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Solution, solid-phase, and fluorous-phase synthesis of carbohydrates and production of carbohydrate microarrays

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Solution, solid-phase, and fluorous-phase synthesis of carbohydrates and production of carbohydrate microarrays

by

Kwang-Seuk Ko

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Chemistry

Program of Study Committee:
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Iowa State University

Ames, Iowa

2006

UMI Number: 3229094

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Kwang-Seuk Ko

has met the dissertation requirements of Iowa State University

Major Professor

For the Major Program

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LIST OF ABBREVIATIONS

A	adenine
ACN	acetonitrile
AcOH	acetic acid
ADP	adenosine 5'-diphosphate
ADP-Glc	adenosine 5'-diphosphate glucose
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CDP	cytidine 5'-diphosphate
CDP-Glc	cytidine 5'-diphosphate glucose
CMP	cytidine 5'-monophosphate
CTP	cytidine 5'-triphosphate
DDQ	2,2-dichloro-5,6-dicyano-*p*-benzoquinone
DNA	deoxyribonucleic acid
DTBP	di-*tert*-butyl pyridne
dTDP	2'-deoxythymidine 5'-diphosphate
dTDP-Glