$$

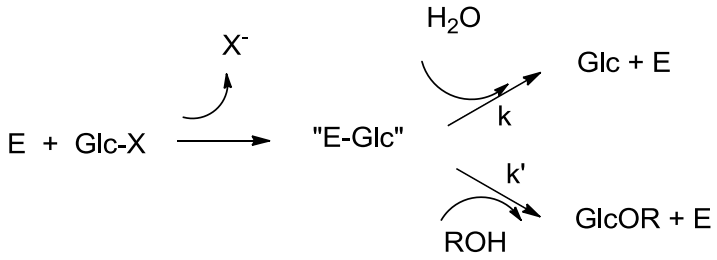
So $dP/dt= k_{obs}[E][Glc][ROH]$

Because the breakdown of "E-Glc" is the rate-limiting step for the reaction, so

$dP/dt= k_{obs}[E\text{-}Glc][ROH]$

k_{obs} is equal to (V/K)r.

The concentration of the enzyme glucose complex "E-Glc" is difficult to measure, so an indirect method was employed. This indirect method can measure the k_{obs} without measuring the concentration of "E-Glc". Using pNPG as a glucose donor, the reaction follows:



If the transfer of the glucosyl residue from the intermediate to the acceptor is first-order in acceptor concentration, then the product ratio is given by the equation:

$[GlcOR]/[Glc] = k'[ROH]/k[H_2O]$

The concentration of $[X^-]$ ([pNP]) is equal to concentration of glucose [Glc] plus [GlcOR]. Thus,

$$\frac{[pNP]-[Glc]}{[Glc]} = \frac{k'[ROH]}{k[H_2O]}$$ so

$$\frac{[pNP]}{[Glc]} = 1 + \frac{k'[ROH]}{k[H_2O]}$$

Thus, the ratio of initial velocity of formation of [pNP] and [Glc] is

$$\frac{V_{[pNP]}}{V_{[Glc]}} = 1 + \frac{k'[ROH]}{k[H_2O]}$$

When [ROH] is zero, $V_{[pNP]}/V_{[Glc]} = 1.0$. Upon addition of alcohols, this value will increase. Using different concentrations of different alcohols and water, the values of k'/k can be determined. In this reaction, k is the first-order rate constant for hydrolysis of pNPG; and k' is k_{obs}. With this method, the value of k' in the reverse reactions can be compared with different alcohols. The reactions were conducted with 1.0 mM pNPG at pH= 5.5 with enzyme concentration of 1.1 mg/mL by adding different concentration of alcohols. The concentration of glucose corrected for absorption of pNPOH at 340nm was determined by a glucose assay (HK+G6PDH) and the concentration of pNP anion was measured via absorption at 400nm. The UV spectra of pNP anion and NADH are well resolved, but there is overlap between the spectra of pNPG, pNPOH and NADH. The glucose assay has pH = 7.56. Thus, according to control experiments, the measurements were conducted at pH 7.56 where ε_{340} (pNPG) = $3.1mM^{-1}cm^{-1,}$ ε_{400} (pNP) = $14.2mM^{-1}cm^{-1}$ and ε_{340} (pNPOH) = $4.3mM^{-1}cm^{-1}$.

Secondary kinetic isotope effect

Isotope effects were determined by measuring the ratio of the initial rates for ^{1}H and ^{2}H substrates of 2, 4-dinitrophenyl-glucoside. The initial rate used for the isotope effect calculation was an average of at least ten rates measured carefully at high substrate concentration ($\approx 5 \times K_m$). 10μL enzyme with the concentration of 1.0 mg/mL (12U/mg) was added in 0.6 mL solution. The solutions of 2, 4-dinitrophenyl-glucoside and 2, 4-dinitrophenyl-[1-^{2}H]-glucoside were treated with the same amount of Amberlyst A-26 ion-exchange resin to remove extra 2, 4-dinitrophenoxide anion. The initial velocity was measured at 400nm with ε_{400} (DNP) = 11.6 $mM^{-1}cm^{-1}$ at pH = 6.3.

RESULTS

K_m study of various substrates

Michaelis constant K_m is an important measurement for enzyme kinetics. The values of Km of various substrates are shown in Figure 9.

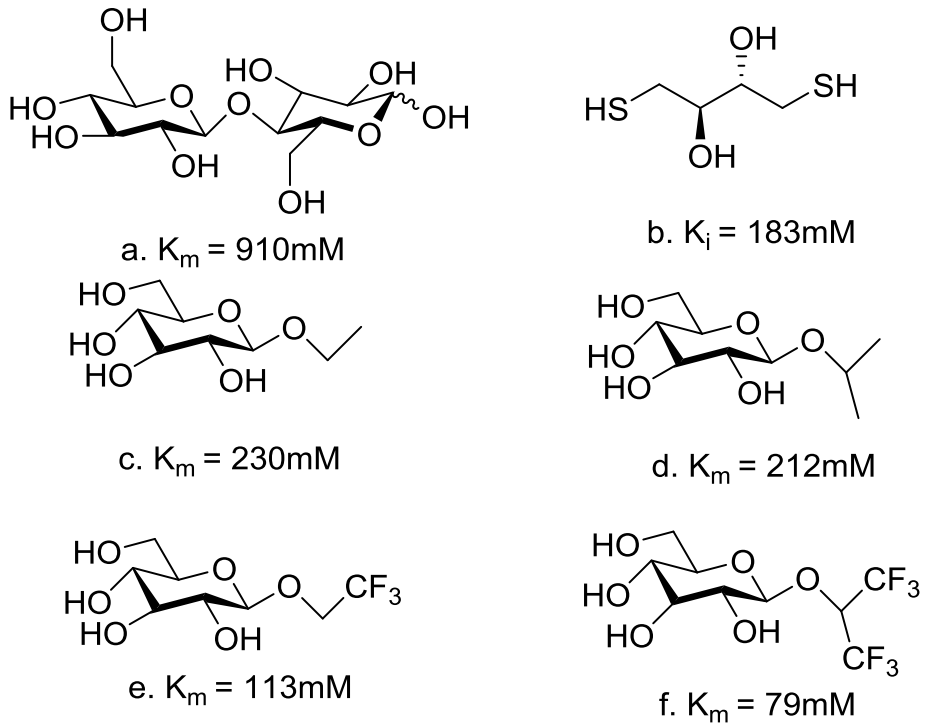


Figure 9. The values of K_m of various substrates and Ki of an inhibitor at pH = 5.5. a. Cellobiose; b. Dithioerythritol; c. Ethyl- β-D-glucopyranoside; d. Isopropyl -β-D-

glucopyranoside; e. Trifluoroethyl β-D-glucopyranoside; f. Hexafluoro isopropyl -β-D-glucopyranoside.

The Michaelis-Menten equation is shown as equation 2, where v_0 is the initial rate of the reaction. This occurs when no appreciable change in the original substrate concentration. V_{max} represents $k_{cat} \times [E]$, where k_{cat} is $(k_2k_3)/ (k_2+k_3)$ (eq. 1). $[S]_0$ represents the initial substrate concentration. K_m is the concentration of substrate required to observe half V_{max}. The initial velocity as a function of the 2, 4-dinitrophenol glucoside concentration is shown in Figure10.

$$v_0 = \frac{V_{\max}[S]_0}{K_m + [S]_0} \qquad \text{eq. 2}$$

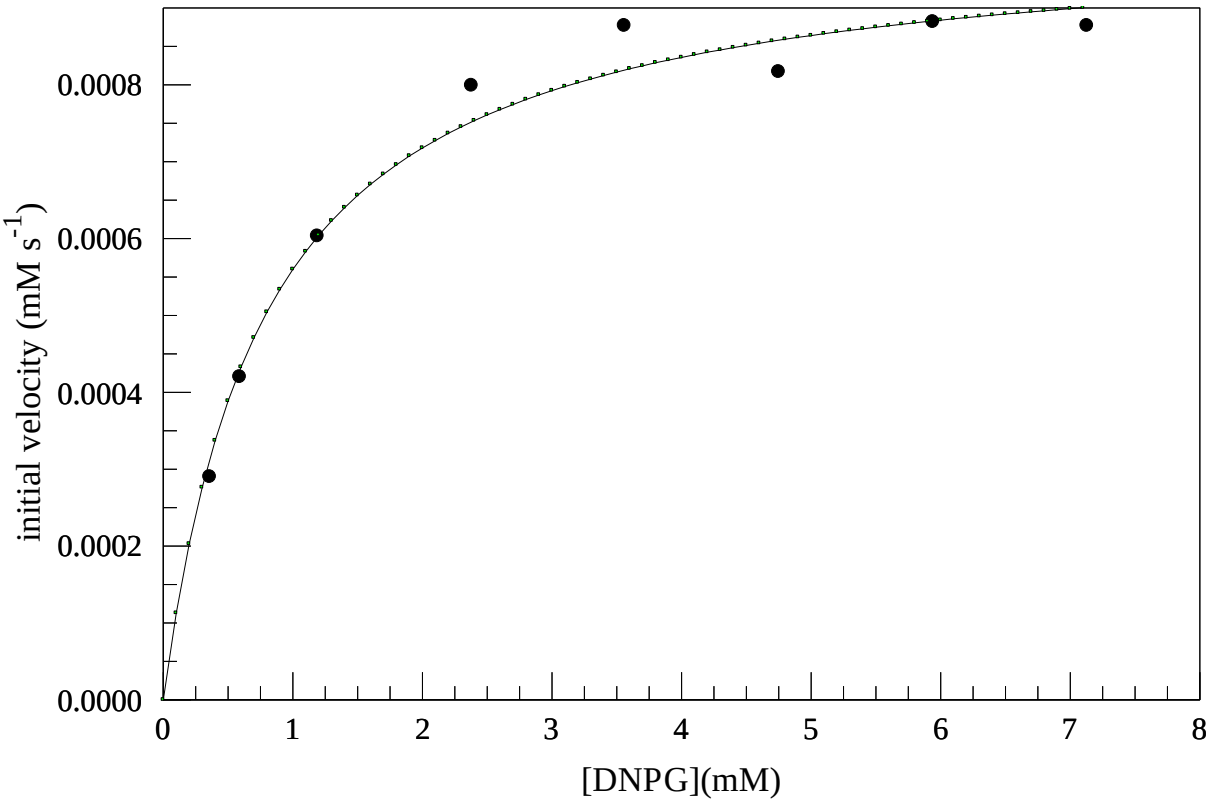


Figure 10. Michaelis-Menten Curve of hydrolysis for 2, 4-DNPG at pH 6.3 with 0.012μM subunits enzyme present. The data are fit to equation 2. V_{max} is 1.0×10^{-3} ($\pm$ 3.3×10^{-5}) mM s^{-1} and K_m is 0.8 ($\pm$ 0.1) mM with a R^2 value of 0.989.

pL dependence of trifluoroethyl β-glucopyranoside

A pL profile of the rate of enzyme catalyzed hydrolysis of trifluoroethyl β-glucopyranoside was garnered at 25 ℃. The results are shown in Figure 11. The corrected second order rate of reaction ($k_{ca}t/K_m$) is plotted against the pL and fit to equation 3.

$$k_{obs} = k^{lim}/[1+10^{(pK1-pH)}+10^{(pH-pK2)}] \qquad \text{eq. 3}$$

k_{lim} represents the rate constant (k_{cat}/K_m) for the reaction of the monoprotonated enzyme with substrate. pK_{a1} and pK_{a2} represent the observed molecular dissociation constants.

In H_2O, $(k_{cat}/K_m)^{lim}$ = 19.03 (±0.41) x 10^2 $M^{-1}s^{-1}$,pK_1 = 2.14 (±0.18), pK_2 =6.27 (±0.06); In D_2O, $(k_{cat}/K_m)^{lim}$ = 15.42 (±0.29) x 10^2 $M^{-1}s^{-1}$,pK_1 = 2.88 (±0.06), pK_2 =7.11 (±0.08). The solvent isotope effect is $^{DOD}(k_{cat}/K_m)^{lim}$ = 1.23 (±0.03).

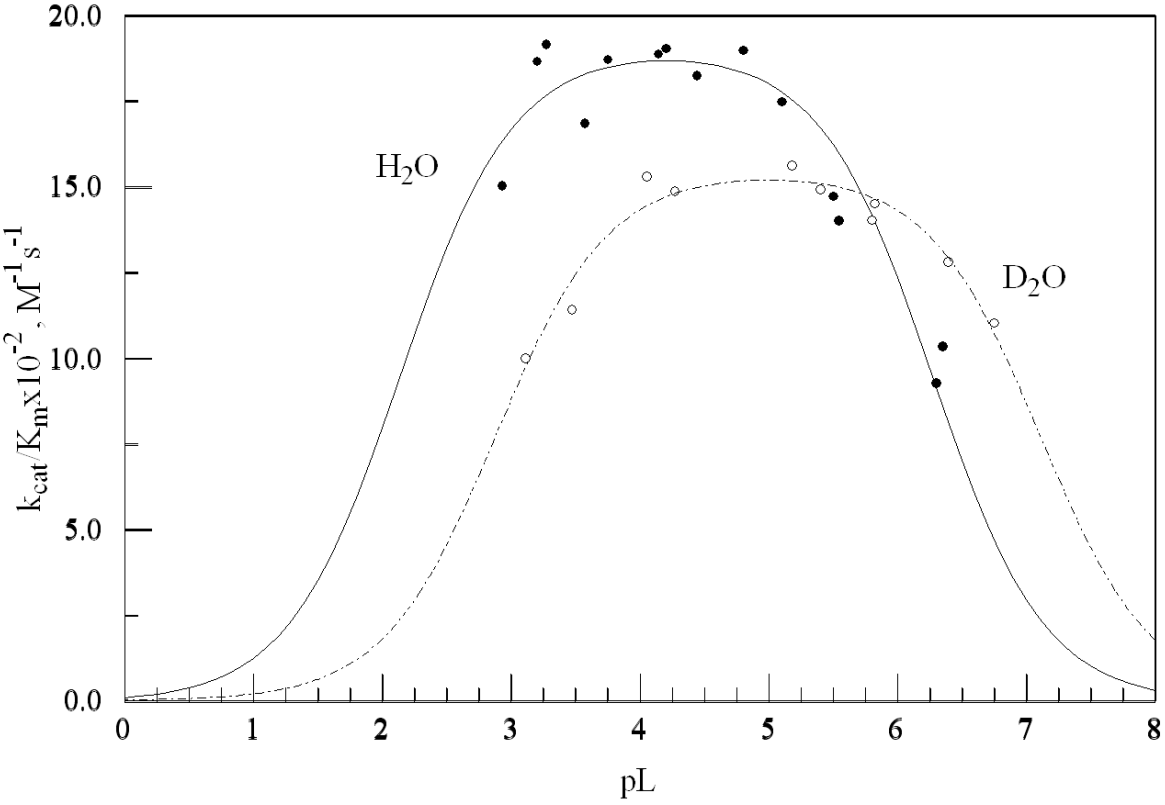


Figure 11. Dependence on pL [= pH (●) or pD (○) as applicable] of the second order rate constant (k_{cat}/K_m) for the hydrolysis of trifluoroethyl β-glucopyranoside by β-glucosidase at 25 °C. Total enzyme present was 0.03 μM subunits and trifluoroethyl β-glucopyranoside concentration was 10 mM.

Alkyl glucosides hydrolysis

The K_m values obtained from the inhibition of enzymatic pNPG hydrolysis of the four alkyl glucosides were shown in Figure 9. Listed in Table 1 are the kinetic parameters for the hydrolysis of the alkyl glucosides with β-glucosidase from sweet almond.

GluOR	pKa of ROH	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
R = $CH(CH_3)_2$	16.5	212	130
R = CH_2CH_3	16.0	230	48
R = CH_2CF_3	12.5	113	1313
R = $CH(CF_3)_2$	9.3	79	8090

Table 1. Michaelis-Menten Kinetic Parameters for the hydrolysis of four different alkyl glucosides by β-glucosidase from sweet almond at 25 ^oC, pH = 5.5, 0.01 M MES/NaCl buffer.

Determining the reverse rate constant (V/K)r

The reverse reaction for hydrolysis of alkyl glucoside follows the equation 3:

$$\frac{V_{[pNP]}}{V_{[Glc]}} = 1 + \frac{k'[ROH]}{k[H_2O]} \qquad \text{eq.3}$$

$V_{[pNP]}/V_{[Glc]}$ is linear dependence to $[ROH]/[H_2O]$, the slope is k'/k. After changing the concentration of alcohol and water, the values of k'/k can be determined. k is the first-order rate constant for hydrolysis of pNPG, which is $4.33 \times 10^{-3}\ s^{-1}$. The results are shown in Figure 12 and Table 2.

GluOR	pKa of ROH	k'/k	k' (s^{-1})
R = $CH(CH_3)_2$	16.5	3.95	1.71×10^{-2}
R = CH_2CH_3	16.0	9.66	4.18×10^{-2}
R = CH_2CF_3	12.5	5.95	2.58×10^{-2}
R = $CH(CF_3)_2$	9.3	7.80	3.38×10^{-2}

Table 2. The values of k'/k and k' of four different alkyl glucosides by β-glucosidase from sweet almond at 25 ^{o}C, pH = 5.5, 0.01 M MES/NaCl buffer.

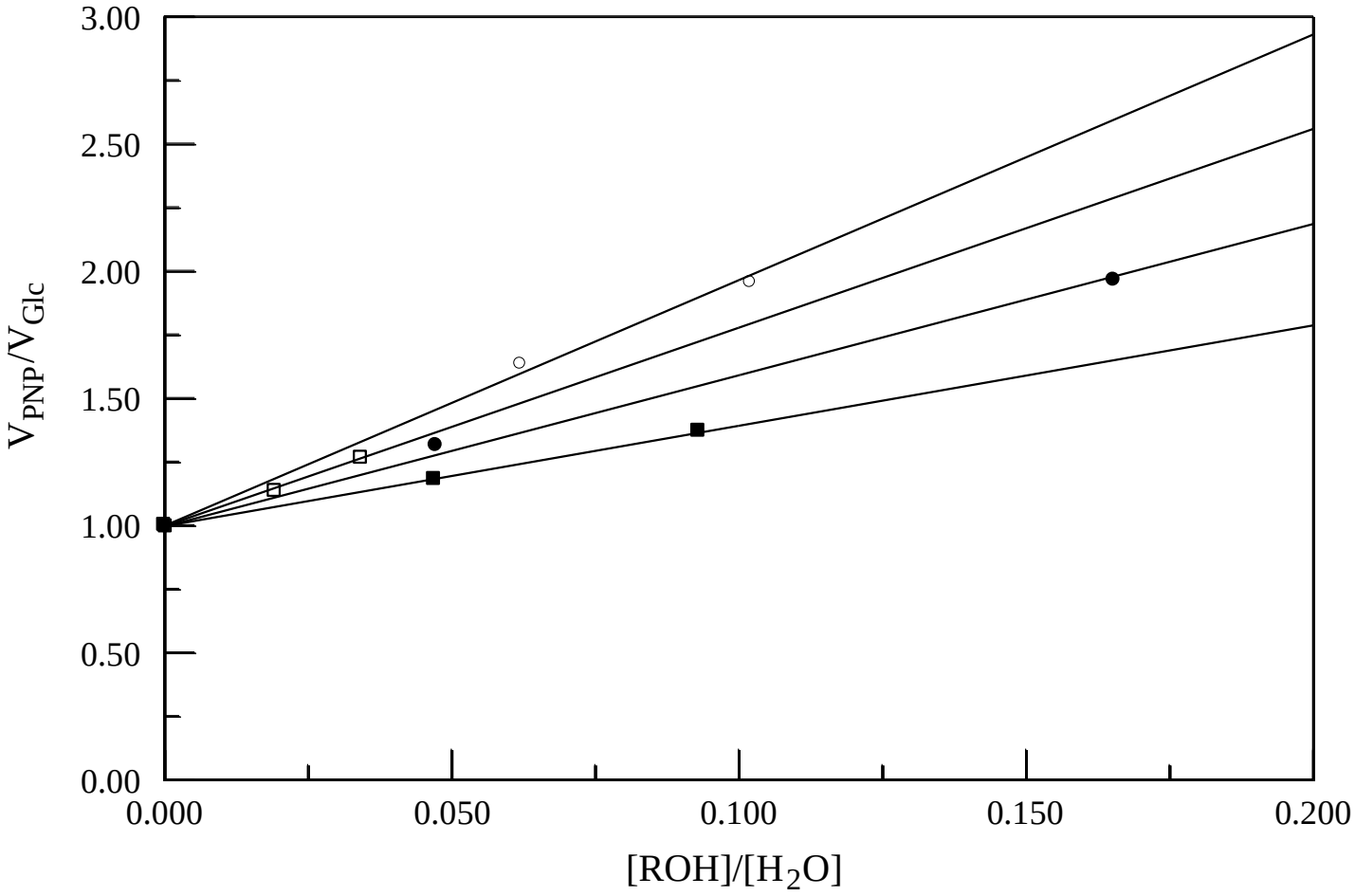


Figure 12. The effect of increasing concentrations of alkyl alcohols on the initial velocity of β-glucosidase-catalyzed cleavage of 1.0 mM pNPG at pH 5.5, monitored by following the formation of 4-nitrophenoxide anion at 400nm. R = [CH_2CH_3 (○), CH_2CF_3 (●), $CH(CH_3)_2$ (■), $CH(CF_3)_2$ (□)].

Brønsted plot of alkyl glucosides hydrolysis

For the reaction:

$$GluOR + E \underset{(k_{cat}/K_m)_r}{\overset{(k_{cat}/K_m)_f}{\rightleftharpoons}} E\,Glu + ROH$$

The equilibrium constant $K = \frac{(k_{cat}/K_m)_f}{(k_{cat}/K_m)_r}$. As shown in Table 1 and Table 2, the values of $(k_{cat}/K_m)_f$ and k' [$k' = (V_{max}/K_m)_r$] are already known for different alkyl glucosides. Thus, the corresponding values of log K can be calculated. The corresponding Brønsted plot is shown in Figure 13. The derived β_{eq} value for the equilibrium constant K is -0.27 (r^2 = 0.84). The corresponding alkyl glucosides GluOR are:

a. R = $CH(CF_3)_2$

b. R = CH_2CF_3

c. R = $CH(CH_3)_2$

d. R = CH_2CH_3

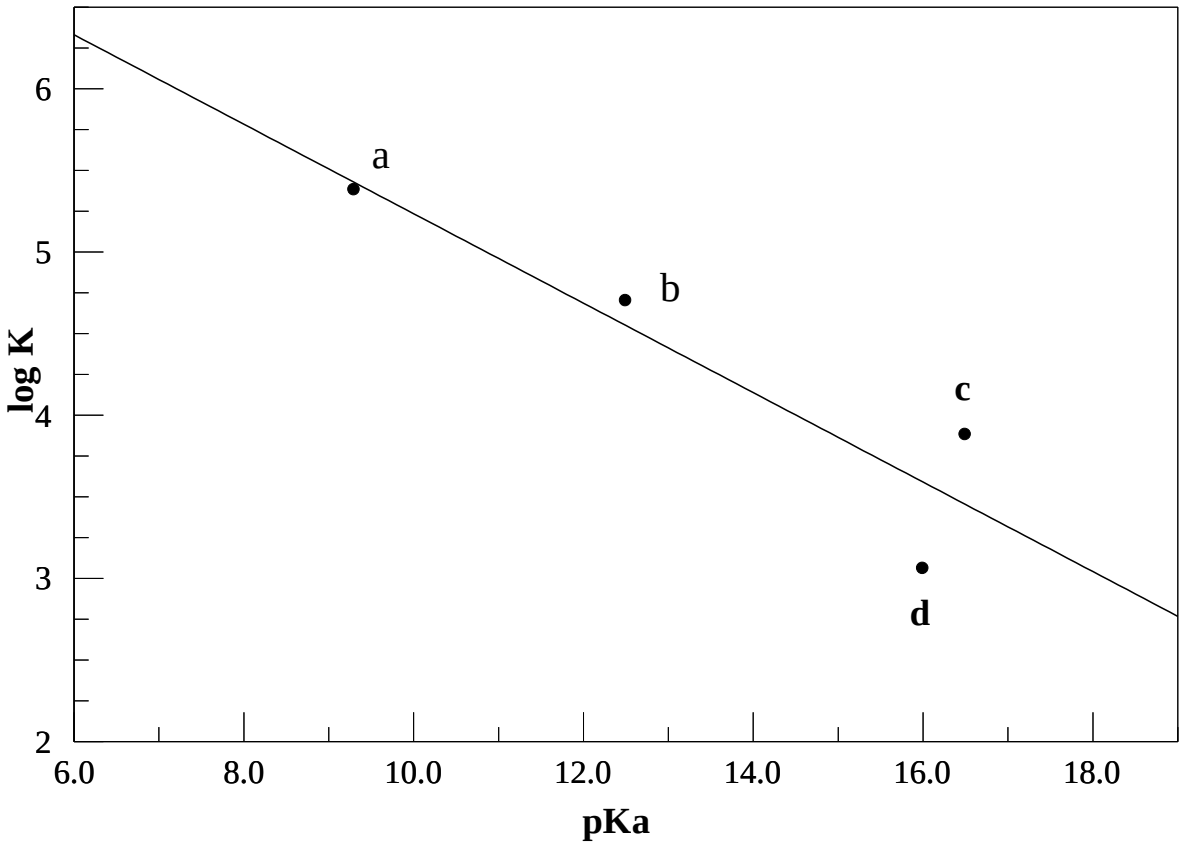


Figure 13. Brønsted plot. Effect of leaving group on the equilibrium constant K for sweet almond β-glucosidase. All experiments were carried out at 25 ^{o}C and pH 5.5. The line is the best linear fit to the data with the slope (β_{eq} = -0.27, r^2 = 0.84).

Secondary kinetic isotope effect

The average initial velocities of 2, 4-dinitrophenyl-glucoside and 2, 4-dinitrophenyl-[1-^{2}H]-glucoside are 2.805 ($\pm$0.042) $\times10^{-2}$ Au/S and 2.554 ($\pm$0.045) $\times10^{-2}$ Au/S, respectively. The secondary rate constant k_{cat}/K_m for 2, 4-dinitrophenyl-glucoside and 2, 4-dinitrophenyl-[1-^{2}H]-glucoside were identical at 3.1 $\times10^{-2}$ $mM^{-1}S^{-1}$. According to the K_m of 2, 4-dinitrophenyl-glucoside, 0.786 ($\pm$0.104) mM and K_m of the 2, 4-dinitrophenyl-[1-^{2}H]-glucoside, 0.701($\pm$0.106) mM. The value of secondary ^{2}H kinetic isotope effects on the k_{cat} corrected by the value of K_m of the 2,4-dinitrophenyl-glucoside with β-glucosidase from sweet almond is 1.12 ($\pm$0.02).

DISCUSSION AND CONCLUSIONS

pL dependence of trifluoroethyl β-glucopyranoside

As shown in Figure 11, the value of pK_2 in H_2O is 6.3. If without any stabilization by the enzyme, the equilibrium constant Keq for the formation of the protonated trifluoroethyl glycoside (pKa≈-8.4) [30] from enzyme residue with pKa of 6.3 is around 4×10^{-15} and the energy of this protonated product is 19.6 kcal/mol higher than that of the ES complex. Based on a value of k_{cat} =230 s^{-1} and the value of $(k_{cat}/K_m)^{lim}$ = 19 $M^{-1}s^{-1}$, the rate constant for cleavage of the protonated substrate is 6×10^{16} s^{-1}. This value is too high according to the stretching vibrational frequency for the β-glycosidic bond. Furthermore, calculation of the Gibbs free energy of activation for hydrolysis, based on the value of k_{cat} = 230 s^{-1}, is 14.5 kcal/mol, which is lower than the energy for protonation. For these two reasons, the result of pL dependence of trifluoroethyl β-glucopyranoside is inconsistent with the explanation for methyl glucoside that protonation occurs in the ground state [24]. However, the SKIE is 1.23 for hydrolysis of trifluoroethyl glycoside; this value is less than the typical SKIE values of general acid/base enzyme catalysis [31]. One possible explanation for this is that the protonated glucoside is stabilized by the enzyme; another possible explanation is that only partial protonation has occurred in the transition state.

Alkyl glucosides hydrolysis

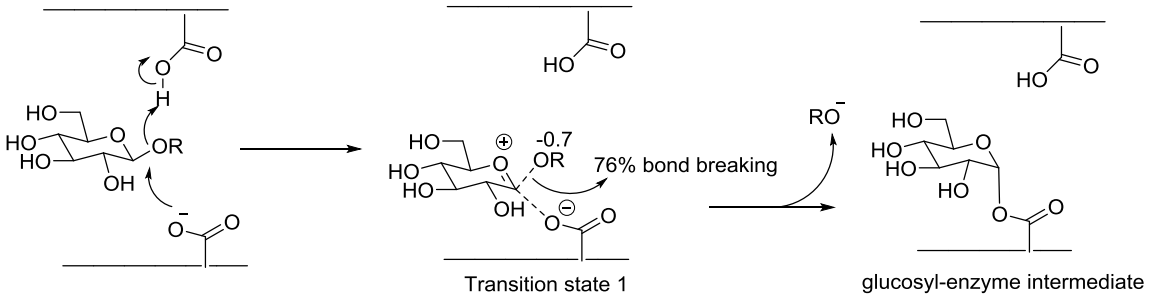
As shown in figure 12, the ratio of initial velocity of formation of [pNP] and [Glc] is linear in the concentration of alkyl alcohols (i.e. [ROH] « K_m for the alcohol under these conditions). Thus the transfer of glucosyl residue is first-order in acceptor concentration.

The derived β_{eq} value for the equilibrium constant K is -0.27 (Figure 13) for the following reaction:

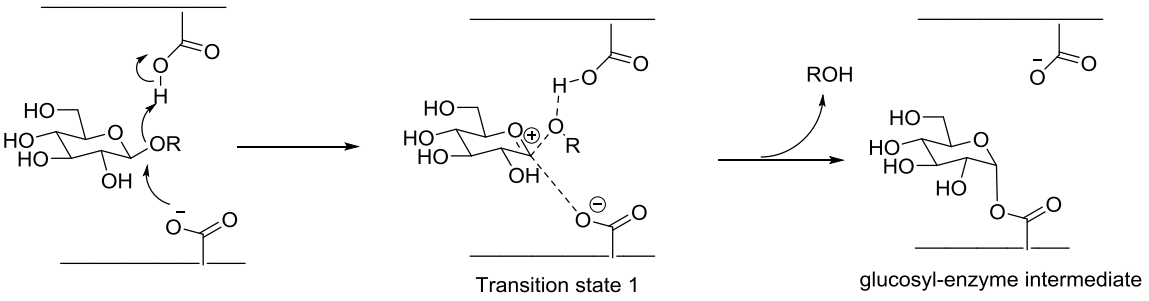
So it can be deduced that the β_{eq} value for the reaction:

the β_{eq} value is -1.27. This is consistent with the electron withdrawing ability of the glucosyl residue (σ= 1.45) [32], meaning the effective charge on oxygen of GluOR is positive 0.27. According to the negative Brønsted coefficient (β_{lg} = -0.97) obtained from k_{cat}/K_m for the hydrolysis of aryl glucosides with pKa>7.1[3], the effective charge on oxygen of GluOR at transition state is minus 0.7. This means there is 76% bond breaking of carbon-oxygen bond in the transition state. This result is similar to the lower limit of C-O bond cleavage in the transition state obtained by Rosenberg and Kirsch [23] based on the ^{18}O-KIE. Considering the steric effect, the β_{lg} values calculated according to

V_{max}/K_m of ethyl glucoside and trifluoroethyl glucoside and of isopropyl glucoside and hexafluoro-isopropyl glucoside are -0.4 and -0.3, respectively. The lower β_{lg} value compared to the β_{lg} value of aryl glucosides (β_{lg} = -0.97) means less negative charge build on the oxygen of GluOR, this result is consistent with the explanation obtained by the solvent kinetic isotope effect of trifluoroethyl glucoside that only partial protonation has occurred in the transition state (Figure 14).



estimated transition state structure of aryl-glucoside hydrolysis

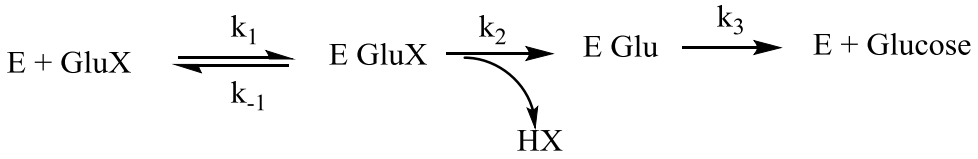


estimated transition state structure of alkyl-glucoside hydrolysis

Figure 14. Estimated transition state structure of aryl and alkyl-glucoside hydrolysis for glucosylation.

Secondary kinetic isotope effect

The Brønsted coefficient of aryl glucosides with pKa >7.1 is -0.97. However, when pKa passes beneath this threshold, the structure-reactivity correlation levels off to a slope of $\beta_{lg} \approx 0$. This implies that the rate-limiting step is changed to the breakdown of the glucosyl-enzyme intermediate (Figure 5). The pKa of 2, 4-dinitrophenol is 4.3, and when this glucoside is used as the substrate, $k_2 \gg k_3$, thus $k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \approx k_3$ for the following reaction:



The value of the secondary kinetic isotope effect on k_{cat} of 2, 4-dinitrophenol glucoside is 1.12 ($\pm$0.02). The large isotope effect indicates that hydrolysis of the glucosyl-enzyme intermediate occurs via an S_N1-like mechanism, indicating that a relatively large amount of carbo-cation character is present in the transition state 2 (Figure 5). This result is consistent with the SKIE on k_{cat} obtained with 2, 4-dinitrophenol glucoside (^{D}V= 1.5) [19]. This is also significantly different from the result obtained with phenyl β-glucopyranoside where there is no secondary deuterium kinetic isotope effect (^{2}H KIE; k_H/k_D=1.01) in glycosylation [21].

In conclusion, for β-glucosidase hydrolysis, the transition state 1 is a concerted S_N2 like mechanism with 76% C-O bond cleavage; the transition state 2 involves an S_N1-like mechanism.

Part II Synthesis of a Disaccharide Building Block for Side-Chain-Branched (1, 3; 1, 6) β-D-Glucans

INTRODUCTION

Description and Natural Sources of (1, 3)-β-glucans

β-(1, 3)-glucans, are a group of polysaccharides found in the cell wall of bacteria, fungi and cereals. These polymers are composed of a backbone chain containing (1, 3)-β-D-linked anhydroglucose repeat units with a glycosidic linkage [33]. The simplest (1, 3)-β-glucans are linear, unbranched chains. In the side-chain-branched members, the (1, 3)-β-glucosyl chain residues are substituted via β-(1, 6)-glycosidic bond by a side chain of varying length (Figure 15).

Although there are a variety of sources of β-glucans, they are most commonly prepared from fungal cell walls. Another abundant source of β-glucan is seaweed, from which it can be isolated. For example, linear β (1, 3)-D-glucan, laminaran, is isolated from a kelp called *Laminaria sp*. β-Glucan is also produced by

bacteria [34]. The methods of isolation of β-glucans are varied [35], the common procedure involves extracting fungal or bacterial cells using dilute acid, base or alcohol. This removes the cell wall material leaving behind the β-glucans. The remaining material is then purified further to give relatively pure β-glucans. Examples of naturally occuring β-glucans are shown in Table 3.

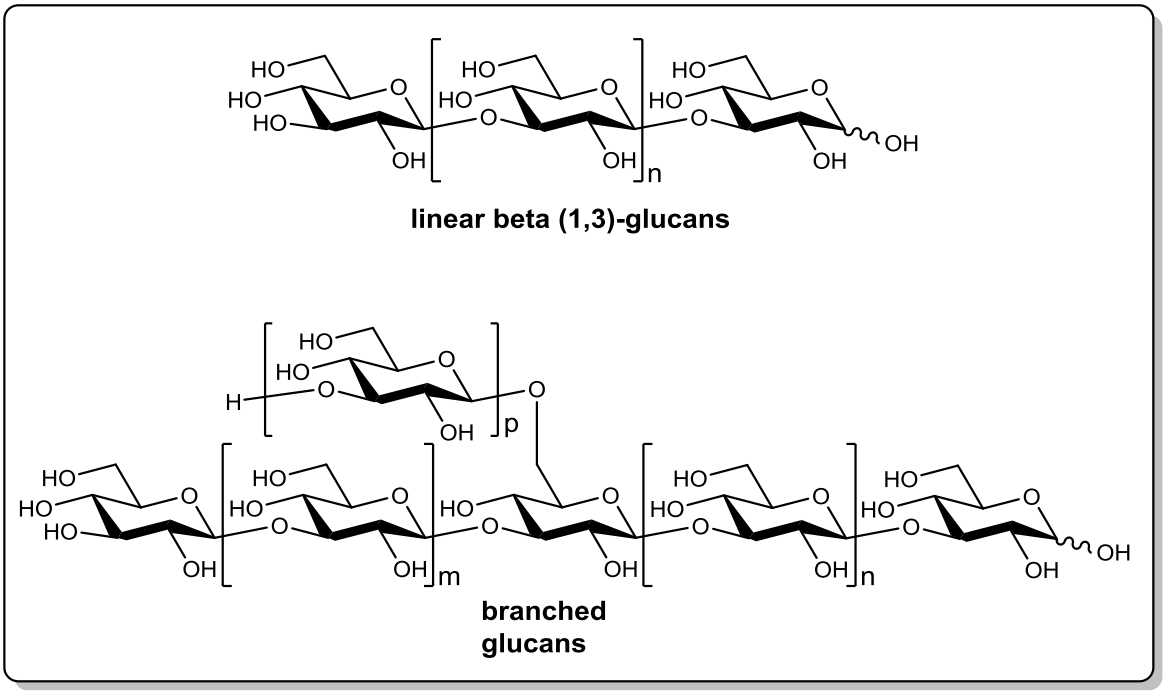


Figure 15. General structures of linear and branched β-glucans

Name	Source	Glycosidic linkage	MW (g/mol)
Curdlan	*Alcaligenes faecalis*	β-(1, 3)	-
Glucan Phosphate	*Saccharomyces cerevisiae*	β-(1, 3),β-(1, 6)	1.57×10^5
Laminaran	*Laminaria digita*	β-(1, 3),β-(1, 6)	7.70×10^3
Pachyman	*Poria cocos*	β-(1, 3),β-(1, 6)	-
Pustulan	*Umbiliaria papullosa*	β-(1, 3)	-
Scleroglucan	*Sclerotium glucanicum*	β-(1, 3),β-(1, 6)	1.02×10^6

Table 3: Examples of natural β-(1, 3)-glucans [36]

Effects on the Immune System and Application of (1, 3)-β-glucans

β-Glucans have attracted a great deal of attention in recent years due to their remarkable effects on the human immune system. β-Glucans can stimulate the immune system to protect individuals from attack of pathogenic microbes and side effects from environmental toxins and carcinogens [37, 38]. β-Glucans are well

known as biological response modifiers [39] (BRMs). These compounds are recognized by the human immune system and arouse the body's response to an infection, enhancing the activity of the immune system to fight off the infectious agent.

β-Glucans have been used in treatment of cancer since the 1980s, primarily in Japan [40]. Animal experiments have demonstrated the remarkable effects of some β-glucans on cancer [37]. Currently, three antitumor mushroom polysaccharides, lentinan, schizophyllan and PSK, isolated from Lentinus edodes, Schizophyllum commune and Coriolus versicolor, have been approved for clinical use in human cancer in Japan [41]. β-Glucans can also be used for treatment of infectious diseases. Certain β-glucans, such as PPG from *Saccharomyces cerevisiae*, effectively treated methicillin-resistant strains of S. aureus and S. epidermidis in guinea pigs [42]. β-Glucans also have been shown to be effective in treatment hypercholesterolaemia [43], diabetes [44], and wound repair [45].

Possible mechanisms of β-glucans action

For multicellular organisms, the ability to detect pathogens is essential for survival. To this end multicellular organisms have developed receptors called ‘pattern

recognition receptors' (PRRs). These receptors can recognize foreign structures which are termed the pathogen associated molecular patterns (or PAMPs) [38]. There are several well described PAMPs, such as lipopolysacharide (LPS), peptidoglycan, lipoteichoic acid (LTA) of bacteria, and β-glucan of fungi. Recognition of these structures by receptors triggers the immune system found in all higher organisms [38]. There are a number of such receptors that have been identified in humans, including Dectin-1, complement receptor 3 (CR3), scavenger receptors and lactosylceramide (LacCer) [38]. Among them, Dectin-1 is the most significant receptor. Dectin-1 is a transmembrane glycoprotein containing two primary domains: a C-type carbohydrate recognition domain (CRD) [46] and an immunoreceptor tyrosine-based activation motif (ITAM) [47] in its cytoplasmic tail, as shown in Figure 16. Dectin-1 is commonly expressed in macrophages and neutrophil lineages [48]. Binding of Dectin-1 with certain β-glucan activates several signal pathways to increase the activity of macrophages and neutrophil lineages and thus promotes the innate immune response [47, 49].

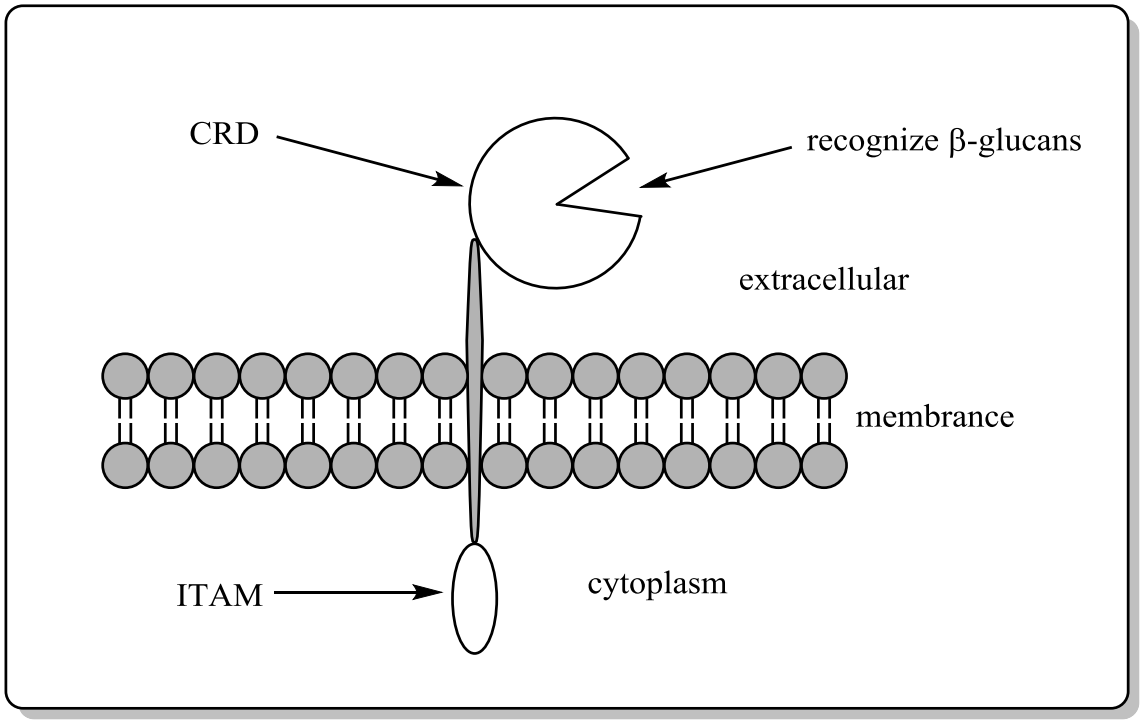


Figure 16. Cartoon representation of Dectin-1

Dectin-1 binds certain glucans preferentially. The β-glucan affinity is relative to primary structure, size and branching frequency of the β-glucan. In order to better understand β-glucan-induced biological events, there is a need to more precisely identify the key structures of β-glucans required for binding to Dectin-1 to trigger Dectin-1 agonist or antagonist activity. In a recent study, Williams and co-workers demonstrate that Dectin-1 bonds highly specifically to (1, 3)-β-D-glucans. They also determined that Dectin-1 recognition of glucans requires a backbone chain

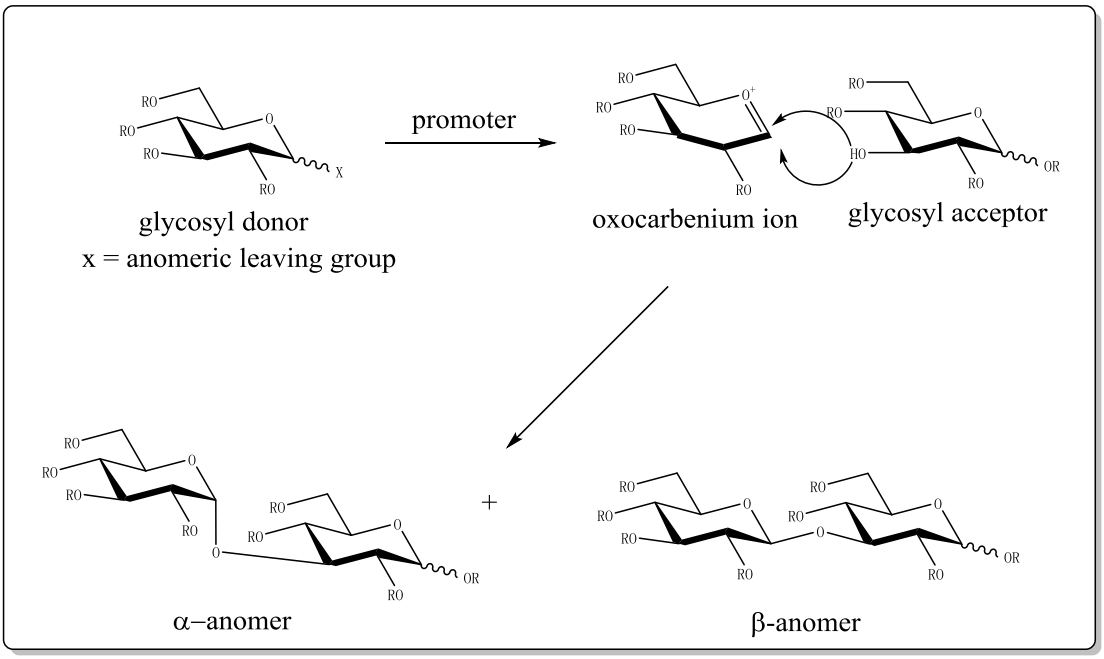
length consisting of at least seven glucose subunits and at least one glucose side-chain branch. In addition, they observed that incorporation of a single (1, 6)-β-linked glucose side-chain subunit increases Dectin-1 binding affinity [50]. Thus, the chemical synthesis of structurally well-defined branching β-glucans with sufficient molecular weight is important to the study of structure-function relationships.

Chemical Synthesis of β-(1, 3)-oligosaccharides

Oligosaccharides have multiple hydroxyl groups and exhibit considerable steric hindrance. Thus, β-(1, 3)-oligosaccharides are challenging targets for laboratory synthesis [51]. However, there are several basic concepts and methods for oligosaccharides synthesis.

The most crucial step in carbohydrate synthesis is the formation of glycosidic bonds. This bond is formed by a nucleophilic displacement of a leaving group (X) attached to the anomeric carbon of a sugar moiety by the OH group of a partially protected sugar moiety. A compound that has only one unprotected hydroxyl group is called a glycosyl acceptor while the glycosyl donor has an anomeric leaving group. The reactions usually involve an appropriate promoter, which helps the leaving group depart from the anomeric carbon to form an oxocarbenium ion intermediate

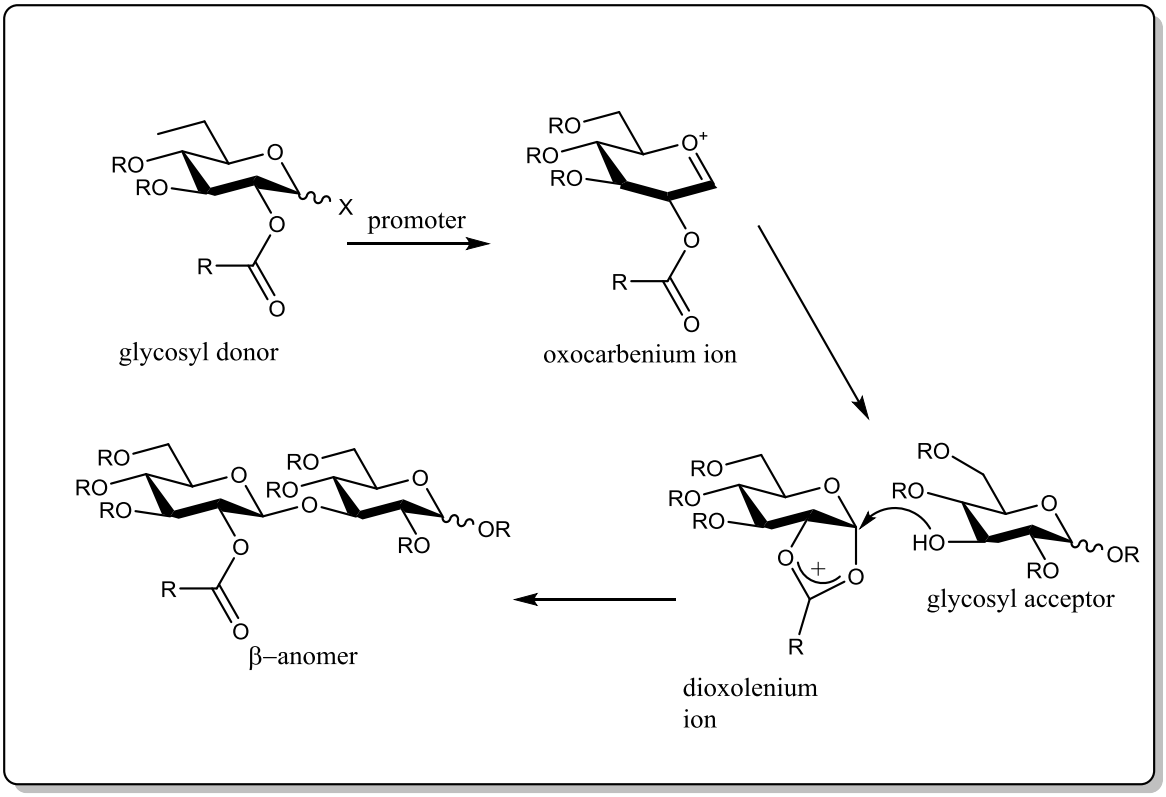
[52]. The glycosyl acceptor attacks the anomeric center to form the glycosidic bond. The products will contain both an α- and β- anomer. (Scheme 5)



Scheme 5. General mechanism of glycosylation

For the synthesis of β-(1, 3)-oligosaccharides, the control of stereochemistry at the anomeric carbon to get only β-anomer is necessary. Otherwise, separation of mixture of the α- and β- anomer increases preparation time and reduces yields. If the donor has an acyl group present at the C-2 position, the β-glycosidic bond will be formed due to the neighboring group participation [53]. When the oxo-carbenium

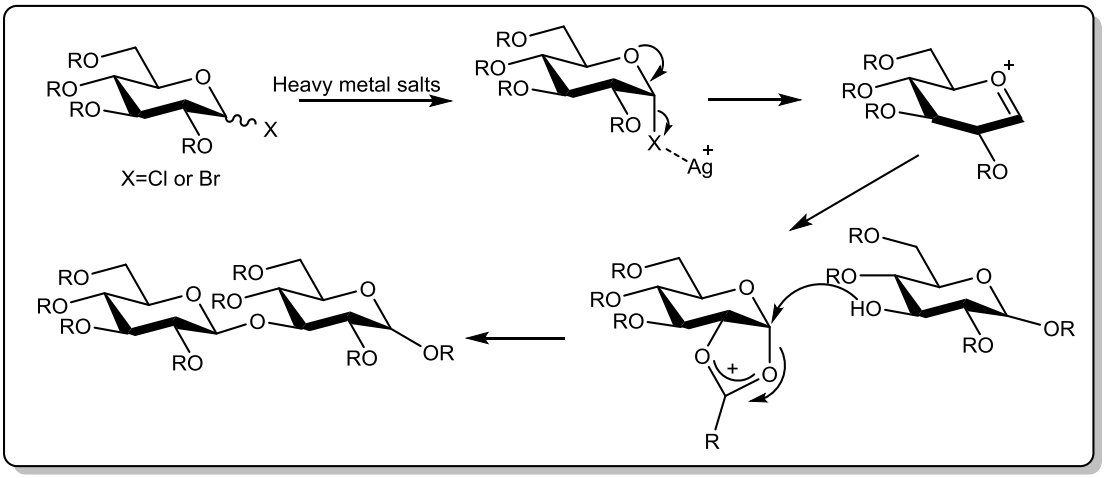
intermediate is formed, the nuclephilic acceptor attacks only from the top face of the anomeric carbon to form the β-anomer. (Scheme 6)



Scheme 6. Mechanism of formation for the β anomer controlled by neighboring group participation

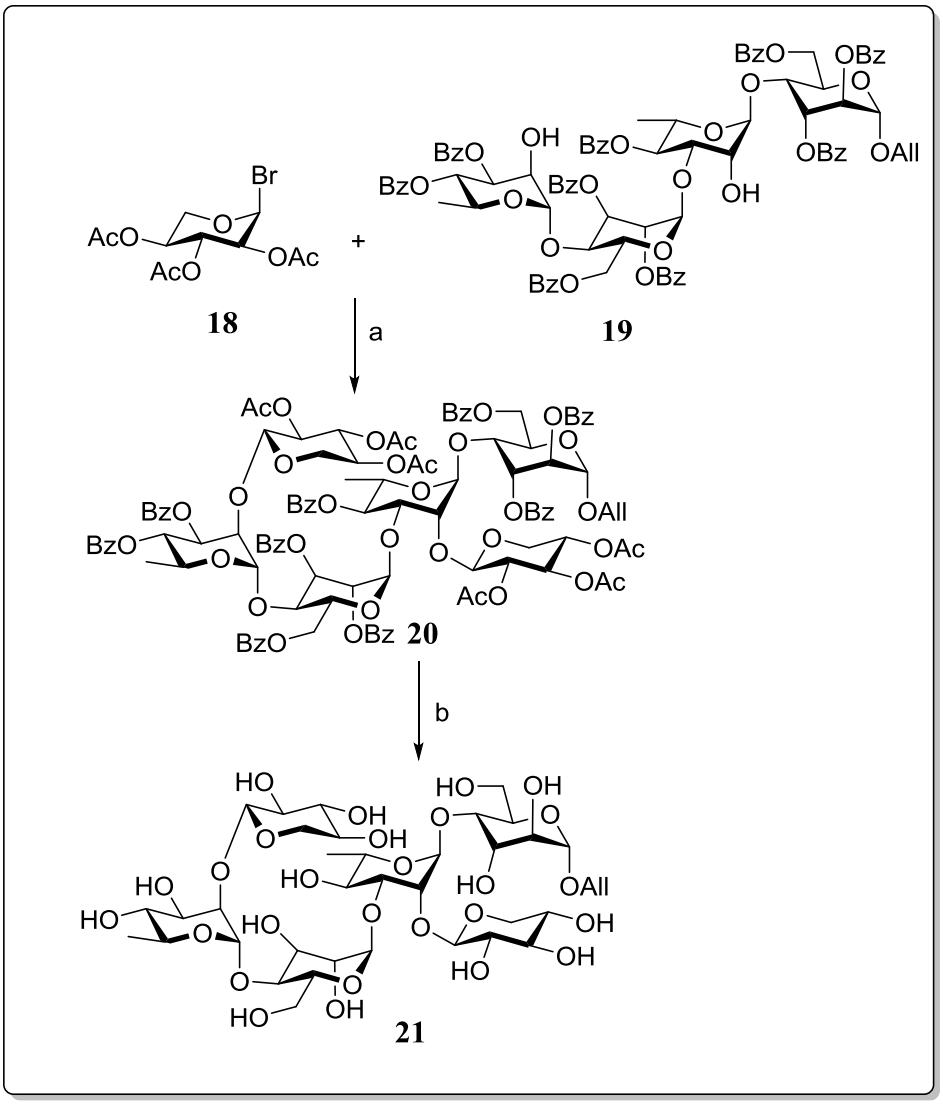
There are several well studied glycosyl donors for the synthesis of carbohydrates, including glycosyl halide, thioglycoside and glycosyl trichloroacetimidate.

Glycosyl halides are widely used as carbohydrate donors for aromatic O-glycosylation. They were first used for this purpose in 1901; Koenigs and Knorr used glycosyl chlorides and glycosyl bromides with silver salt catalysis to prepare glycoside derivatives [54]. Glycosyl bromides are most commonly used and are more reactive than other glycosyl halides. Additionally, glycosyl bromides are usually isolated as the thermodynamically favored α-anomer, thus the glycosylation process generally results in the inversion of stereochemistry. In addition to silver salts, such as AgOTf [55], mercuric salts [56] also can be used to catalyze this reaction (Scheme 7).



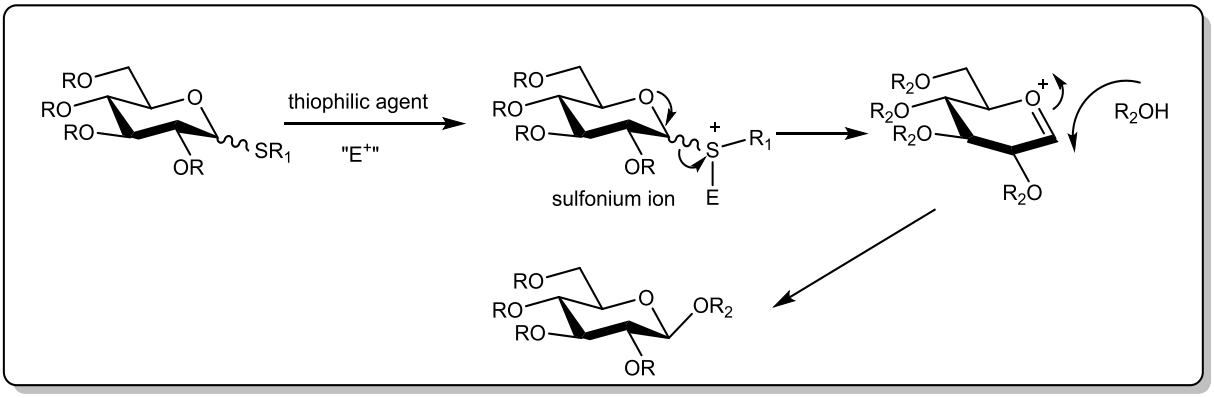
Scheme 7. General mechanism of activation of glycosyl halides

Kong and coworkers [57] synthesized a hexasaccharide, which is a dimer of the repeating unit of the antigen O2 polysaccharide of *Stenotrophomonas maltophilia* (Scheme 8). The target was synthesized through coupling of 2, 3, 4-tri-O-acetyl-α-L-xylopyranosyl bromide **18** with the tetrasaccharide **19** in the presence of AgOTf. The desired hexasaccharide **20** was deacylated to achieve the targeted product **21**.



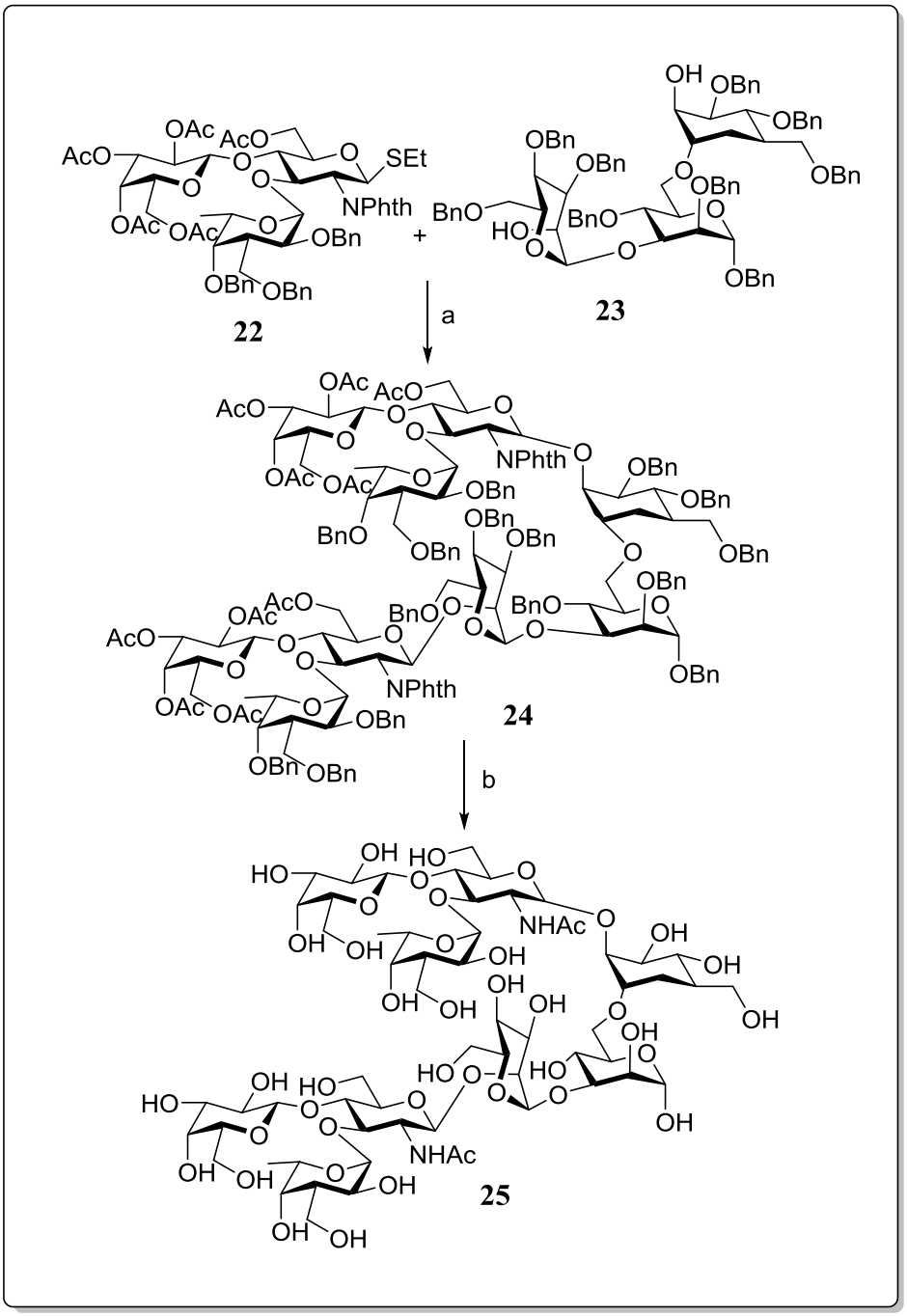
Scheme 8. Kong and coworkers synthesize the hexasaccharide using the Koenigs-Knorr method [57]. a. AgOTf, CH_2Cl_2, 4Å MS, -5^oC for 2h, 67%; b. NaOMe/MeOH, rt-40^oC, overnigh-8h, 97%.

Thioglycosides are one of the most popular and versatile donors in carbohydrate synthesis. They were first introduced as glycosyl donors by Ferrier and coworkers who used thioglycoside and mercuric sulfate in the preparation of several glycoside derivatives [58]. The sulfur atom in a thioglycoside is a soft nucleophile and is able to react selectively with soft electrophiles suchs as heavy metal cations, halogens, and alkylating or acylating reagents. Thioglycosides are relatively stable carbohydrate derivatives that can be activated by thiophilic reagents. After the intermediate sulfonium ion is formed, the molecule can cleave to give oxocarbenium ion intermediate that reacts with the acceptor giving the desired product. The thiophilic reagents include N-iodosuccinimide and triflic acid (NIS/TfOH) [59], MeOTf [60] or NIS/AgOTf [61] (Scheme 9).



Scheme 9. General mechanism of activation of thioglycosides

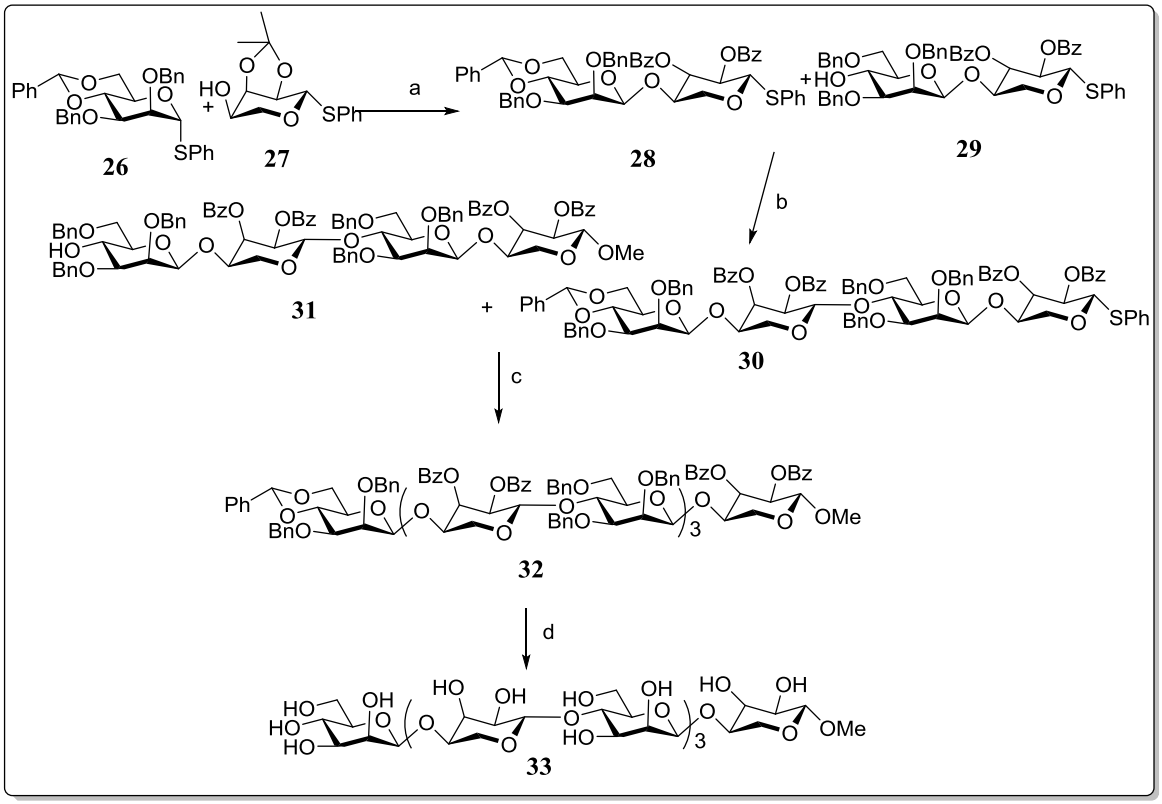
Lönn [62] synthesized a nonasaccharide, which is a structural element of glycopeptides found in the urine from patients with fucosidosis. The synthesis of the nonasaccharide **25** involved the thioglycoside **22** and the trimannoside **23**, which were condensed, using methyl triflate as promoter, to give the nonasaccharide derivative **24**. Removal of the protecting groups and chromatographic separation then yielded the nonasaccharide **25** (Scheme 10).



Scheme 10. Synthesis of a nonasaccharide using thioglycoside as a donor [62]. a. MeOTf, Et_2O, 4Å MS, 61%. b. (1) hydrazine hydrate, aq. ethanol, acetic anhydride-pyridine, 72%. (2) NaOMe/MeOH, AcOH, rt, 90% AcOH, 10% Pd/C, 400kPa, overnight, 80%.

Crich and coworkers [63] synthesized an octasaccharide, a subunit of the $[\rightarrow 4)$-β-D-Manp-$(1\rightarrow 4)$-β-D-Xylp-$(1\rightarrow]_n$ xylomannan motif, which is proposed as the

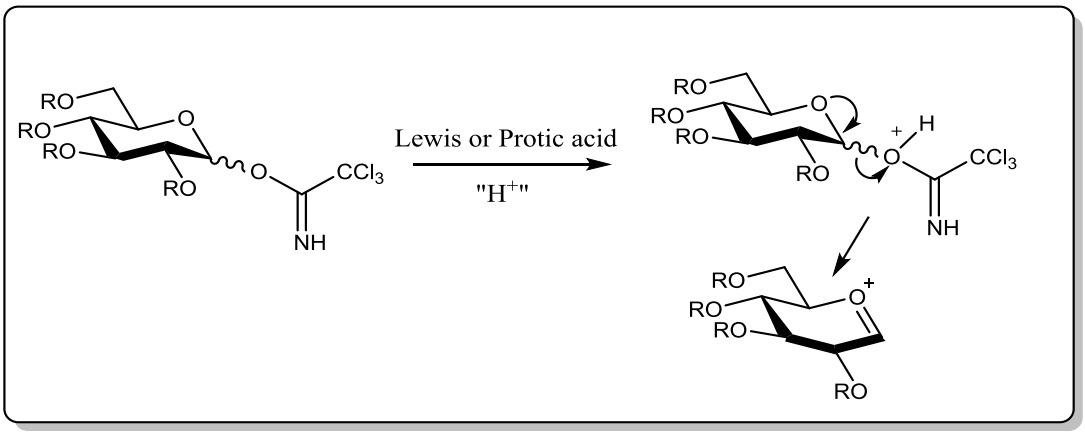
structure of a novel nonprotein, thermal hysteresis-producing antifreeze substance from a freeze-tolerant Alaskan beetle. They first prepared the disaccharide units **28** and **29** by assembling thioglycoside donor **26** and xylopyranosyl acceptors **27**. The tetrasaccharides **30** and 3**1** were then synthesized through coupling reactions between the disaccharide donors and acceptors. Finally, the octasaccharide **32** was obtained and deprotected to get the desired target **33** (Scheme 11).



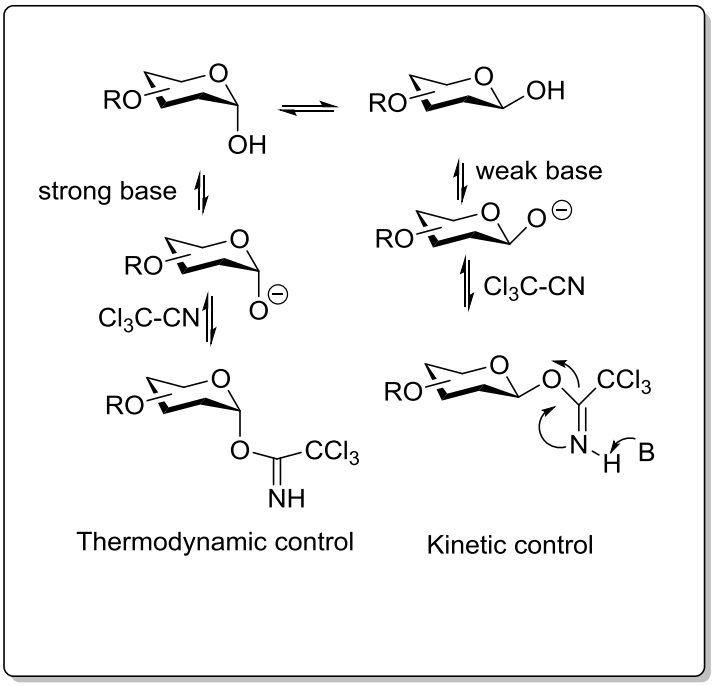
Scheme 11. Synthesis of an octasaccharide using thioglycoside as a donor [63]. a. (1) BSP, TTBP, Tf_2O, CH_2Cl_2, -60^oC, 86%. (2) AcOH, then Bzcl, Py, 87% b. BSP, Tf_2O, CH_2Cl_2, -60^oC, 79%. c. BSP, Tf_2O, CH_2Cl_2, -60^oC, 63%. d. (1) NaOMe/MeOH. (2) H_2,, Pd/C, MeOH, 92%.

In 1980, Schmidt [64] developed the method using glycosyl trichloroacetimidates as glycosyl donors in oligosaccharide synthesis. Trichloroacetimidate donors can be activated even at low temperatures by catalytic

amounts of Lewis acid, such as TMSOTf or BF_3/OEt_2 [65]. This reaction can be carried out under very mild conditions, making a good choice for sensitive or highly functionalized targets (Scheme 12). Another strength of the trichloroacetimidate methodology is good stereochemical control of glycosidic bond formation [66]. Trichloroacetimidates can be synthesized from the corresponding hemiacetals by treatment with trichloroacetonitrile in CH_2Cl_2 with addition of a suitable base. The choice of base can affect the configuration of the anomeric carbon. In general, weak bases such as potassium carbonate [67] produce the kinetically more stable β-anomer. However, this product anomerizes in a strong base catalysed reaction through retro-anomerization of the 1-oxide anion. The thermodynamically more stable α-anomer is formed through a new trichloroacetonitrile addition. Examples of strong bases used are sodium hydride [68] or DBU [69] (Scheme 13).



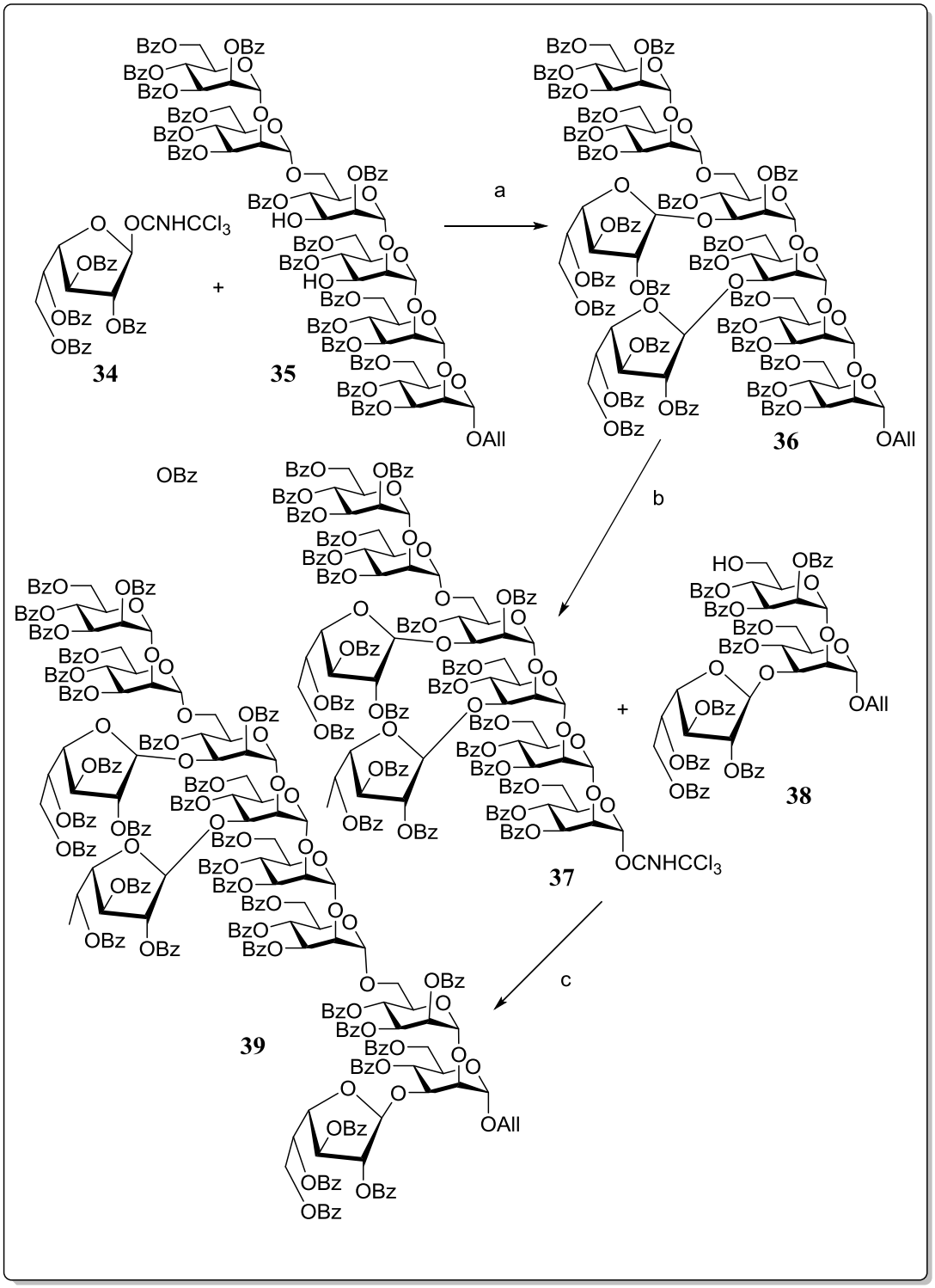
Scheme 12. General mechanism of trichloroacetimidates activation



Scheme 13. Proposed mechanism for formation of the α- and β-glycosyl trichloroacetimidate

Kong and coworkers [70] reported a convergent synthesis of an undecasaccharide, the repeating unit of the polysaccharide of T. mentagrophytes IFO 5466 and T. rubrum IFO 5467. Coupling of **35** with 2, 3, 5, 6-tetra-O-benzoyl-β-D-galactofuranosyl trichloroacetimidate **34** gave octasaccharide **36**. Subsequent deallylation and trichloroacetimidate formation afforded octasaccharide donor **37**. Coupling of trisaccharide acceptor **38** with octasaccharide

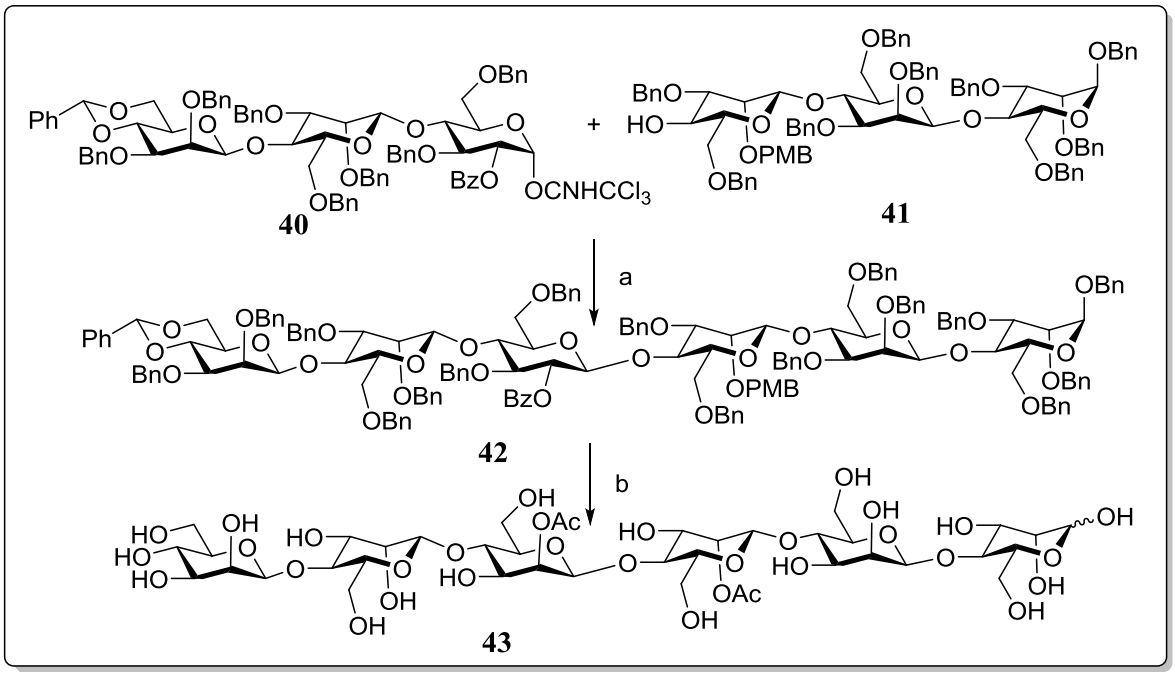
donor **37** afforded the undecasaccharide **39**. Finally, debenzoylation in a saturated solution of NH_3 in methanol yielded the target undecasaccharide (Scheme 14).



Scheme 14. Synthesis of an undecasaccharide using glycosyl trichloroacetimidates as glycosyl donors [70]. a. TMSOTf , CH_2Cl_2, 20 to 0 ^oC, 2-4 h, 73%. b. (1) $PdCl_2$,

CH_3OH, rt, 4 h, 80%. (2) CCl_3CN, DBU, CH_2Cl_2, 2 h, 89%. c. TMSOTf , CH_2Cl_2, 20 to 0 ^oC, 2-4 h,79%.

Most recently, Wong and coworkers [71] synthesized a partially acetylated β-(1→4)-linked hexamannose and investigated its bioactivity. They first prepared the trimannoside acceptor **41** with a trisaccharide donor **40**. Having the two trisaccharides in hand, assembly of these fragments was accomplished by treatment with a catalytic amount of TMSOTf to afford hexasaccharide **42**. Finally, the target compound **43** was obtained after selective deprotection and acylation (Scheme 15).

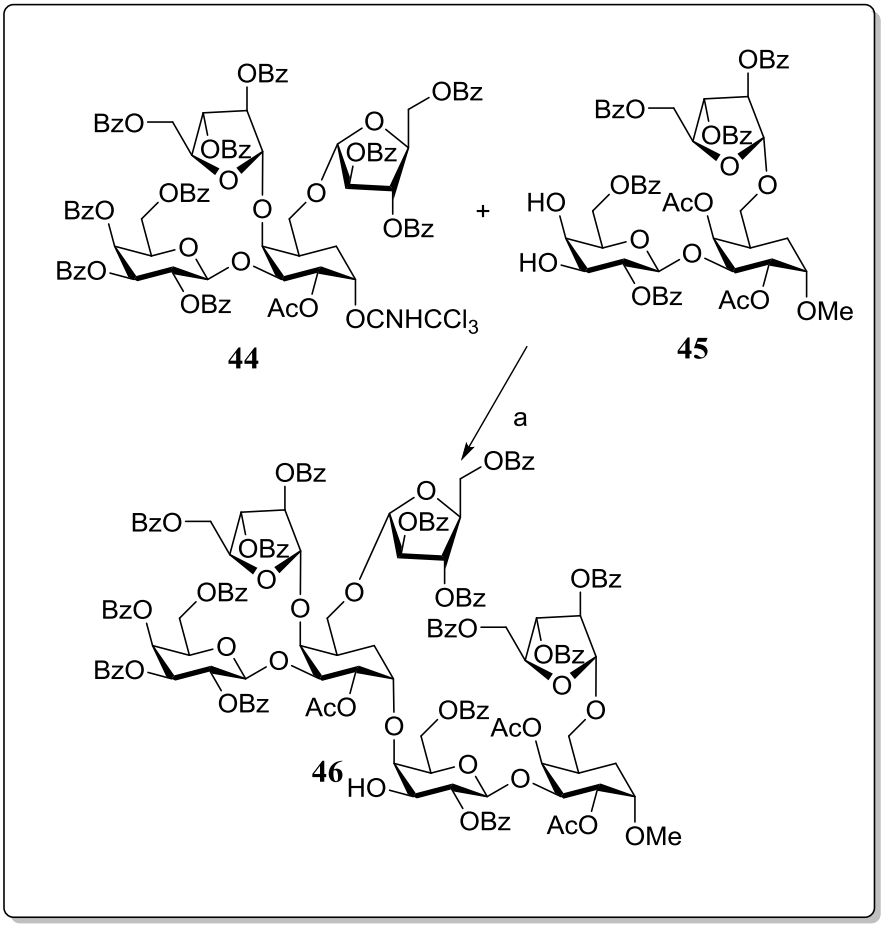


Scheme 15. Synthesis of a hexamannose by glycosyl trichloroacetimidates as glycosyl donors [71]. a. TMSOTf, CH_2Cl_2, 4ÅMS, −75 to 0 ^oC, 6.5 h, 80%. b. (1) $LiAlH_4$, Et_2O, −40 to 0 ^oC, 30 min. 71%. (2) CAN, CH_3CN/toluene/H_2O (18:1:1, v/v/v), 0 ^oC to rt, 1.5 h; 58%. (3) Ac_2O, DMAP, pyr, rt, 3.5−4.0 h, 66%. (4) H_2, Pd/C, MeOH/THF/AcOH (10:20:3, v/v/v), rt, 23 h, 74%.

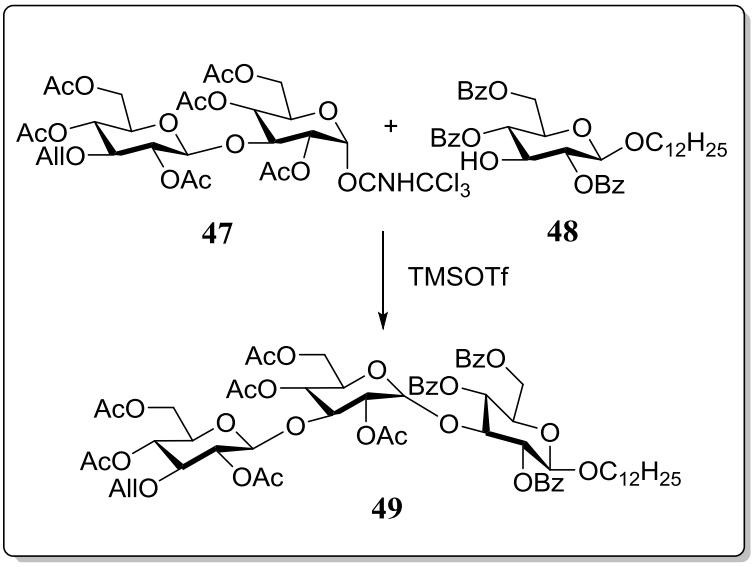
RESULTS AND DISCUSSION

Background

The key to synthesis of β-(1, 3)-glucans achieving high stereoselectivity in the formation of the glycosidic bond. The ideal result is that the coupling reactions give only the β anomer. As mentioned earlier, the acetate on the C-2 position was employed as a neighboring participating group that controls the stereochemical outcome of the coupling reaction. However, the undesirable α-anomer could be the major product despite the influence of acyl group at C-2 position. For example, Du[72] and coworkers reported that coupling of tetrasaccharide donor **44** and trisaccharide acceptor **45** in dry CH_2Cl_2 at −20 ^oC gave only α products **46** (Scheme 16) even with an acetate protecting group at the C-2 position. Surprisingly, Kong [73] and coworkers also found that the coupling reaction of donor 3-*O*-allyl-2, 4, 6-tri-*O*-acetyl-β-D-glucopyranosyl-(1, 3)-2, 4, 6-tri-*O*-acetyl-D-glucopyranosyl trichloroacetimidate **47** and accepor lauryl 2, 4, 6-tri-*O*-benzoyl-β-D-glucopyranoside **48** gave pure α product **49** (Scheme 17).



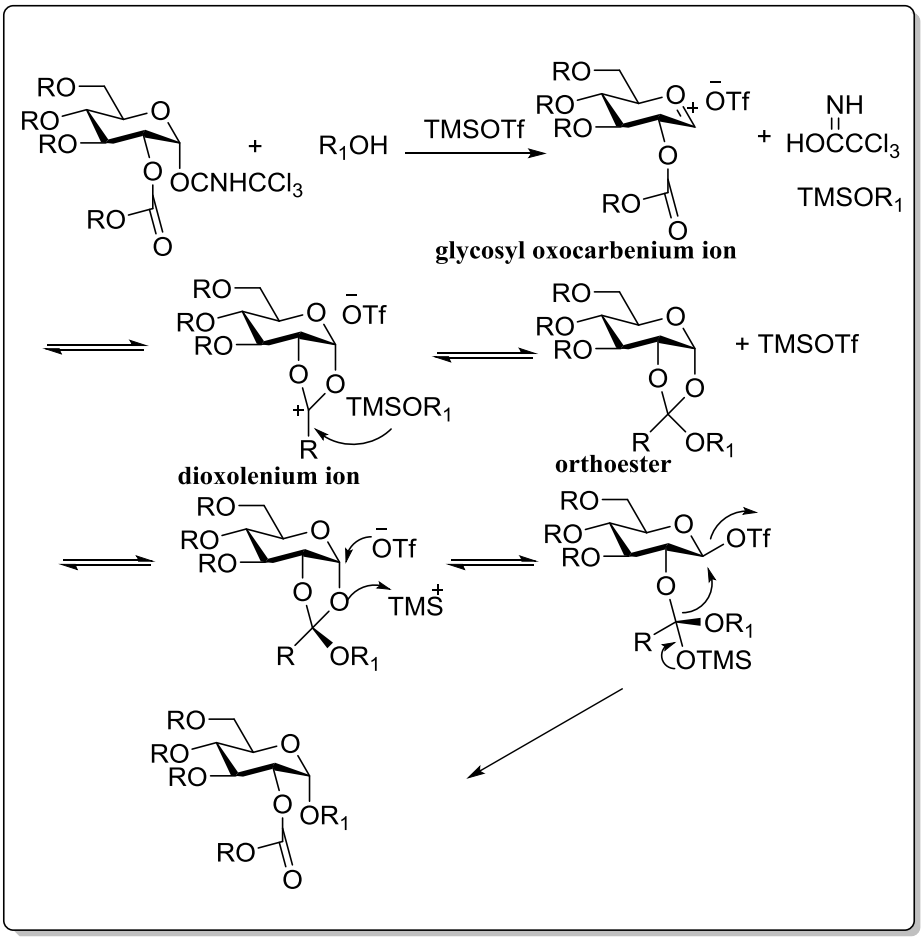
Scheme 16. Unexpected α-stereochemical outcomes reported by Du [72] and coworkers. a. TMSOTf, CH_2Cl_2, 55%.



Scheme 17. Unusual α-stereochemical outcomes reported by Kong [73] and coworkers

Kong proposed that the orthoester formed as a by-product of the glycosylation reactions was the reason for the unexpected formation of the α-anomer [73]. Orthoesters are usually formed due to trapping of the intermediate bridging cation by the nucleophile as opposed to attack at the anomeric carbon. Kong and coworkers found that orthoesters were formed in the initial stage of the reaction by monitoring TLC and they were later able to isolate the orthoesters [73]. The glycosyl donor was activated by a promoter to form a glycosyl oxocarbenium ion. After that, a

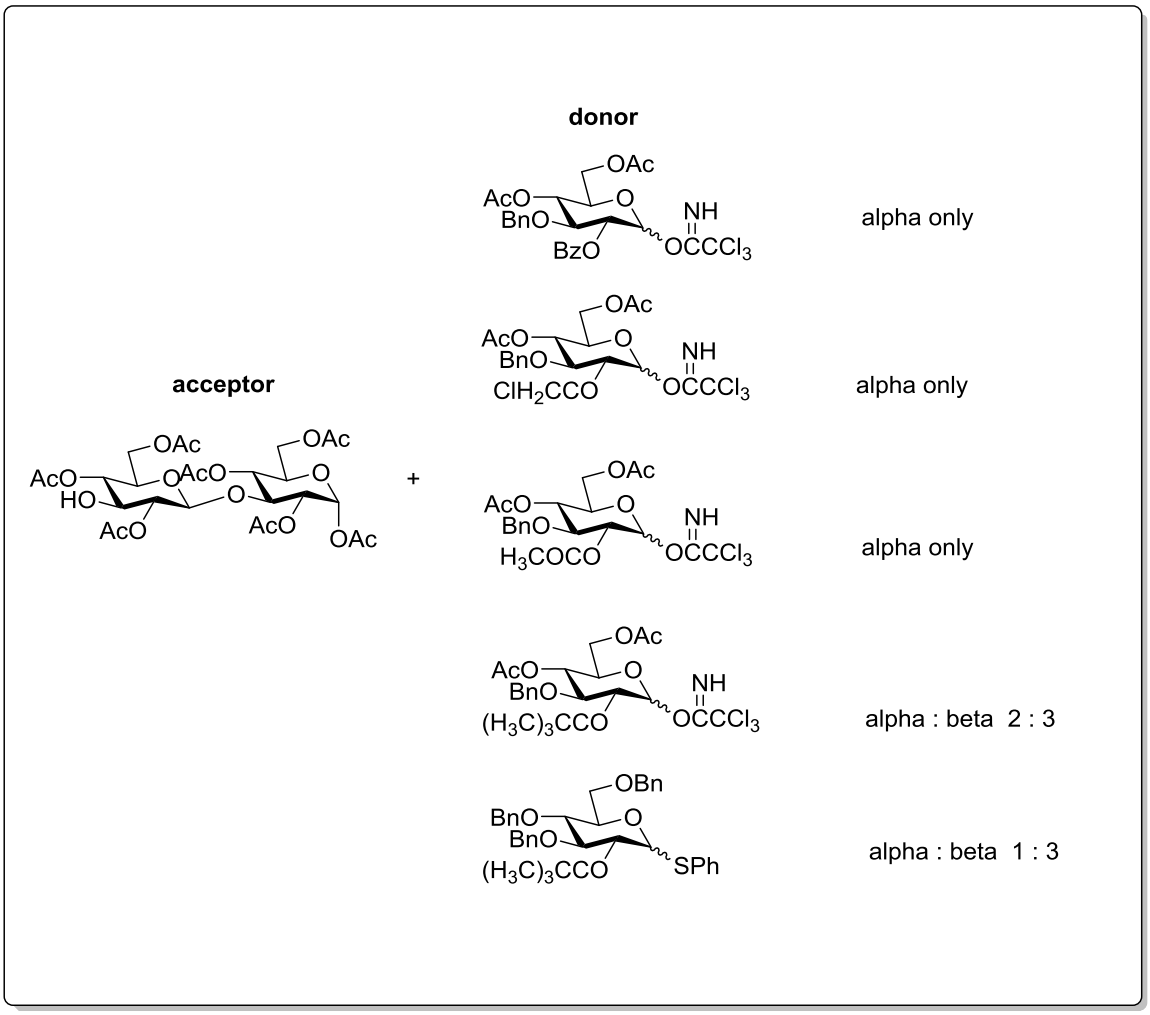
dioxolenium ion was formed. If the nucleophile attacks the C-1 of dioxolenium ion, the normal β-linkage is obtained. However, the nucleophiles can attack the carbonyl carbon of the dioxolenium ion instead of the anomeric center to form the undesired orthoester which rearranges in the presence of a Lewis acid to give the α-anomer (Scheme 18).



Scheme 18. Formation of the otrthoester and proposed mechanism of the orthoester rearrangement.

ADMB Protecting Group

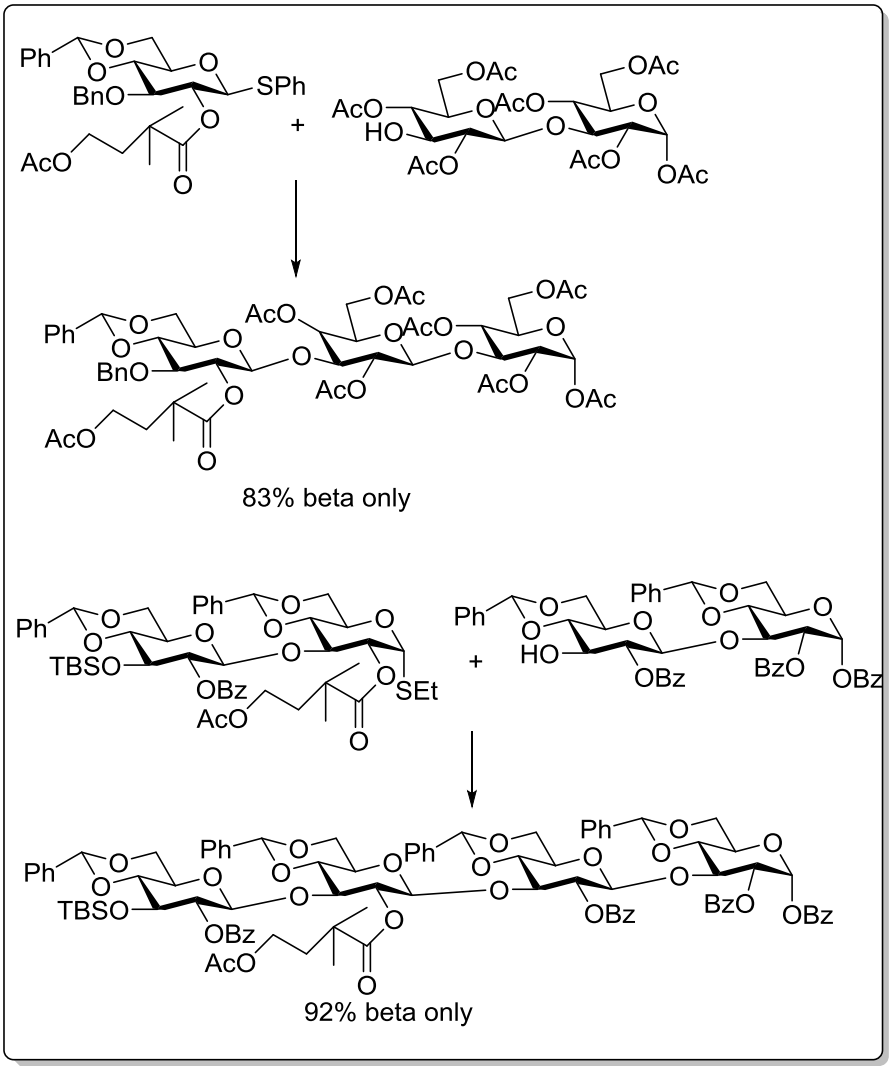
In order to eliminate the formation of α-anomer during the coupling reactions, Yu [74] and coworkers preformed several glycosylations using various glycosyl donors with different C-2 protecting groups (Scheme 19). Clearly, the sterically hindered pivaloate ester at C-2 aids in the formation of the β-glycosidic bond. However, the pivaloyl ester protecting group cannot completely prevent the formation of the unwanted α-anomer. In addition, the deprotection of pivaloate esters requires harsh conditions [75].



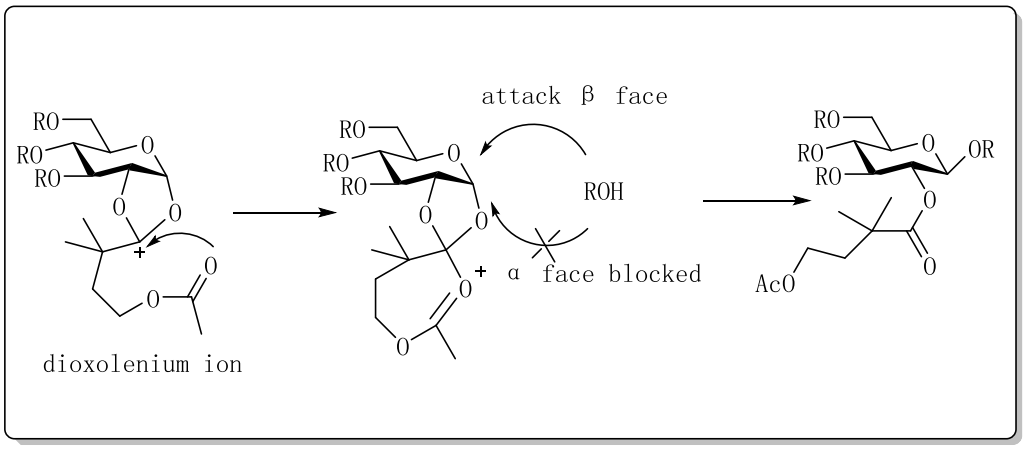
Scheme 19. Glycosylation using different glycosyl donors with various C-2 ester-protecting groups.

In order to retain the advantages of the bulky pivaloate esters, while avoiding the harsh conditions needed for deprotection, Yu [74] and coworkers prepared a new type of C-2 acyl protecting group, 4-aceoxy-2,2-dimethylbutanoate

(ADMB). With ADMB ester as protecting group at the C-2 position, Yu found that the problem of formation of α-anomers was solved. The coupling afforded good yields of β-glycosides (Scheme 20). Although it is uncertain why the ADMB ester is beneficial to β-glycoside formation, Yu proposed that the presence of the ADMB group could stabilize the dioxolenium ion and sterically prevent the formation of orthoesters (Scheme 21).



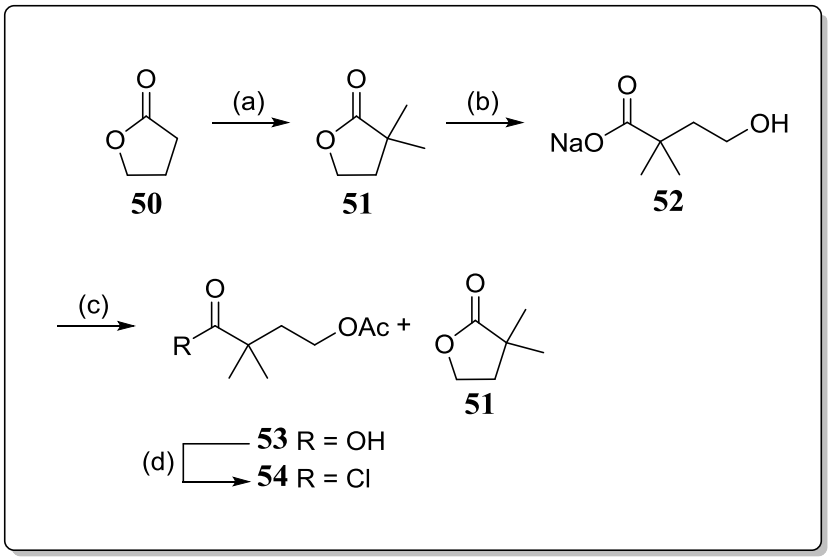
Scheme 20. The Glycosylation using the ADMB ester protecting group at C-2 to give only β-glycosides.



Scheme 21. Proposed mechanism of β-glycosides formation using ADMB ester as protecting group.

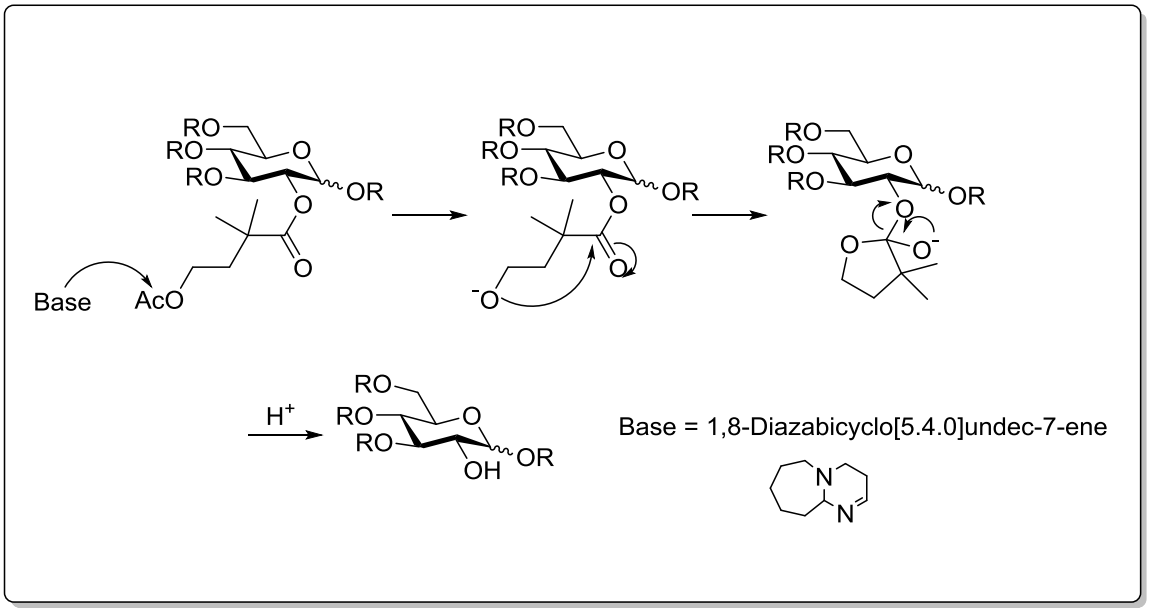
Preparation of ADMB chloride **54** is shown in Scheme 22 [76]. Dimethylation of *γ*-butyrolactone **50** was achieved through treatment with excess CH_3I and NaH under reflux in THF to give the *α*, *α*-dimethyl-*γ*-butyrolactone **51** in 55% yield [77]. Saponification of **51** with 1M NaOH afforded the sodium salt **52**

quantitatively. Acetylation of **52** gave the corresponding ADMB acid **53** and *α*, *α*-dimethyl-*γ*-butyrolactone **51** in 1:1 ratio. These two products are easily separated by extraction to recover **51** and pure **53**. The ADMB acid **53** was converted to the ADMB chloride **54** on treatment with oxalyl chloride and catalytic DMF in benzene.



Scheme 22. Preparation of the ADMB chloride. (a) CH_3I, NaH, THF, reflux, 55%; (b) $NaOH_{(aq)}$ (1M), MeOH, rt, 100%; (c) Ac_2O, DMAP, Py, 0^o C to rt, **64**, 45%; (d) oxalyl chloride, benzene, DMF (cat.), rt, 76%.

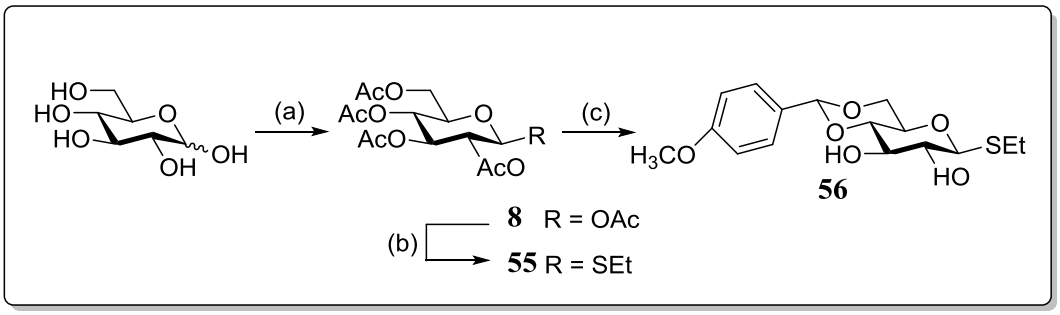
The ADMB ester can be removed under very mild conditions using a catalytic amount of 1, 8-diazabicyclo [5.4.0] undec-7-ene (DBU) in methanol at room temperature [78]. The mechanism of the base catalyzed deprotection of the ADMB group is shown in Scheme 23.



Scheme 23. Mechanism for the deprotection of the ADMB ester

Synthesis of monosaccharide building blocks

The synthesis started from 4,6-*O*-p-methoxybenzylidene-1-thio-β-D-glucopyranoside **56** which could be prepared according to the literature procedure [79]. As shown in Scheme 24, acetylation of D-glucose using acetic anhydride with sodium acetate catalyst under reflux gave the β-anomer **8** with a 65% yield. Thioglycosylation of **8** by treatment with ethanethiol and catalytic $SnCl_4$ at 0^o C generated the kinetic product, β-thioglucoside **55**. Zemplén deacetylation [80] of **55** gave the tetra-alcohol thioglucoside. Then the tetra-alcohol thioglucoside was reacted with anisaldehyde dimethyl acetal and catalytic tosylic acid under reduced pressure to arrive at the thioglucoside **56**.

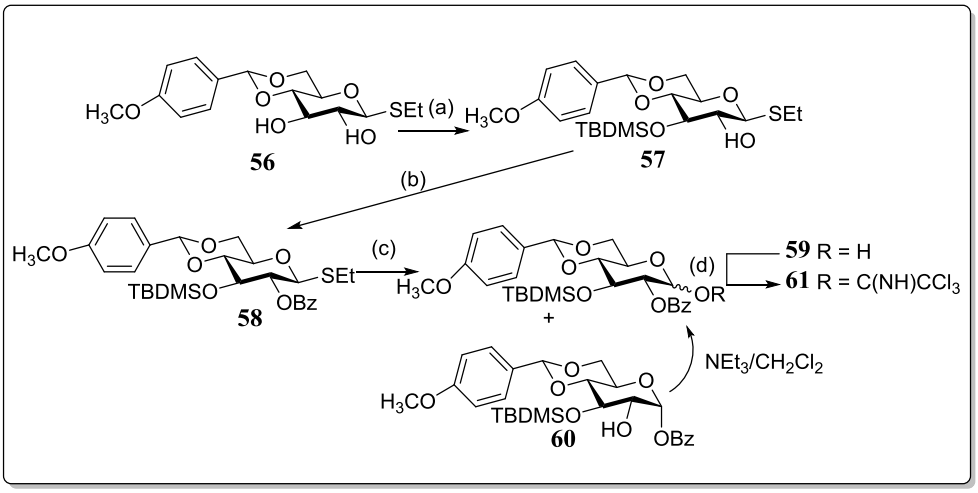


Scheme 24. Reagents and conditions: (a) Ac_2O, NaOAc, 135^o C, 65%; (b) EtSH, $SnCl_4$, CH_2Cl_2, 0^o C, 60%; (c) i. NaOMe/MeOH, rt, ii. anisaldehyde dimethyl acetal, TsOH, DMF, 15mmHg, 50^o C, 75% for two steps.

After that, the C-2 and C-3 alcohols of thioglucoside **56** can be selectively protected by orthogonal protecting groups (Scheme 25). It is generally accepted that the C-2 hydroxyl group is more reactive than the C-3 alcohol of glucose molecule because it is closer to the anomeric carbon. However, the β-configuration of the thioethyl functionality of **56** sterically deactivates the reactivity of the C-2 alcohol. Thus, thioglucoside **56** reacted with *tert*-butyldimethylsilyl chloride (TBDMSCl) almost exclusively at the C-3 alcohol to give **57** with a 79% yield. After that, benzoylation at the C-2 hydroxyl group of **57** afforded the monosaccharide thioglucoside **58**.

Hydrolysis of the thioglucoside **58** according to the BSP method was developed by Crich's group [81]. To the thioglucoside **58** was added 1-benzenesulfunyl piperidine (BSP), a non-nucleophilic base 2, 6-di-tert-butylpyridine and Tf_2O and allowed to react for 1 and half hours to give **59** and **60** with a ratio of 2:1. Treatment of crude **60** with NEt_3/CH_2Cl_2 promoted the migration of the C-1 benzoate back to the C-2 hydroxyl giving the thermodynamically more stable hemiacetal **59**. Treatment of **59** under standard Schmidt conditions [64]

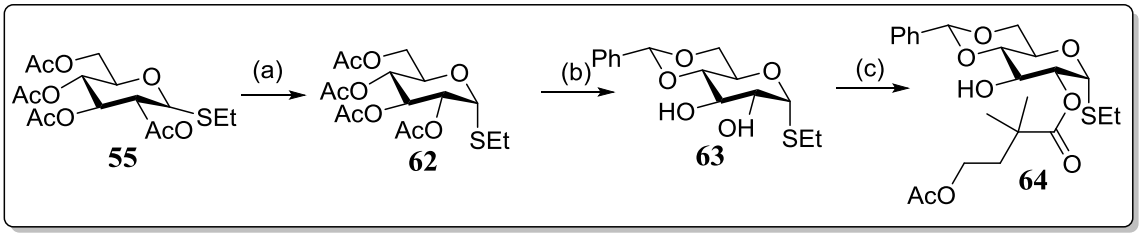
with trichloroacetonitrile and DBU provided the trichloroacetimidate donor **61** as the building block (Scheme 25).



Scheme 25. Reagents and conditions: (a) TBDMSCl, Imidazole, DMF, rt, 79%; (b) BzCl, DMAP, Py, 80^{o} C, 97%; (c) BSP, 2, 6-di-tert-butylpyridine, Tf_2O, CH_2Cl_2/H_2O, 65%; (d) Cl_3CCN, DBU, CH_2Cl_2, 0^{o} C to rt, 90% (α/β = 8/1).

The glycosyl acceptor was synthesized by anomerization of β-thioglucoside **55** under thermodynamically controlled conditions using one equivalent of $SnCl_4$ at room temperature. This gave a mixture of α- and β-anomers in a ratio of 3:2, and the isolated yield of the desired α-thioglucoside **62** by recrystallization was 36% (Scheme 23). The mother liquor can be re-subjected to the

isomerization process to obtain more of the α-anomer providing a higher yield. Zemplén deacetylation [80] of **62**, followed by protection of the C-4 and C-6 alcohols using benzaldehyde dimethyl acetal yielded the α-thioglucoside **47** [82].The thioethyl group no longer sterically hinders the approach of the electrophile to the C-2 hydroxyl group due to the α configuration. Therefore, C-2 OH is now more reactive than the C-3 alcohol. As a result, selective acylation of **63** at the C-2 hydroxyl group by ADMB chloride in pyridine afforded **64** in 87% yield (Scheme 26).

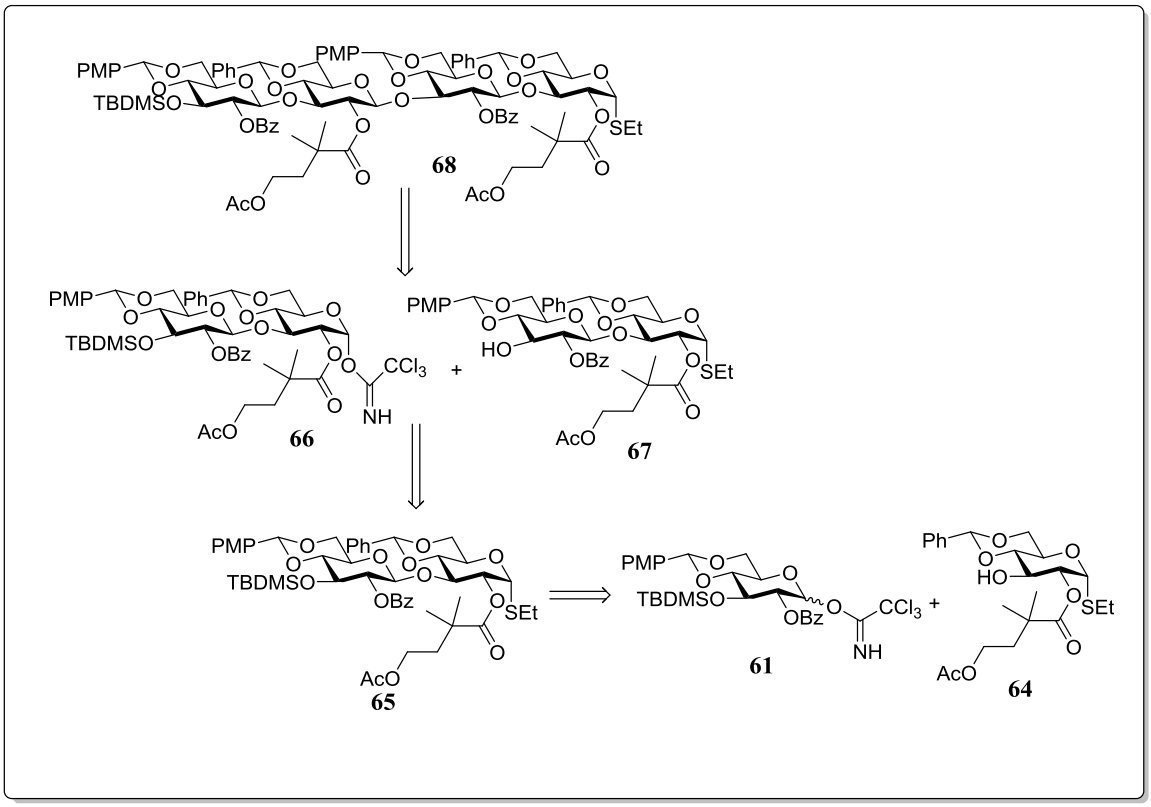


Scheme 26. Reagents and conditions: (a) $SnCl_4$, rt, 36%; (b) i. NaOMe/MeOH, rt, ii. $PhCH(OMe)_2$, TsOH, DMF, 15mmHg, 50^o C, 77% for two steps; (c) ADMB chloride, Py, rt, 87%.

PMP group and the proposed use of the disaccharide building block

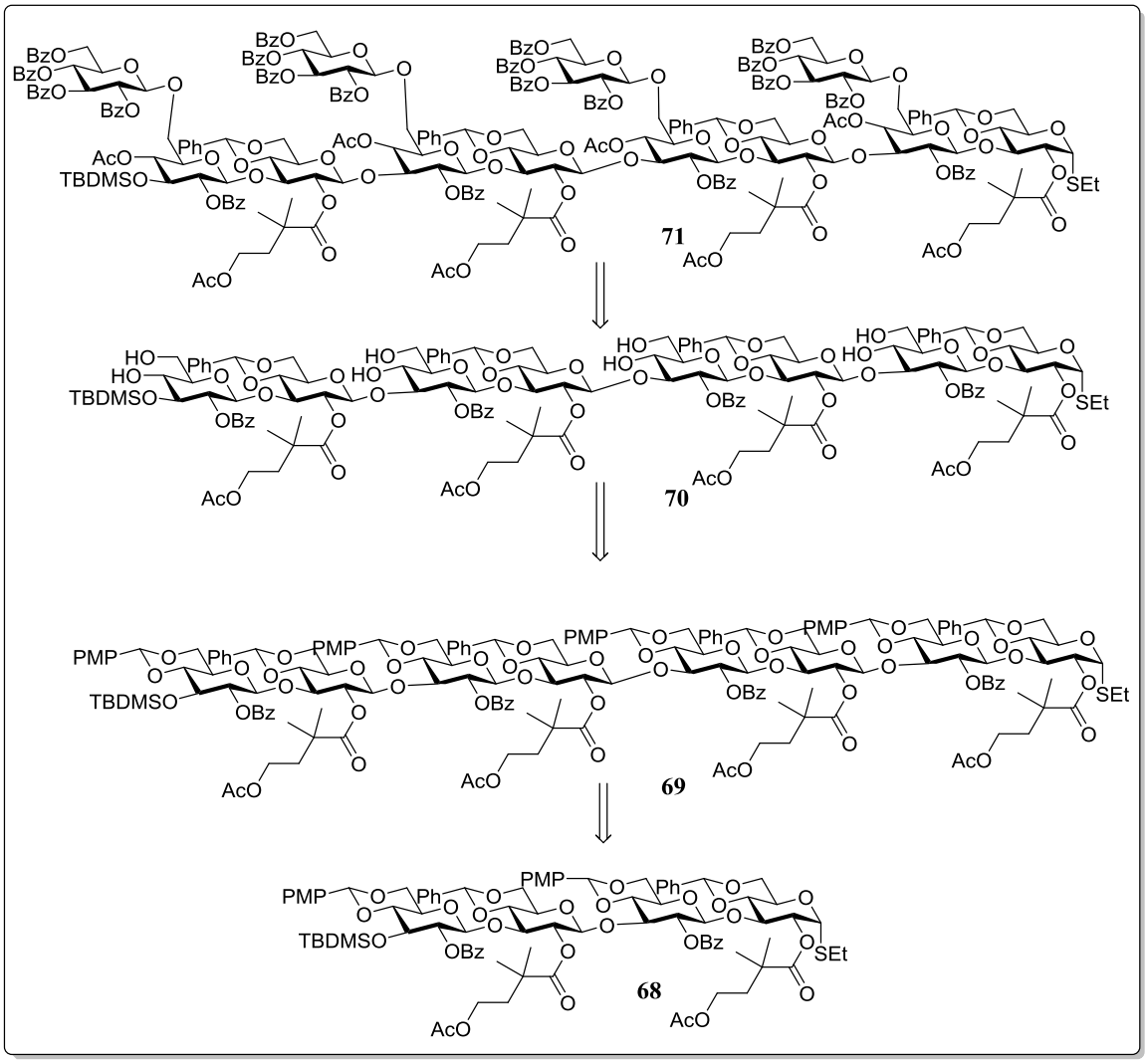
The *p*-methoxybenzylidene (PMP) acetal is a versatile protecting group for diols. This protecting group undergoes acid hydrolysis 10 times faster than the customary benzylidene group [83]. Thus, if the PMP groups can be removed without affecting the benzylidene group, then the disaccharide containing one PMP group and one benzylidene group can be used as a building block for synthesis of branched oligosaccharides.

The proposed synthetic strategy is shown in Scheme 27. The disaccharide building block **65** was synthesized by coupling of the glycosyl donor **61** and acceptor **64**. After that, the disaccharide **65** can be converted to disaccharide donor **66** and acceptor **67**, and then the tertasaccharide **68** can be formed.



Scheme 27. Retrosynthetic analysis of tertasaccharide **68**.

The octasaccharide **69** can be synthesized in the same manner as the tertasaccharide **68**. After the PMP groups were removed, the branched oligosaccharide **71** could be synthesized in two steps from partially deprotected octasaccharide **70** (Scheme 28).



Scheme 28. Retrosynthetic analysis of branched oligosaccharide **71**.

CONCLUSIONS

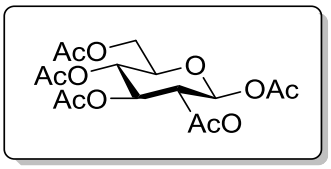
In conclusion, successful synthesis of the β (1, 3) - disaccharide building block for branched oligosaccharide has been carried out. This disaccharide building block is conveniently converted to the disaccharide donor or acceptor forming the tetrasaccharide and octasaccharide. This disaccharide building block containing the 4-acetoxy-2, 2-dimethylbutanoate (ADMB) group makes it a good substrate for coupling reactions due to the exclusive stereoselectivity brought about by neighboring group participation. The more sensitive protective *p*-methoxybenzylidene groups can be removed without affecting the benzylidene group. Using this method, it is possible to synthesize the branched oligosaccharide in a faster, simpler way. This disaccharide building block is valuable for further study.

EXPERIMENTAL

General methods

^{1}H NMR spectra were recorded on a Varian Unity Inova 400 (400 MHz) spectrometer using $CDCl_3$ as solvent unless otherwise noted; multiplicities are quoted as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), doublet of triplets (dt), or multiplet (m). Carbon nuclear magnetic resonance (^{13}C) spectra were recorded on a Varian Unity Inova 400 (100 MHz) spectrometer using $CDCl_3$ unless otherwise noted. Spectra were assigned using COSY, DEPT and HMQC experiments. All chemical shifts are quoted on the δ-scale in parts per million (ppm). Residual solvent signals were used as an internal reference. Low- and high-resolution (HRMS) electrospray (ESI) mass spectra were recorded using a Bruker Microtof Electrospray instrument.

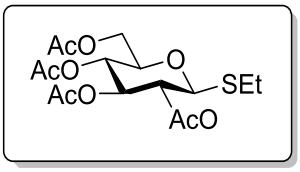
Thin-layer chromatography (TLC) was carried out on Sorbent Technologies UV254 Silica Gel plates. Flash column chromatography was carried out on silica gel (40-63 μm, Sorbent Technologies). Unless otherwise noted, all reactions were carried out in oven-dried glassware with magnetic stirring. Reagents were obtained from commercial sources and used without further purification. Molecular sieves were flame dried under high vacuum prior to use. All solvents were dried and freshly distilled using standard procedures.



8

1, 2, 3, 4, 6-Penta-*O*-acetyl-β-D-glucopyranose

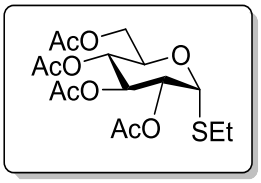
Anhydrous sodium acetate (5.0 g, 0.06mol) was added to a stirred solution of D-glucose (10.0 g, 0.055 mol) in acetic anhydride (67 mL, 0.71 mol) at room temperature. The reaction was heated to 135 ^{o}C under reflux with N_2 atmosphere for 2 hrs, the reaction mixture was poured onto ice (250g) and a white solid formed. The precipitate was filtered and washed with water until an acetic acid smell could no longer be detected. The resulting crude product was purified by recrystallization from 95% ethanol to give **8** (15 g, 65% yield) as a white solid: mp 129-131^{o}C; ^{1}H NMR δ 2.00 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 2.07 (s, 3H), 2.10 (s, 3H), 3.82 (ddd, *J* = 2.4 Hz, 4.4 Hz and 10.0 Hz, 1H, H-5), 4.09 (dd, *J* = 2.2 Hz and 12.6 Hz, 1H, H-6e), 4.27 (dd, *J* = 4.4 Hz and 12.8 Hz, 1H, H-6a), 5.09-5.14 (m, 2H, H-2 and H-4), 5.23 (t, *J* = 9.4 Hz, 1H, H-3), 5.69 (d, *J* = 8.4 Hz, 1H, H-1); ^{13}C NMR δ 20.68, 20.82, 20.93, 61.50 (C-6), 67.77 (C-4), 70.27 (C-2), 72.76 (C-5), 72.84 (C-3), 91.75 (C-1), 169.07, 169.35, 169.50, 170.20, 170.70.



55

Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside

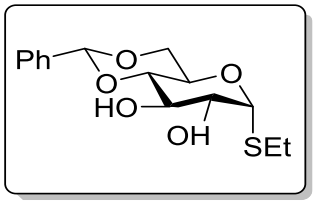
Ethanethiol (2.3 mL, 0.03 mol) and $SnCl_4$ (0.7 mL, 6 mmol) were added to a stirred solution of **8** (9.0 g, 0.023 mol) in anhydrous CH_2Cl_2 (70 mL) at 0 ^oC under N_2 atmosphere. After being stirred at 0 ^oC under N_2 for 3 hrs, the reaction mixture was poured into ice water (100 mL) to quench. The organic layer was separated, washed successively with saturated $NaHCO_3$ (3 × 100 mL), water (2 × 100 mL) and brine (50 mL), and dried over Na_2SO_4. Removal of solvent under reduced pressure afforded a white solid which was purified by recrystallization from 95% ethanol to give **55** (5.4 g, 60%) as a white solid: mp 79-81^o C; ^{1}H NMR δ 1.25 (t, *J* = 7.4 Hz, 1H), 1.99 (s, 3H), 2.00 (s, 3H), 2.03 (s, 3H), 2.06 (s, 3H), 2.61-2.76 (m, 2H), 3.69 (ddd, *J* = 2.4 Hz, 5.0 Hz and 10.2 Hz, 1H, H-5), 4.11 (dd, *J* = 2.4 Hz and 12.4 Hz, 1H H-6e), 4.22 (dd, *J* = 4.8 Hz and 12.4 Hz, 1H, H-6a), 4.47 (d, *J* = 10.0 Hz, 1H, H-1), 4.99-5.09 (m, 2H, H-2 and H-4), 5.20 (t, *J* = 9.4 Hz, 1H, H-3); ^{13}C NMR δ 14.99, 20.77, 20.80, 20.92, 24.35, 62.30 (C-6), 68.44 (C-4), 69.95 (C-2), 74.04 (C-3), 75.99 (C-5), 83.67 (C-1), 169.59, 170.31, 170.85.



62

Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranoside

$SnCl_4$ (23.8 mL, 0.204 mol) was added to a stirred solution of **55** (80.0 g, 0.204 mol) in anhydrous CH_2Cl_2 (700 mL) at 0 ^oC under N_2. After being stirred at room temperature under N_2 for 24 hrs, the reaction mixture was quenched with ice water (600 mL) and the organic layer was separated, washed successively with saturated $NaHCO_3$ (3 × 600 mL), water (2 × 600 mL) and brine (400 mL), and dried over Na_2SO_4. Removal of solvent under reduced pressure afforded a white solid which was purified by recrystallization from 95% ethanol twice to give **62** (28.8 g, 36%) as a white solid: mp 91-93 ^oC; ^1H NMR δ 1.25 (t, *J* = 7.6 Hz, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 2.05 (s, 3H), 2.07 (s, 3H), 2.48-2.60 (m, 2H), 4.06 (dd, *J* = 2.4 Hz and 12.4 Hz, 1H, H-6e), 4.28 (dd, *J* = 4.8 Hz and 12.4 Hz, 1H, H-6a), 4.42 (ddd, *J* = 2.3 Hz, 4.7 Hz and 10.2 Hz, 1H, H-5), 4.99-5.05 (m, 2H, H-2 and H-4), 5.34 (t, *J* = 9.8 Hz, 1H, H-3), 5.67 (d, *J* = 6.0 Hz, 1H, H-1); ^{13}C NMR δ 14.75, 20.66, 20.71, 20.74, 20.79, 24.25, 61.96 (C-6), 67.52 (C-5), 68.60 (C-4), 70.55 (C-3), 70.72 (C-2), 81.72 (C-1), 169.66, 169.91, 169.95, 170.60.

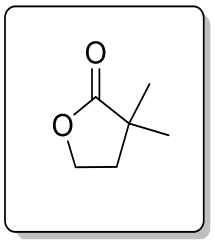


63

Ethyl 4, 6-*O*-benzylidene-1-thio-α-D-glucopyranoside

Catalytic NaOMe/MeOH solution (1.0M, 0.5 mmol) was added to a stirred solution of **62** (5.0 g, 12.7 mmol) in methanol (40 mL) and the reaction mixture was stirred at room temperature overnight. The reaction mixture was neutralized with Dowex 50W-X8 (0.6 g). After filtration, the filtrate was concentrated in vacuo by toluene (3 ×20 mL). The crude product was dissolved in anhydrous DMF (20 mL). Benzaldehyde dimethyl acetal (2.3 mL, 15.2 mmol) and TsOH ·H_2O (20 mg, 0.1 mmol) were added at room temperature. After heating to 50^o C under reduced pressure (15mmHg) for 2 hrs, the reaction was quenched by addition of Et_3N (0.5 mL) and diluted with ethyl EtOAc (40 mL). The organic solution was washed with water (2 × 30 mL) and brine (20 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was recrystallized from CH_2Cl_2/hexanes to give **63** (2.7 g, 68%, 2 steps) as a white solid:: mp 139-141^{o}C; ^{1}H NMR δ 1.29 (t, *J* = 7.6 Hz, 3H), 2.60-2.69 (m, 2H), 3.44 (t, *J* = 9.4 Hz, 1H, H-4), 3.69-3.75 (m, 2H, H-3 and H-6a), 3.81, (dd, *J* = 5.4 Hz and 9.4 Hz, 1H, H-2), 4.16 (dt, *J* = 4.9 Hz and 9.7 Hz, 1H, H-5),

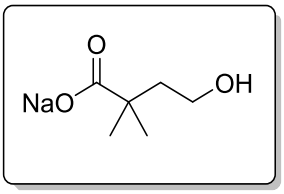
4.23 (dd, *J* = 4.8 Hz and 10.4 Hz, 1H, H-6e), 5.31 (d, *J* = 5.6 Hz, 1H, H-1), 5.49 (s, 1H), 7.34-7.49 (m, 5H), ^{13}C NMR δ 15.42, 25.95, 63.57 (C-5), 68.92 (C-6), 72.22 (C-3), 72.36 (C-2), 81.23 (C-4), 87.35 (C-1), 102.18, 126.55, 128.55, 129.51, 137.14.



51

Dihydro-3, 3-dimethyl-2(3H) furanone (α,α-Dimethyl-γ-butyrolactone)

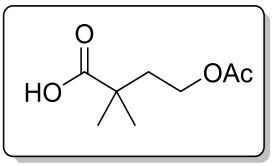
A mixture of γ-butyrolactone (15.4 mL, 0.200 mol) and iodomethane (34.9 mL, 0.560 mol) was added over a period of 45 minutes via a slow drip from a dropping funnel to a vigorously stirred refluxing suspension of NaH (20g, 60% in mineral oil, 0.5 mol) in THF (200 mL). After the addition, the reaction mixture was refluxed for 2 hrs and the reaction was poured into ice water (150 mL) to quench. The solution mixture was acidified with 1M HCl (100 mL), and extracted with CH_2Cl_2 (3 × 400 mL). Removal of solvent under reduced pressure afforded a yellow oil. The crude product was distilled (bp. 10 mmHg, 72-74 ^{o}C) to give pure **51** (12.5 g, 55%) as a colorless oil: ^{1}H NMR δ 1.24 (s, 6H), 2.09 (t, *J* = 6.8 Hz, 2H), 4.24 (t, *J* = 6.8 Hz, 2H).



52

2, 2-Dimethyl-4-hydroxybutanoic acid sodium salt

A solution of **51** (10.0 g, 87.6 mmol) and 1M NaOH (89 mL, 89 mmol) in methanol (50 mL) was stirred at room temperature for 18 hours. Removal of solvent under reduced pressure afforded a white solid residue which was further dried by co-evaporation with toluene (3 ×200 mL) to give **52** (13.5g, 100%) as a white powder. The crude product was used without further purification.

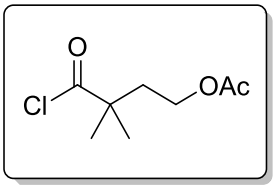


53

4-Acetoxy-2, 2-dimethylbutanoic acid

Acetic anhydride (7.02g, 68.8mmol) was added to a vigorously stirred

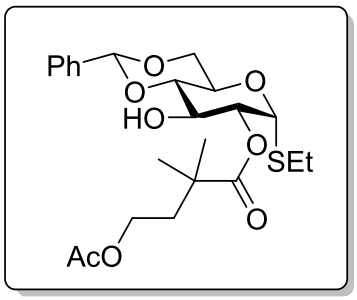
suspension of **52** (5.30 g, 34.4 mmol) and DMAP (0.84 g, 6.9 mmol) in anhydrous pyridine (20 mL) via a slow drip through a dropping funnel at 0 ^{o}C. After being stirred at room temperature for 18 hrs, the reaction mixture was cooled to 0 ^{o}C and acidified with 6 M HCl (30 mL). The resulting solution was saturated with brine (30 mL), and the solution was extracted with CH_2Cl_2 (3 × 200 mL). The combined extracts were washed with brine (2 × 300 mL) and dried over Na_2SO_4. Removal of solvent under reduced pressure afforded a yellow oil which is a mixture of **51** and **53** (1.2: 1). The crude product was dissolved in ether (50 mL) and the resulting organic solution was extracted with saturated $NaHCO_3$ (5 × 60 mL). Removal of the ether solution under reduced pressure afforded **51** (2.12 g) as a yellow oil which could be re-subjected to saponification. The combined aqueous extracts were acidified with 6M HCl until pH ~ 2-3, and the resulting solution was extracted with CH_2Cl_2 (3 × 400 mL). The combined CH_2Cl_2 extracts were dried over $MgSO_4$ and concentrated under reduced pressure to give **53** (2.69, 45%) as a yellow oil. The crude product was used without further purification. ^{1}H NMR δ 1.23 (s, 6H), 1.92 (t, *J* = 6.9 Hz, 2H), 1.99 (s, 3H), 4.12 (t, *J* = 6.9 Hz, 2H).



54

4-Acetoxy-2, 2-dimethylbutyrylchloride [76]

Oxalyl chloride (4.0 mL, 44.2 mmol) was added to a stirred solution of **53** (7.0 g, 42 mmol) in anhydrous benzene (25 mL), followed by catalytic DMF (4 drops). After stirring at room temperature for 1 hr, the reaction mixture was concentrated under reduced pressure to afford a dark brown solution. The crude product was distilled (bp. 0.5mmHg, 72-74 ^{o}C) to give pure **54** (6.13 g, 76%) as a colorless oil: ^{1}H NMR δ 1.33 (s, 6H), 2.01 (s, 3H), 2.03 (t, *J* = 6.5 Hz, 2H), 4.14 (t, *J* = 6.5 Hz, 2H).



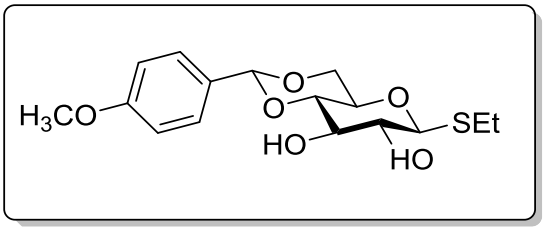
64

Ethyl 2-*O*-(4-acetoxy-2, 2-dimethylbutanoyl)-4, 6-*O*-benzylidene-1-thio-α-D-glucopyranoside

4-Acetoxy-2, 2-dimethylbutyryl chloride (1.2 g, 6.2 mmol) was slowly added to a stirred solution of the diol **63** (1.71 g, 5.45 mmol) in anhydrous pyridine (15 mL) at

0 ^{o}C. The reaction mixture was stirred at room temperature for 24 hrs. Upon removal of solvent under reduced pressure, the residue was dissolved in ethyl acetate (150 mL)

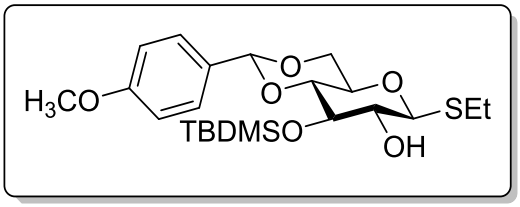
and water (30 mL). The organic layer was washed with saturated $NaHCO_3$ (2 × 130 mL), brine (100 mL), and dried over Na_2SO_4. Removal of the solvent under reduced pressure afforded a yellow oil. The crude product was purified by flash chromatography over silica gel (EtOAc/hexanes; 1/3) to give pure **64** (2.20 g, 87%) as a white solid: mp 75-77 ^oC; R_f = 0.27 (EtOAc/hexanes; 1/3); ^1H NMR δ 1.21 (s, 3H), 1.23 (t, *J* = 7.4 Hz, 3H), 1.27 (s, 3H), 1.75-1.82 (m, 1H), 2.00 (s, 3H), 2.02-2.10 (m, 1H), 2.47-2.58 (m, 2H), 3.19 (d, *J* = 2.8 Hz, 1H, hydroxyl proton) 3.57-3.81 (m, 1H, H-4), 3.75-3.81 (m, 1H, H-6a), 3.98-4.05 (m, 1H, one of methylene protons in AcO-CH_2), 4.11 (dt, *J* = 2.7 Hz and 10.4 Hz, 1H, H-3), 4.18-4.30 (m, 3H, H-5, H-6e and one of methylene protons in AcO-CH_2), 4.90 (dd, *J* = 5.8 Hz and 9.8 Hz, 1H, H-2), 5.55 (s, 1H), 5.61 (d, *J* = 6.0 Hz, 1H, H-1), 7.33-7.37 (m, 3H), 7.48-7.50 (m, 2H); ^{13}C NMR δ 14.94, 20.98, 24.18, 24.63, 26.11, 38.20, 40.67, 61.61, 62.55 (C-5), 68.59 (C-6), 68.87 (C-3), 73.67 (C-2), 81.06 (C-4), 82.39 (C-1), 101.85, 126.38, 128.26, 129.18, 137.12, 171.31, 176.30; ESI-TOF-HRMS calcd for $C_{23}H_{32}O_8SNa$ [M + Na]$^+$ *m/z* 491.1716, found *m/z* 491.1783.



56

Ethyl 4,6-*O*-*p*-methoxybenzylidene-1-thio-β-D-glucopyranoside

Catalytic NaOMe/MeOH solution (1.0M, 2.5 mmol) was added to a stirred solution of **55** (24.4 g, 62.2 mmol) in methanol (200 mL) and the reaction mixture was stirred at room temperature overnight. The reaction mixture was neutralized with Dowex 50W-X8 (3.0 g). After filtration, the filtrate was concentrated in vacuo by toluene (3 ×100 mL). After that, the crude product was dissolved in anhydrous DMF (100 mL). Anisaldehyde dimethyl acetal (12.7 mL, 74.6 mmol) and TsOH Ħ$_2$O (100 mg, 0.5 mmol) were added at room temperature. After heating at 50 ^{o}C under reduced pressure (15mmHg) for 2 hrs, the reaction was quenched by addition of Et_3N (3.0 mL) and diluted with ethyl EtOAc (200 mL). The organic solution was washed with water (2 × 200 mL) and brine (100 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was recrystallized from CH_2Cl_2/hexanes to give **56** (16.4g, 75%, 2 steps) as a white solid: mp 146-150 ^{o}C; ^{1}H NMR δ 1.31 (t, *J* = 7.6 Hz, 3H), 2.71-2.77 (m, 2H), 3.45-3.52 (m, 2H, H-2 and H-5), 3.54 (t, *J* = 9.2Hz, 1H, H-4), 3.73 (t, *J* = 10.0Hz, 1H, H-6a), 3.78 (s, 3H), 3.81 (dt, *J* = 8.8 Hz and 2.4Hz, 1H, H-3), 4.31 (dd, *J* = 10.4Hz and 4.8Hz, 1H, H-6e), 4.45 (d, *J* = 10.0 Hz, 1H, H-1), 5.48 (s, 1H), 6.87 (d, *J* = 8.8Hz, 2H), 7.39 (d, *J* = 9.2Hz, 2H); ^{13}C NMR δ 15.40, 24.85, 55.45, 68.67 (C-6), 70.62 (C-5), 73.32 (C-2), 74.63 (C-3), 80.40 (C-4), 86.57 (C-1), 101.95, 113.85, 127.81,129.53,160.36. (NMR spectrum at the end)

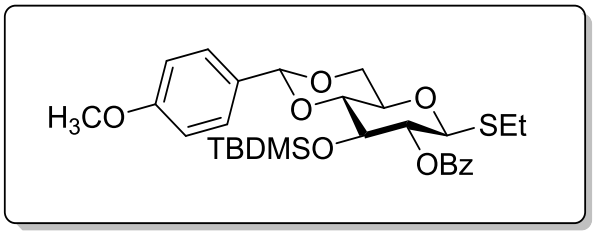


57

Ethyl 4,6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)-1-thio-β-D-glucopyranoside

To a stirred solution of **56** (11.0 g, 32 mmol) and imidazole (3.28 g, 48 mmol) in anhydrous DMF (50 mL) was added *t*-butyldimethylsilyl chloride (5.23 g, 35.2 mmol) in small portions at 0 ^{o}C. After the solution was stirred at room temperature overnight, the reaction mixture was quenched by addition of ethyl acetate (300 mL) and saturated NH_4Cl (100 mL). The organic layer was washed with saturated $NaHCO_3$ (2 × 300 mL) and brine (150 mL), and dried over Na_2SO_4. Removal of solvent under reduced pressure afforded a white solid which was purified by crystallization from hexane to give pure **57** (11 g, 79%) as a white solid: mp 92-95 ^{o}C; R_f = 0.23 (EtOAc/hexanes; 1/8); ^{1}H NMR δ 0.01 (s, 3H), 0.08 (s, 3H), 0.85 (s, 9H), 1.30 (t, *J* = 7.4 Hz, 3H), 2.70-2.76 (m, 2H), 3.40-3.48 (m, 3H, H-2, H-4 and H-5), 3.68-3.75 (m, 2H, H-3 and H-6a), 3.78 (s,3H), 4.29 (dd, *J* = 4.4 Hz and 10.4 Hz, 1H, H-6e), 4.43 (d, *J* = 9.6 Hz, 1H, H-1), 5.45 (s, 1H), 6.86 (d, *J* = 8.8Hz), 7.38 (d, *J* = 8.8Hz); ^{13}C NMR δ -4.58, -4.13, 15.46, 18.50, 24.84, 26.00, 55.43, 68.76 (C-6), 71.00

(C-5), 74.27 (C-2), 76.06 (C-3), 81.25 (C-4), 86.71 (C-1), 101.76, 113.62, 127.64, 129.85, 160.13; ESI-TOF-HRMS calcd for $C_{22}H_{36}O_6SSiNa$ $[M + Na]^+$ *m/z* 479.1892, found *m/z* 479.1886. (NMR spectrum at the end)



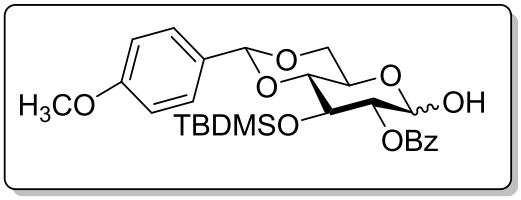
58

Ethyl 2-*O*-benzoyl-4, 6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)-1-thio-β-D-glucopyranoside

Benzoyl chloride (0.7 mL, 6.2 mmol) was added to a stirred solution of the alcohol **57** (1.0 g, 2.2 mmol) and DMAP (133 mg, 1.1 mmol) in anhydrous pyridine (10 mL) at 0^o C. The reaction mixture was heated to 80 ^{o}C for 24 hrs under N_2. After removal of solvent under reduced pressure, the residue was dissolved in EtOAc (50 mL) and extracted with saturated NH_4Cl (20 mL). The organic layer was washed with saturated $NaHCO_3$ (2 × 50 mL), brine (30 mL), and dried over Na_2SO_4. Removal of solvent under reduced pressure afforded a yellow oil which was purified by flash chromatography over silica gel (EtOAc/hexanes; 1/15) to give pure **58** (1.2 g, 97%) as a white solid: mp 89-91

^{o}C; R_f = 0.2 (EtOAc/hexanes; 1/14); ^{1}H NMR δ -0.16 (s, 3H), -0.07 (s, 3H), 0.67 (s,

9H), 1.20 (t, *J* = 7.4 Hz, 3H), 2.67-2.74 (m, 2H), 3.52 (dt, *J* = 4.8 Hz and 9.6 Hz, 1H, H-5), 3.60 (t, *J* = 9.2 Hz, 1H, H-4), 3.76 (t, *J* = 10.0 Hz, 1H, H-6a), 3.78 (s, 3H), 4.02 (t, *J* = 8.8 Hz, 1H, H-3), 4.35 (dd. *J* = 4.8 Hz and 10.4 Hz, 1H, H-6e), 4.60 (d, *J* = 10.0 Hz, 1H, H-1), 5.26 (dd, *J* = 8.8 Hz and 10.0 Hz, 1H, H-2), 5.49 (s, 1H), 6.86 (d, *J* =8.8Hz, 2H), 7.40 (d, *J* =8.8Hz, 2H); ^{13}C NMR δ -4.83, -4.00, 14.94, 18.07, 24.08, 25.68, 55.40, 68.74 (C-6), 70.01 (C-5), 73.40 (C-2), 74.36 (C-3), 81.54 (C-4), 84.33 (C-1), 101.90, 113.61, 127.71, 128.11, 128.51, 129.71, 129.99, 130.17, 133.27, 160.17, 165.42; ESI-TOF-HRMS calcd for $C_{29}H_{40}O_7SSiNa$ $[M + Na]^+$ *m/z* 583.2172, found *m/z* 553.2248. (NMR spectrum at the end)

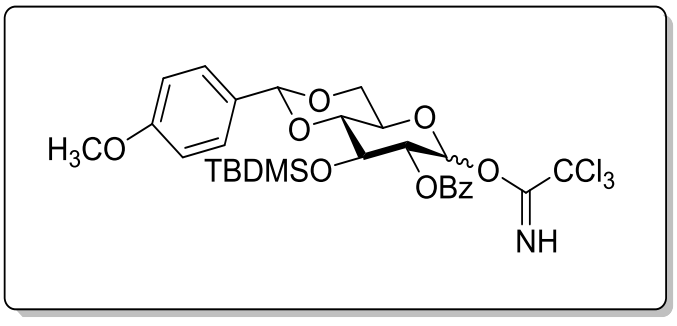


59

2-*O*-Benzoyl-4, 6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)-α/β-D-Glucopyranose

A stirred solution of **58** (300 mg, 0.53 mmol) and 1-benzenesulfinyl piperidine (**BSP**, 140mg, 0.64mmol) and 2, 6-Di-tert-butylpyridine (350uL, 1.55mmol) in wet CH_2Cl_2 [H_2O-CH_2Cl_2 (1/200, v/v)] (5 mL) was kept at -42 ^oC for 15 min. Then Tf_2O (180uL, 1.06mmol) was added in one portion and the reaction mixture was stirred at the same temperature for 1 and half hours. After the reaction was completed,

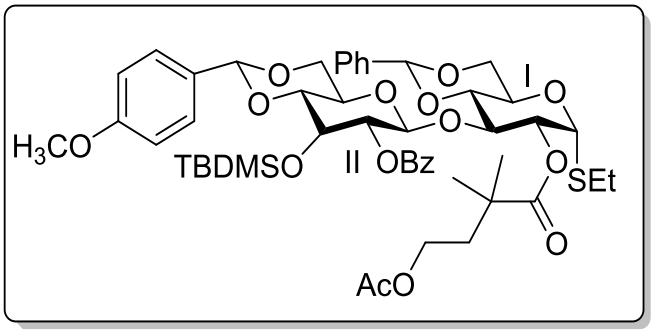
saturated $NaHCO_3$ was added to quench the reaction. After warmed to rt, the reaction mixture was washed with saturated $NaHCO_3$ (2 × 50 mL) and brine (30mL), and dried over Na_2SO_4. Removal of solvent under reduced pressure afforded a yellow oil which was purified by flash chromatography over silica gel (EtOAc/hexanes; 1/8) to give pure **59** (170 mg, 65%, α/β = 2.1/1) as a white solid: (α anomer) R_f = 0.24 (EtOAc/hexanes; 1/6); ^{1}H NMR δ -0.06 (s, 3H), -0.03 (s, 3H), 0.68 (s, 9H), 3.56 (t, *J* = 9.6 Hz, 1H, H-4), 3.73 (t, *J* = 10.4 Hz, 1H, H-6a), 3.80 (s, 3H), 4.11 (dt, *J* = 4.8 Hz and 10.0 Hz, 1H, H-5), 4.26 (dd, *J* = 4.8 Hz and 10.0 Hz, 1H, H-6e), 4.33 (t, *J* = 9.2 Hz, 1H, H-3), 5.11 (dd, *J* = 3.8 Hz and 9.4 Hz, 1H, H-2), 5.47 (t, *J* = 4.0 Hz, 1H, H-1), 5.50 (s, 1H), 6.87 (d, *J* = 8.8Hz, 2H), 7.41 (d, *J* = 8.8Hz, 2H), 7.48 (t, *J* = 7.6Hz, 2H), 7.60 (t, *J* = 7.6Hz, 1H), 8.07 (d, *J* = 8.0Hz, 2H); ^{13}C NMR ($CDCl_3$) δ -4.70, -4.00, 18.11, 25.74, 55.43, 62.50 (C-5), 69.01 (C-6), 69.57 (C-3), 74.88 (C-2), 82.24 (C-4), 91.24 (C-1), 101.95, 113.71, 127.56, 128.49, 129.45, 129.81, 130.08, 133.45, 160.18, 166.32; ESI-TOF-HRMS calcd for $C_{27}H_{36}O_8SiNa$ $[M + Na]^+$ *m/z* 539.2070, found *m/z* 539.2098. (NMR spectrum at the end)



61

***O*-[2-*O*-benzoyl-4, 6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)]-α/β-D-glucopyranosyl trichloroacetimidate**

To a stirred solution of the hemiacetal **59** (2.4 g, 4.73 mmol) in anhydrous CH_2Cl_2 (20 mL) was added 1, 8-diazabicyclo[5.4.0]undec-7-ene (**DBU**, 50 μL). The resulting solution was cooled to 0 ^oC and trichloroacetonitrile (2.37 mL, 23.67 mmol) was added in one portion. After stirring at room temperature for 5 hrs, the reaction mixture was concentrated under reduced pressure to afford a yellow oil which was purified by flash chromatography over silica gel (EtOAc-hexanes; 1/8) to give a mixture of α- and β-**61** (2.8 g, 90%, α/β =8/1) as a white solid: (α anomer) mp 91-95 ^oC; R_f = 0.37 (EtOAc-hexanes; 1/6); ^1H NMR δ -0.08 (s, 3H), 0.00 (s, 3H), 0.70 (s, 9H), 3.69 (t, *J* = 9.4 Hz, 1H, H-4), 3.77 (t, *J* = 10.4 Hz, 1H, H-6a), 3.80 (s, 3H), 4.06 (dt, *J* = 4.9 Hz and 9.9 Hz, 1H, H-5), 4.32 (dd. *J* = 4.8 Hz and 10.4 Hz, 1H, H-6e), 4.39 (t , *J* = 9.4 Hz, 1H, H-3), 5.32 (dd, *J* = 3.6 Hz and 9.2 Hz, 1H, H-2), 5.53 (s, 1H), 6.54 (d, *J* = 4.0 Hz, 1H, H-1), 6.87 (d, *J* = 8.4Hz, 2H), 7.38-7.43 (m,4H), 7.54 (t, *J* = 7.2Hz, 1H), 8.01 (d, *J* = 7.2Hz, 2H), 8.50 (s, 1H); ^{13}C NMR δ -4.70, -4.01, 18.15, 25.73, 55.44, 65.53 (C-5), 68.81 (C-6), 69.87 (C-3), 73.48 (C-2), 81.69 (C-4), 91.13, 94.35 (C-1), 102.01, 113.74, 128.12, 128.56, 129.24, 129.39, 130.07, 133.54, 160.21, 160.98, 165.83; ESI-TOF-HRMS calcd for $C_{29}H_{36}Cl_3NO_8SiNa$ $[M + Na]^+$ *m/z* 682.1163, found *m/z* 682.1225. (NMR spectrum at the end)



65

Ethyl 2-*O*-(4-acetoxy-2,2-dimethylbutanoyl)-4,6-*O*-benzylidene-3-*O*-[2-*O*-benzoyl-4,6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)-β-D-glucopyranosyl]-1-thio-α-D-glucopyranoside

A mixture of the glucosyl acceptor **64** (912 mg, 1.95 mmol), glucosyl donor **61** (1.42 g, 2.16 mmol) and 4 Å molecular sieves (1.5 g) in CH_2Cl_2 (20 mL) was stirred at room temperature under N_2 for 1 hr. The reaction mixture was cooled to -70^o C and a TMSOTf solution (39 μL in 2.16 mL CH_2Cl_2, 0.1 M, 0.216 mmol) was added. After being stirred at -70 ^{o}C under N_2 for 6 hrs, the reaction mixture was quenched by addition of Et_3N (2 mL). The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure to afford a yellow oil which was purified by flash chromatography over silica gel (EtOAc/hexanes; 1/5) to give pure **65** (1.88 g, 89%) as a white solid: mp 92-95 ^{o}C; R_f = 0.29 (EtOAc/hexanes; 1/4); ^{1}H

NMR δ -0.16 (s, 3H), -0.08, (s, 3H), 0.70 (s, 9H), 1.13 (s, 6H), 1.19 (t, *J* = 7.4 Hz, 3H), 1.83 (dd, *J* = 6.6 Hz and 8.2 Hz, 2H), 2.00 (s, 3H), 2.41-2.53 (m, 2H), 3.41 (dt, *J* = 4.8 Hz and 9.8 Hz, 1H, H-5^{II}), 3.67-3.73 (m, 3H, H-4^{I}, H-4^{II} and H-$6a^{II}$), 3.78 (t, *J* = 10.0 Hz, 1H, H-$6a^{I}$), 3.81 (s, 3H), 3.89 (dd, *J* = 7.4 Hz and 8.6 Hz, 1H, H-3^{II}), 4.03-4.11 (m, 2H, AcO-CH_2), 4.21-4.33 (m, 4H, H-3^{I}, H-5^{I}, H-$6e^{I}$ and H-$6e^{II}$), 4.89 (dd, *J* = 6.0 Hz and 9.6 Hz, 1H, H-2^{I}), 5.02 (d, *J* = 6.8 Hz, 1H, H-1^{II}), 5.15 (t, *J* = 7.0 Hz, 1H, H-2^{II}), 5.17 (s, 1H), 5.54 (s, 1H), 5.61 (d, *J* = 5.6 Hz, 1H, H-1^{I}), 6.88 (d, *J* =9.2 Hz, 2H), 7.39-7.56 (m, 10H), 8.06 (d, *J* = 9.2 Hz, 2H); ^{13}C NMR δ -4.87, -4.16, 14.88, 18.03, 21.17, 24.19, 25.09, 25.21, 25.69, 38.31, 40.82, 55.47, 61.49, 62.88 (C-5^{I}), 66.17 (C-5^{II}), 68.83 (C-6^{I} and C-6^{II}), 73.28 (C-3^{II}), 73.70 (C-3^{I}), 74.06 (C-2^{I}), 75.59 (C-2^{II}), 79.68 (C-4^{I}), 81.15 (C-4^{II}), 82.17 (C-1^{I}), 99.49 (C-1^{II}), 101.42, 101.91, 113.92, 126.37, 128.15, 128.42, 128.44, 129.03, 129.43, 129.85, 129.97, 133.22, 137.31, 160.37, 165.13, 170.97, 176.18; ESI-TOF-HRMS calcd for $C_{49}H_{64}O_{14}SSiNa$ $[M + Na]^+$ *m/z* 989.3785, found *m/z* 989.3756. (NMR spectrum at the end)

REFFERENCES

1. B. Henrissat. *Biochem. J*. **(1991)** 280, 309-316.

2. (a) B.L. Cantarel; P.M. Coutinho; C. Rancurel; T. Bernard; V. Lombard; B. Henrissat. *Nucleic Acids Res*. **(2009)** 37, D233–D238; (b) R. Brunner; W. Wirtz; J.K.C. Rose; A.G. Darbill; F. Govers; D. Scheel; T. Nurnberger. *Phytochemistry*. **(2002)**, 59, 689–696; (c) R. Opassiri; B. Pomthong; T. Akiyama; M. Nakphaichit; T. Onkoksoong; M. Ketudat-Cairns; J. R. Ketudat Cairns. *Biochem J*. **(2007)** 408, 241–249.

3. E. Beutler; G.A. Grabowski, Gaucher disease, in: C.R. Scriver, A.L. Beaudet, D. Valle, W.S. Sly (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, **(2001)**, pp. 3635–3668.

4. T.D. Butters. *Curr Opin Chem Biol* **(2007)** 11, 412–418.

5. H. Dvir; M. Harel; A. McCarthy; L. Toker; I. Silman; A. Futerman; J. Sussman. *EMBO Reports*. **(2003)** 4, 704–709.

6. R. James; K. Cairns; A. Esen. *Cell. Mol. Life Sci.* **(2010)** 67, 3389–3405.

7. A.V. Morant; K. Jørgensen; C. Jørgensen; S.M. Paquette; R. Sánchez-Pérez; B.L. Møller; S.Bak. *Phytochemistry*. **(2008)** 69, 1795–1813.

8. H. Niemeyer. *Phytochemistry*. **(1988)** 27, 3349–3358.

9. J. Poulton. *Plant Physiol.* **(1990)** 94, 401–405.

10. G. Wiese; H.J. Grambow. *Phytochemistry*. **(1986)** 25, 2451–2455.

11. B. Brzobohaty; I. Moore; P. Kristoffersen; L. Bako; N. Campos; J. Schell; K. Palme. *Science.* **(1993)** 262, 1051–1054.

12. K.H. Lee; H.L. Piao; H.Y. Kim; S.M. Choi; F. Jiang; W. Hartung; I. Hwang; J.M. Kwak; I.J. Lee; I. Hwang. *Cell*. **(2006)** 126, 1109–1120.

13. (a) M.K. Bhat; S. Bhat. *Biotechnol. Adv*. **(1997)** 15. 583–620; (b) P. Béguin. *Annu. Rev. Microbiol*. **(1990)** 44, 219–248.

14. H. Gilbert, H. Stalbrand, H. Brumer. *Curr Opin Plant Biol.* **(2008)** 11, 338–348.

15. (a) E. Lymar; B. Li; V. Renganathan. *Appl Environ Microbiol.* **(1995)** 61, 2976 – 2980; (b) K. Igarashi; T. Tani; R. Kawal; M. Samejima. *J Biosci Bioeng.* **(2003)** 95, 572–576; (c) T. Tsukada; K. Igarashi; M. Yoshida; M. Samejima. *Appl Microbiol Biotechnol.* **(2006)** 73, 807–814.

16. F. Wöhler; J. Liebig. *Annu. Pharm.* **(1837)** 22, 1-24.

17. S. He; S.G. Withers. *J. Biol. Chem.* **(1997)** 272, 24864-24867.

18. (a) C.S. Hudson; H.S. Paine. *J.Am.Chem.Soc.* **(1909)** 31, 1242-1248; (b) D.E. Eveleigh; A.S. Perlin. *Carbohydrate. Research*. **(1969)** 10, 87-95.

19. M.P. Dale; W.P. Kopfler; I. Chait; L.D. Byers. *Biochemistry* **(1986)** 25, 2522-2529.

20. Y. Li; L. Chang; H. Shu; J. Chir. *Journal of Chinese Chemical Society.* **(1997)** 44, 81-87.

21. F.W. Dahlquist; T.Rand-Meir; M.A. Raftery. *Biochemistry* (**1969**) 8, 4214-4221.

22. J.K. Lee; A.D. Bain; P.J. Berti. *J. Am. Chem. Soc.* (**2004**) 126, 3769-3776.

23. S. Rosenberg; J.F. Kirsch. *Biochemistry.* **(1981)** 20, 3196–3204.

24. E.B. Golden; L.D. Byers. *Biochimica et Biophysica Acta*. **(2009)** 1974, 1643-1647.

25. A.K. Covington; M. Paabo; R.A. Robinson; R.G. Bates. *Anal. Chem.* **(1968)** 40. 700 – 706.

26. L.A. Berven; S.G. Withers. *Carbohydrate Research.* **(1986)** 156, 282-285.

27. J. L. Xue; S. Cecioni; L. He; S. Vidal; J. Praly. *Carbohydrate Research*. **(2009)** 344, 1646-1653.

28. W. Lu; G. Lin; H. Yu; A. Tong; J. Xu. *Journal of Molecular Catalysis B: Enzymatic.* **(2007)** 44, 72–77.

29. D. Gueyrard; P. Rollin; T. Nga; M. Ourevitch; J. Begue; D. Bonnet-Delpon. *Carbohydrate Research.* **(1999)** 318, 171–179.

30. A. Kankaanperä *Acta Chem. Scand.* **(1969)** 23, 1723-1727.

31. K.B. Schowen, Solvent hydrogen isotope effect, in: R.D. Gandour, R.L. Schowen (Eds), Transition states of Biochemical processes, Plenum, 225-283 **(1978)**.

32. E.A. Avegno; S.J. Hasty; A.R. Parameswar; G.S. Howarth; A.V. Demchenko; L.D. Byers. *Archives of Biochemistry and Biophysics.* **(2013)** 537, 1-4.

33. D. Williams; D. Lowman; H. Ensley. *Toxicology of 1→3-Beta-Glucans, S. Young; V. Casranova, CRC Press*. **(2005)** pp 1-34.

34. M. Novak; V. Vetvicka. *Jounal of Immunotoxicology*. **(2008)** 5, 47-57.

35. (a) J. Bohn; J. BeMiller*;* *Carbohydrate Polymers*. **(1995)** 28, 3-14;
(b) R. Kollár; B. Reinhold; E. Petráková; H. Yeh; G. Ashwell; J. Drgonová; J. Kapteyn; F. Klis; E. Cabib. *J. Biol. Chem*. **(1997)** 272, 17762-17775;
(c) V. Lépagnol-Descamps; C. Richard; M. Lahaye; P. Potin; J. Yvin; B. Kloareg. *Carbohydr. Res*. **(1998)** 310, 283-289.

36. Y. Kim; E. Kim; C. Cheong; D. Williams; C. Kim; S. Lim. *Carbohydr. Res.* **(2000)** 328, 331-341.

37. V. Vetvicka; J. Yvin. *International Immunopharmacology.* **(2004)** 4, 721–730.

38. G. Brown; S. Gordon. *Cellular Microbiology.* **(2005)** 7, 471–479.

39. D. Williams; A. Mueller; W. Browder. *J. Endotoxin Res.* **(1995)** 2, 203-208.

40. G. Chihara; E. Mihich. *Advances in Immunopharmacology, L. Chedid, J. Haddends Pergamon Press, New York.* **(1985)** 397-402.

41. V. E. Ooi; F. Liu. *Curr Med Chem*. **(2000)** 7, 715-729.

42. D. Kernodle; H. Gates; A. Kaiser. *Antimicrobial Agents and Chemotherapy.* **(1998)** 42, 545–549.

43. R. Nicolosi; S. Bell; B. Bistrian; I. Greenberg; R. Forse; G. Blackburn. *American Journal of Clinical Nutrition.* **(1999)** 70, 208–212.

44. Y. Kim; K. Kim; H. Choi; D. Lee. *Biotechnological Letters.* **(2005)** 27, 483–487.

45. D. Wei; L. Zhang; D. Williams; I. Browder. *Wound Repair and Regeneration* **(2002)** 10, 161–168.

46. (a) G. Brown; P. Taylor; D. Reid; J. Willment; D. Williams; L. Martinez-Pomares; S. Wong; S. Gordon. *J Exp Med.* **(2002)** 196, 407-412;
(b) G. Brown; J. Herre; D. Williams; J. Willment; A. Marshall; S. Gordon. *J Exp Med.* **(2003)** 197, 1119-1124.

47. J. Willment; S. Gordon; G. Brown. *J Biol Chem.* **(2001)** 276, 43818-43823.

48. P. Taylor; G. Brown; D. Reid; J. Willment; L. Martinez-Pomares; S. Gordon; S. Wong. *J Immunol.* **(2002)** 169, 3876-3882.

49. F. Grunebach; M. Weck; J. Reichert; P. Brossart. *Experimental Hematology.* **(2002)** 30, 1309–1315.

50. E. Adams, P. Rice, B. Graves, H. Ensley, H. Yu, G. Brown, S. Gordon, M. Monteiro, E. Papp-Szabo, D. Lowman, T. Power, M. Wempe, D. Williams. *J Pharmacol Exp Ther.* **(2008)** 325, 115-123.

51. (a) F. Jamois, V. Ferrières, J. Guégan, J. Yvin, D. Plusquellec, V. Vetvicka. *Glycobiology.* **(2005)** 15, 393-407.
(b) K. Mo, H. Li, J. Mague, H. Ensley. *Carbohydrate Research.* **(2009)** 344, 439–447.

52. R. Schmidt, W. Kinzy. *Adv Carbohydr Chem Biochem.* **(1994)** 50, 21-123.

53. B. Ernst, G. Hart, P. Sinaÿ *Carbohydrates in Chemistry and Biology Wiley-VCH 2000.*

54. W. Koenigs, E. Knorr. *Chem. Ber.* **(1901)** 34, 957-981.

55. S. Hanessian, J. Banoub, *Carbohydr Res.* **(1977)** 53, C13-C16.

56. (a) J. Jacquinet, D. Duchet, M. Milat, P. Sinay. *J. Chem. Soc., Perkin Trans. 1* **(1981)** 326-330;
(b) A. Lipták, P. Nánási, A. Neszmélyi, H. Wagner. *Tetrahedron.* **(1980)** 36, 1261-1268.

57. W. Wang, F. Kong. *Carbohydrate Research.* **(1999)** 315, 128–136.

58. R. Ferrier, R. Hay, N. Vethaviyasar. *Carbohydrate Research.* **(1973)** 27, 55-61.

59. G.Veeneman, S. van Leeuwen, J.van Boom. *Tetrahedron Letters.* **(1990)** 31, 1331-1334.

60. H. Lönn. *Carbohydr. Res.* **(1985)** 139, 105-113.

61. H. Yin, T. Lowary. *Tetrahedron Letters.* **(2001)** 42, 5829-5832.

62. H. Lönn. *Carbohydr. Res.* **(1985)** 139, 115-121.

63. D. Crich, Y. Rahaman. *J. Org. Chem.* **(2011)** 76, 8611−8620.

64. R. Schmidt. J. Michel. *J. Angew. Chem. Int. Ed. Engl.* **(1980)** 19, 731 – 732.

65. R. Schmidt. *Angew Chem Int Ed Engl.* **(1986)** 25, 212-235.

66. R. Schmidt, J. Michel. *J. Carbohydr. Chem.* **(1985)** 4, 141–169.

67. R. Schmidt, J. Michel. *Tetrahedron Lett.* **(1984)** 25, 821-824.

68. F. Urban, B. Moore, R. Breitenbacn. *Tetrahedron Lett.* **(1990)** 31, 4421-4424.

69. (a) G. Grundler, R. Schmidt. *Carbohydr Res.* **(1985)** 135, 203-218.
(b) K. Sadozai, T. Nukada, Y. Ito, Y. Nakahara, T. Ogawa, A. Kobata. *Carbohydr Res.* **(1986)** 157, 101-123.

70. Z. Ma, J. Zhang, F. Kong, *Tetrahedron: Asymmetry.* **(2004)** 15, 1517–1525.

71. K. Ohara, C. Lin, P. Yang, W. Hung, W. Yang, T. Cheng, J. Fang, C. Wong . *J. Org. Chem.* **(2013)** 78, 6390−6411.

72. F. Yang, H. He, Y. Du, M. Lu. *Carbohydrate Research.* **(2002)** 337, 1165–1169.

73. Y. Zeng, J. Ning, F. Kong. *Tetrahedron Letters.* **(2002)** 43, 3729–3733.

74. H. Yu, D. L. Williams, H. E. Ensley. *Tetrahedron Letters.* **(2005)** 46, 3417–3421.

75. K. K. Ogilvie, D. J. Iwacha. *Tetrahedron Letters.* **(1973)** 14, 317-319.

76. W. F. Hoffman. *U.S. Patent 4665091*. May 12, **1987**; CA 107: 96514.

77. J. L. Baas, A. Davies-Fidder, H. O. Huisman. *Tetrahedron.* **(1966)** 22, 285.

78. L. B. H. Baptistella, J. F. D. Santos, K. C. Ballabio, A. J. Marsaioli. *Synthesis*. **(1989)** 21, 436-438.

79. P. J. Garegg, J. Kvarnstrom, A. Niklasson, S. Svensson. *J. Carbohydr. Chem.* (**1993)** 12, 933-953.

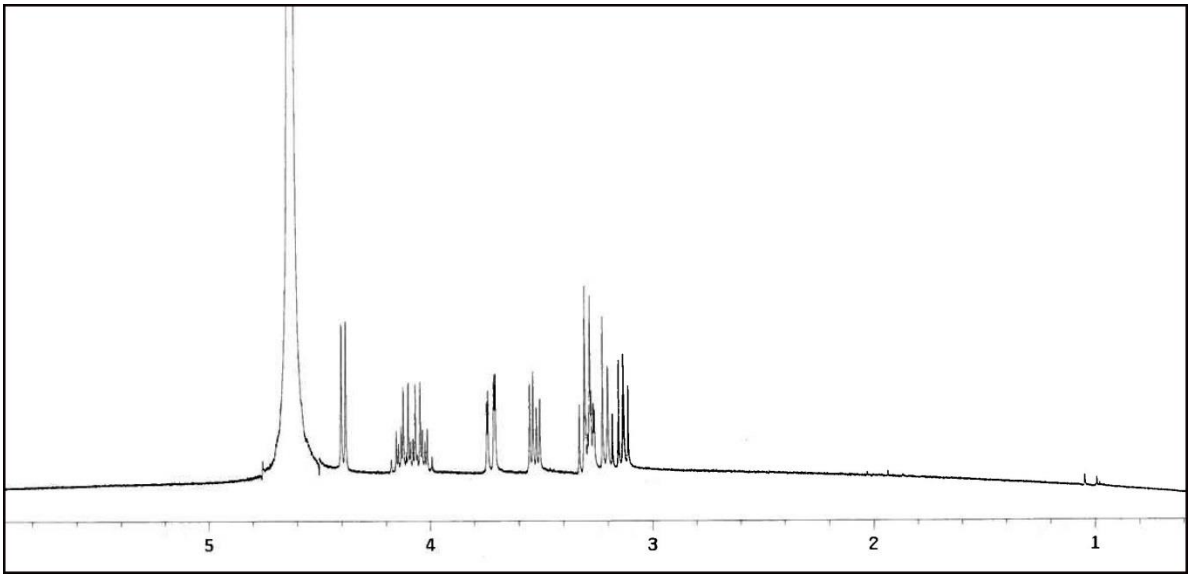
80. G. Zemplén, E. Pacsu. *Ber. Dtsch. Chem. Ges*. **(1929)** 62, 1613.

81. D. Crich, M. Smith. *J. Am. Chem. Soc*. **(2001)** 123, 9015-9020.

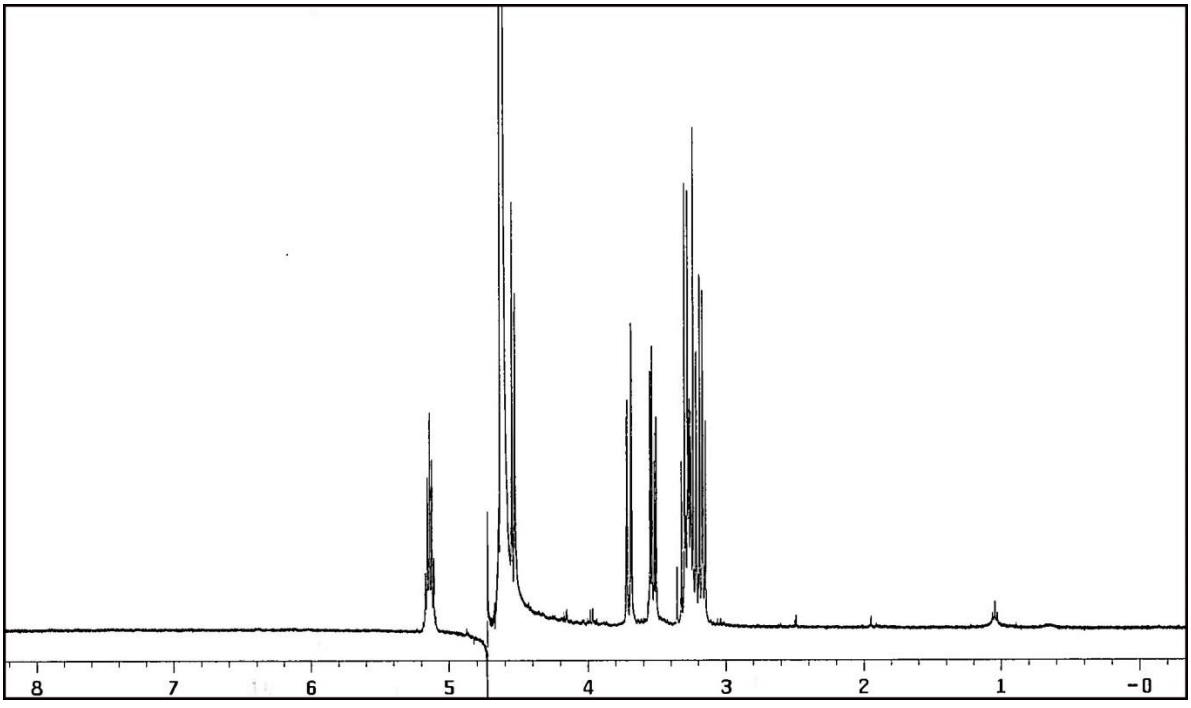
82. K. Takeo, K. Maki, Y. Wada, S. Kitamura. *Carbohydr. Res*. **(1993)** 245, 81-96.

83. M. Smith, D. H. Rammler, I. H. Goldberg, H. G. Khorana. . *J. Am. Chem. Soc*. **(1962)** 84, 430-440.

NMR Data

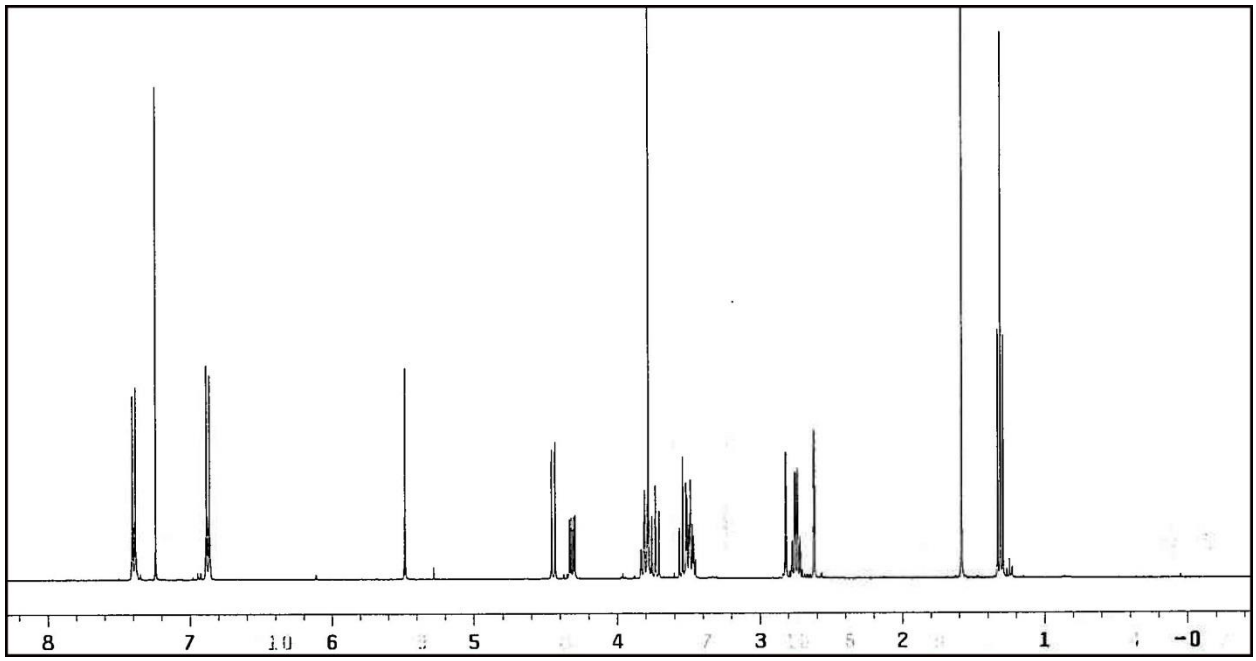


^{1}H NMR: 2, 2, 2-trifluoroethyl β-D-glucopyranoside (10)

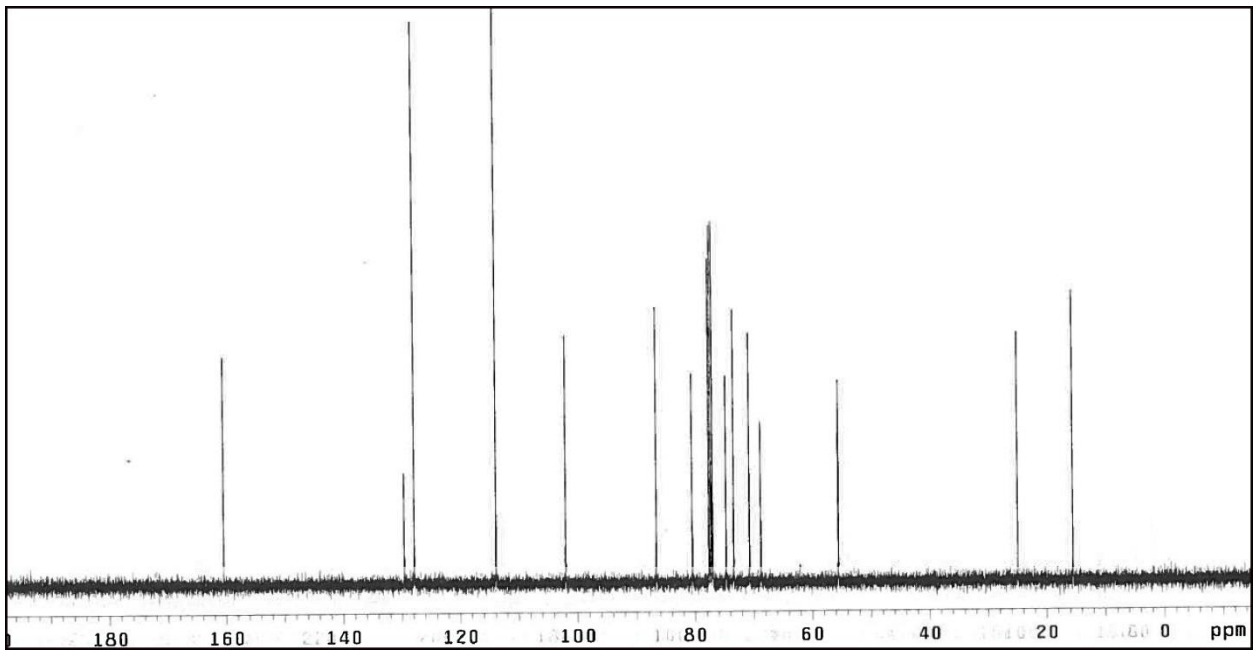


^{1}H NMR: 1, 1, 1, 3, 3, 3-hexafluoro isopropyl -β-D-glucopyranoside (17)

Ethyl 4,6-*O*-*p*-methoxybenzylidene-1-thio-β-D-glucopyranoside (56)

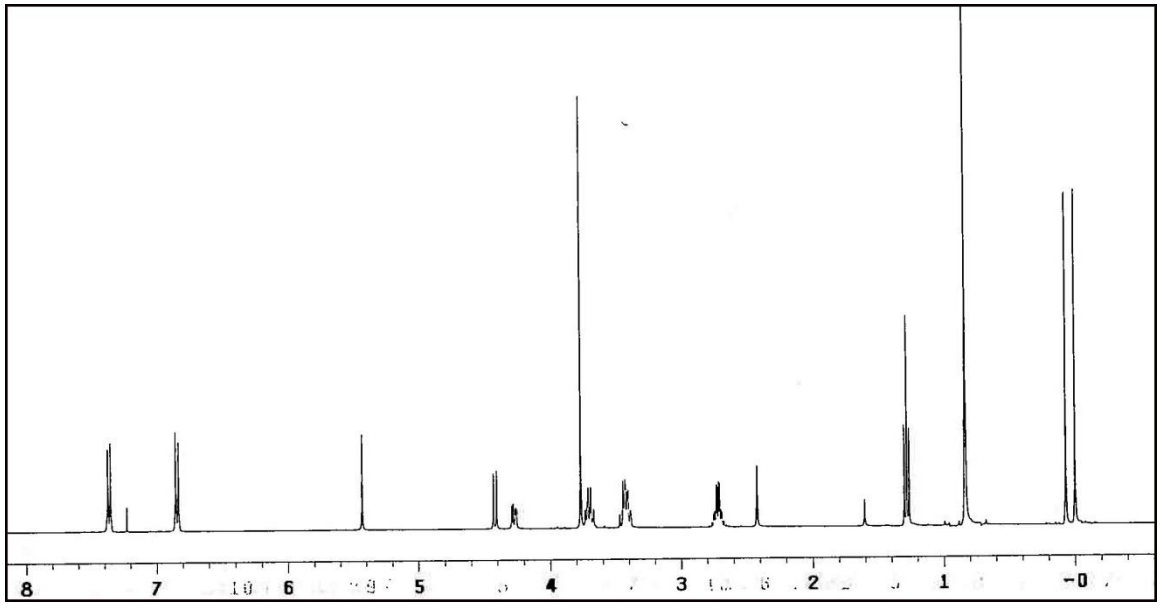


^{1}H NMR

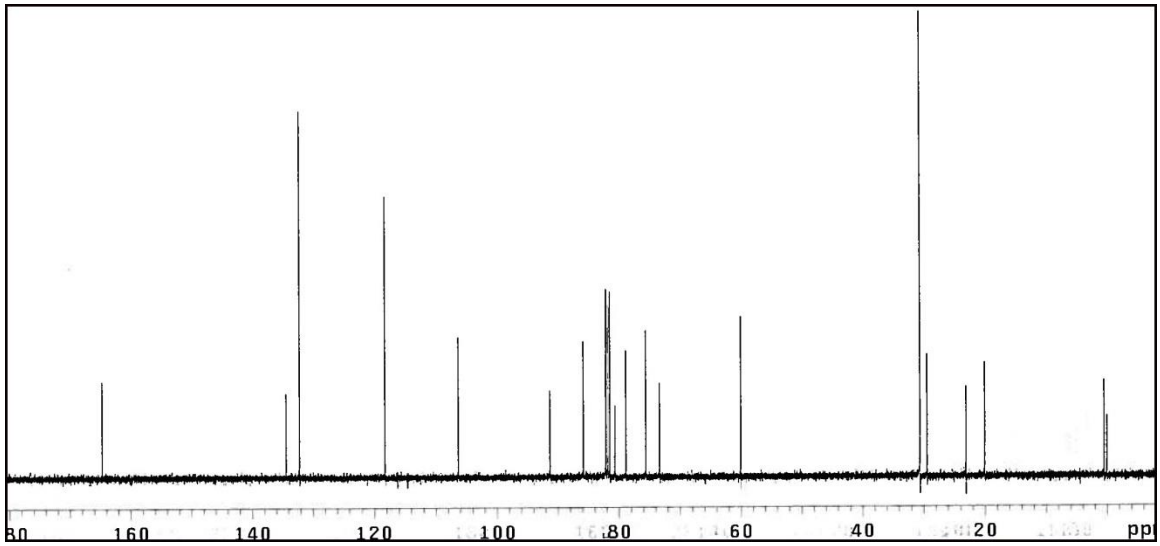


^{13}C NMR

Ethyl 4,6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)-1-thio-β-D-glucopyranoside (57)

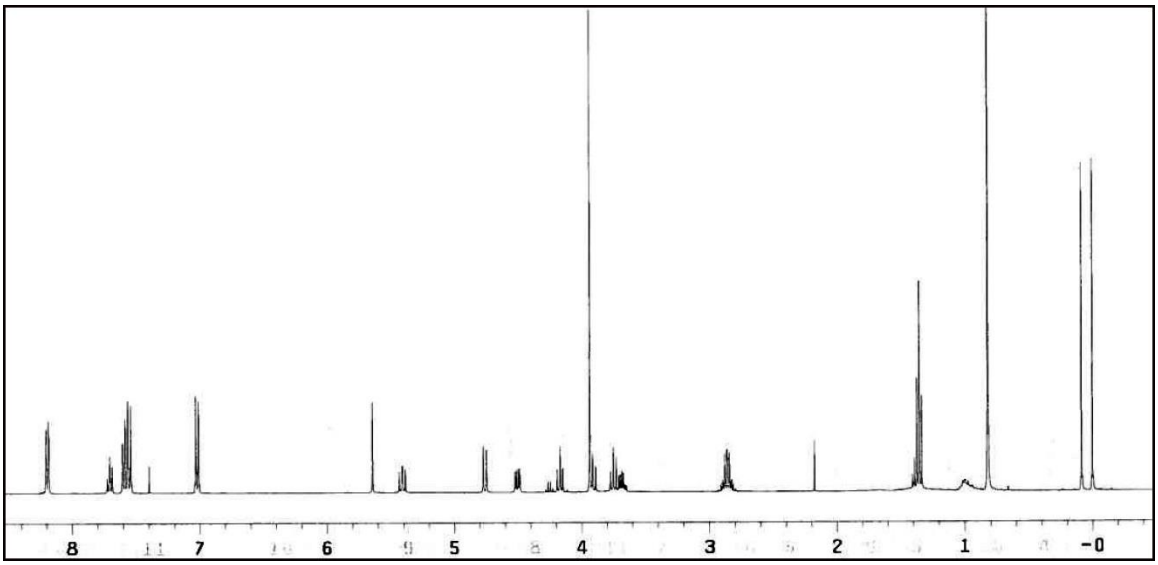


^{1}H NMR

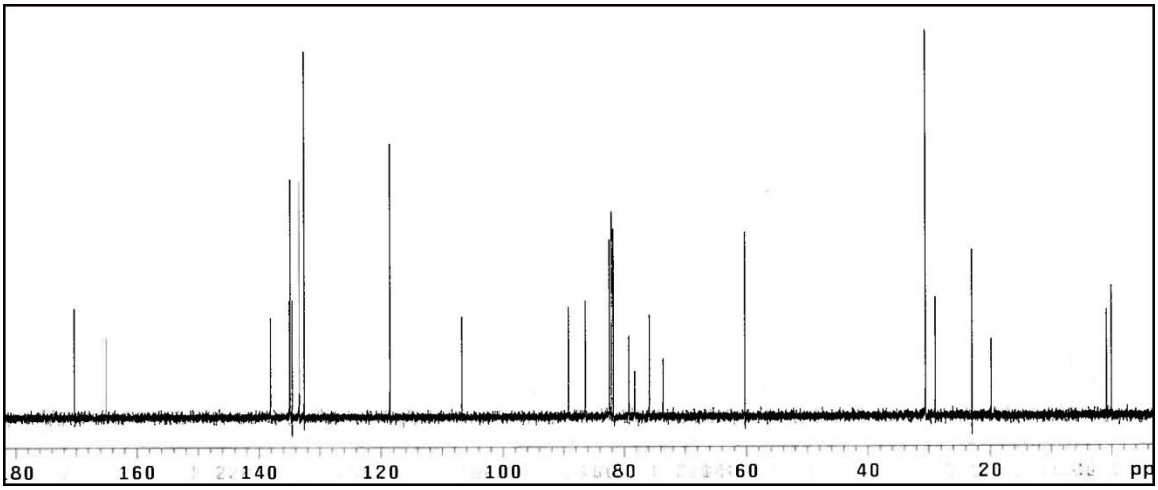


^{13}C NMR

Ethyl 2-*O*-benzoyl-4, 6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)-1-thio-β-D-glucopyranoside (58)

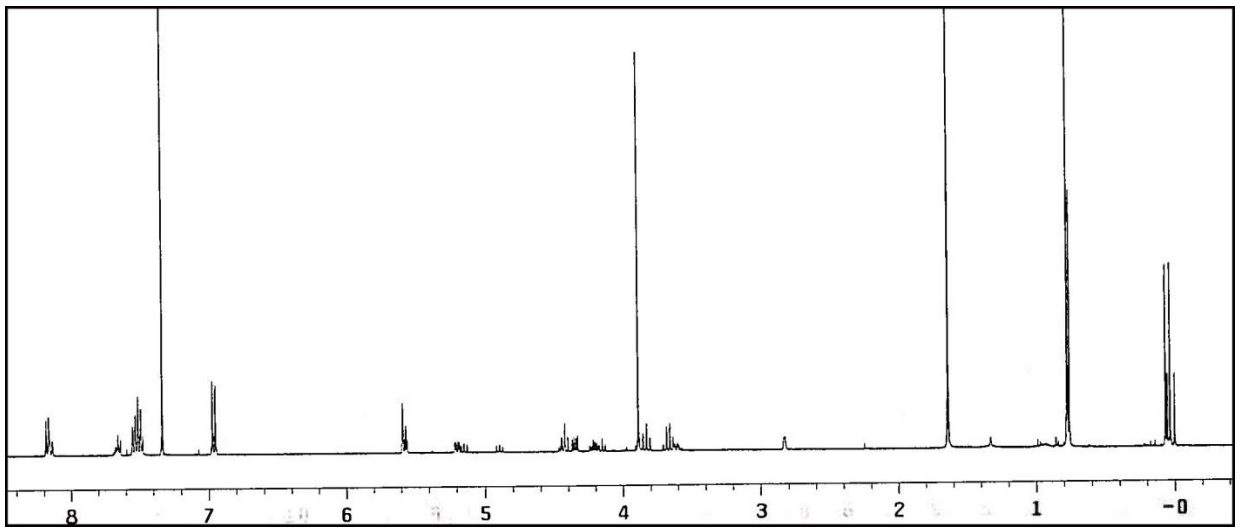


^{1}H NMR

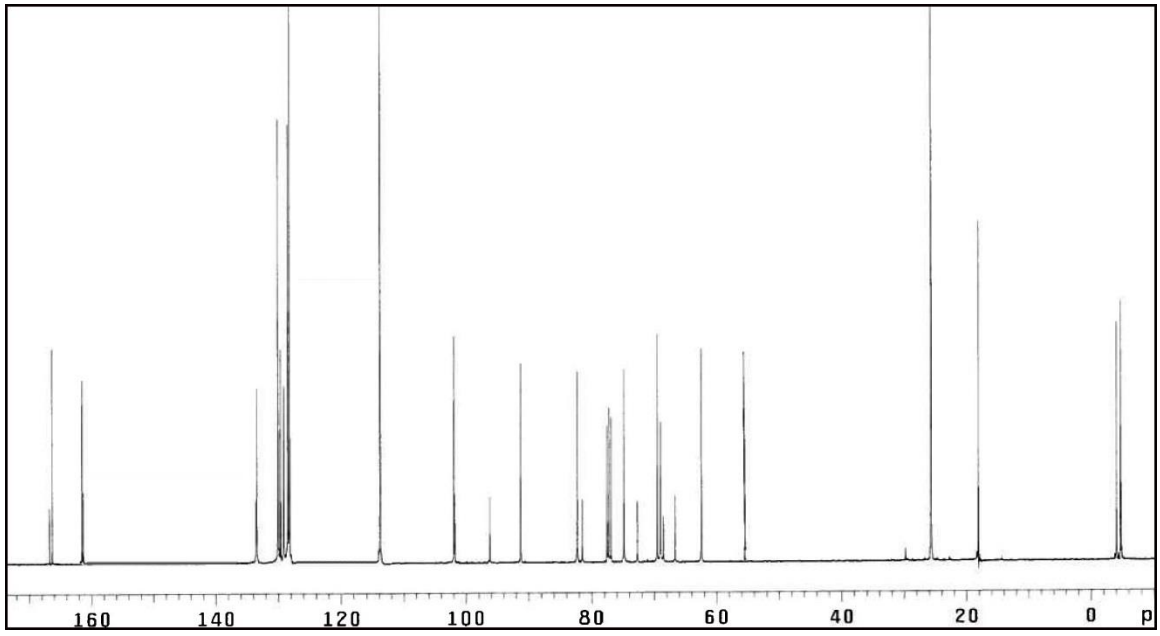


^{13}C NMR

2-*O*-Benzoyl-4, 6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)-α/β-D-Glucopyranose (59)

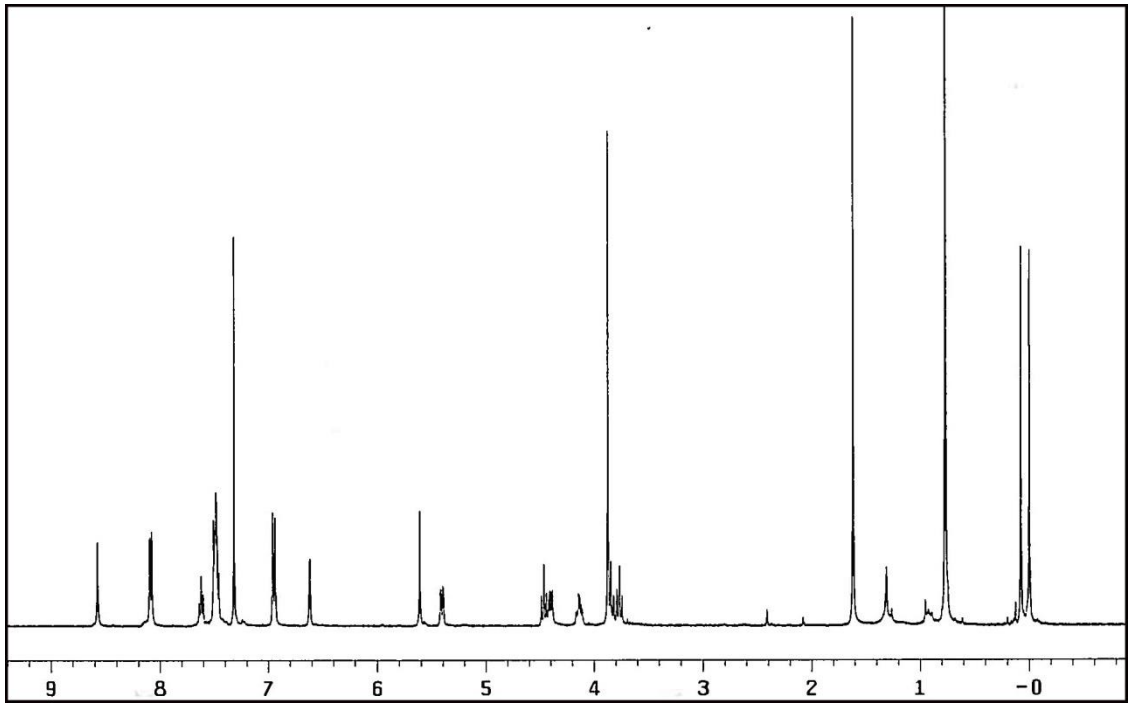


^{1}H NMR

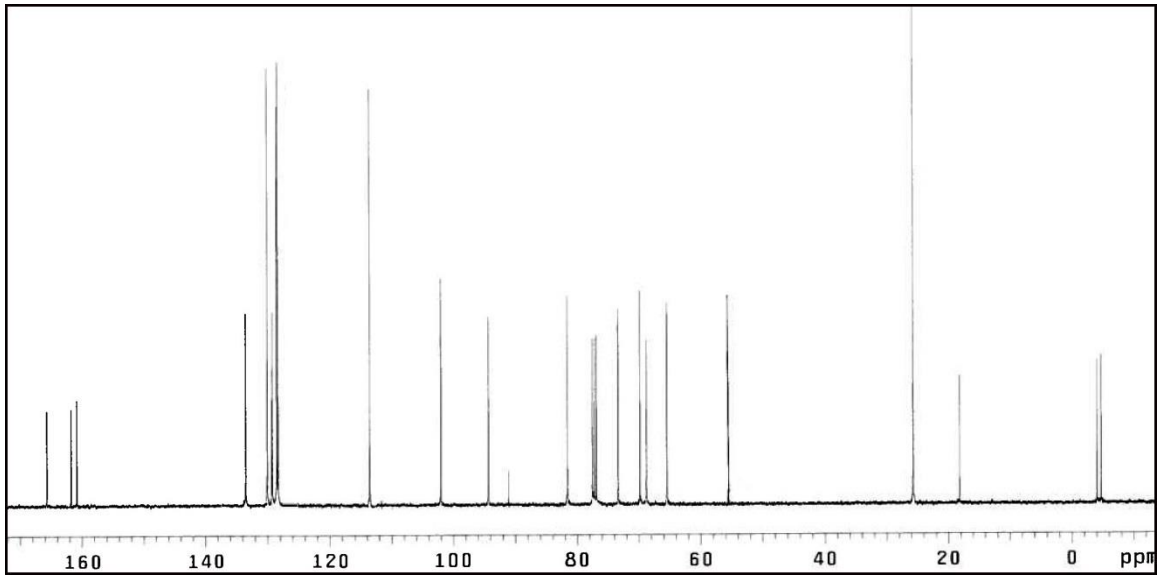


^{13}C NMR

***O*-[2-*O*-benzoyl-4, 6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)]-α/β-D-glucopyranosyl trichloroacetimidate (61)**

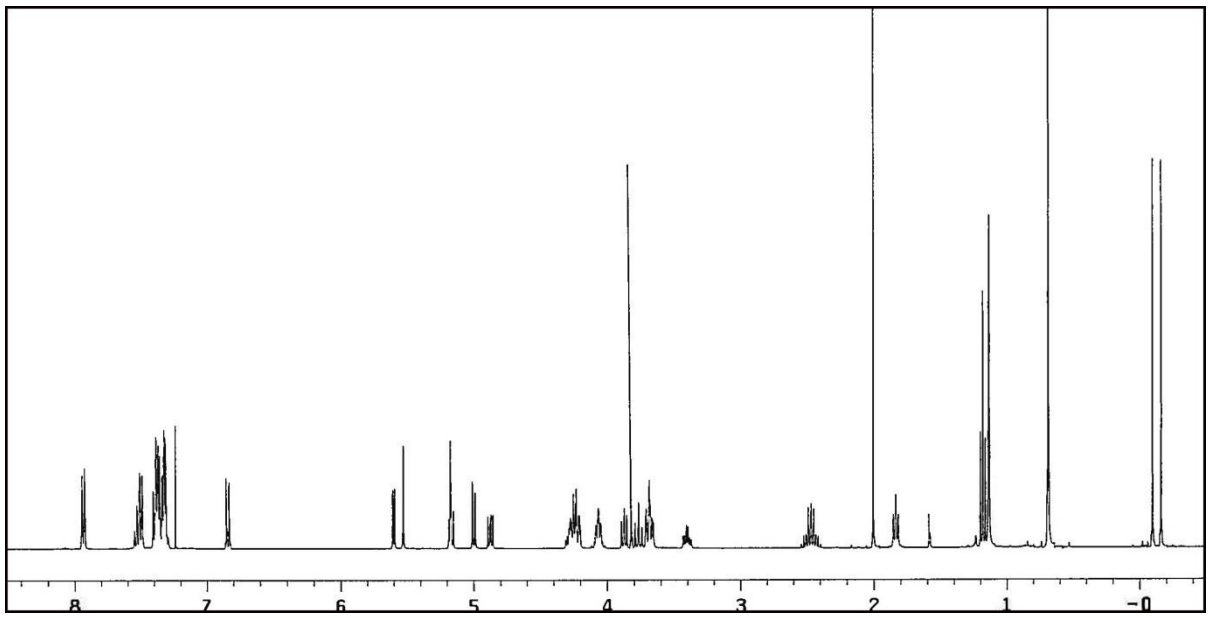


^{1}H NMR

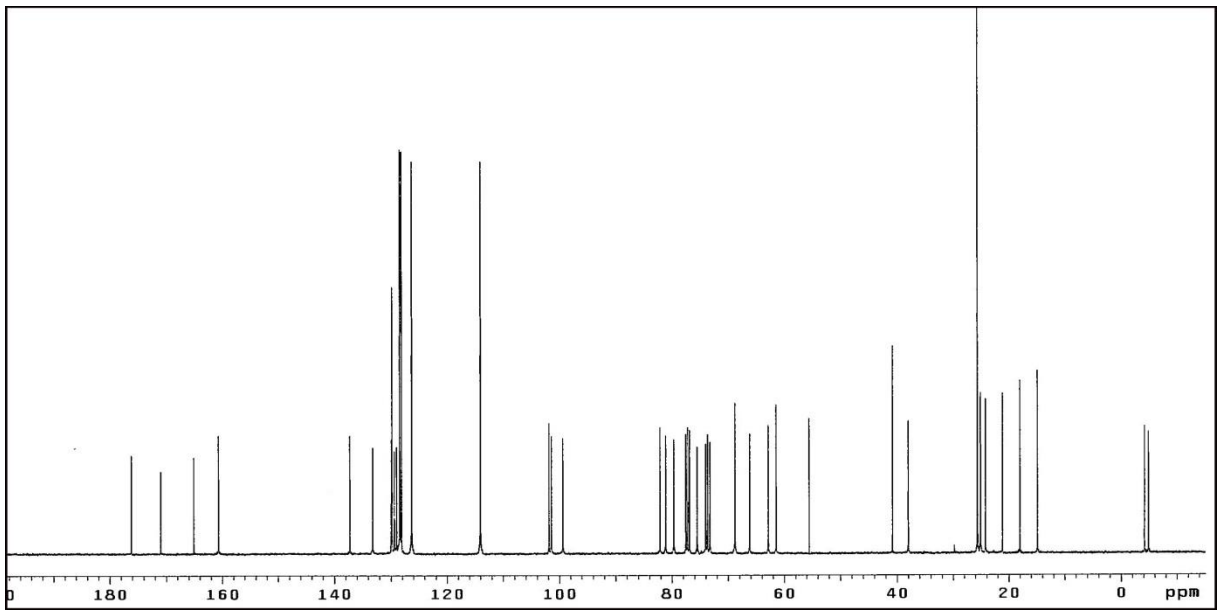


^{13}C NMR

Ethyl 2-*O*-(4-acetoxy-2,2-dimethylbutanoyl)-4,6-*O*-benzylidene-3-*O*-[2-*O*- benzoyl-4,6-*O*-*p*-methoxybenzylidene-3-*O*-(*t*-butyldimethylsilyl)-β-D-glucopyranosyl]-1-thio-α-D-glucopyranoside (65)



^{1}H NMR



^{13}C NMR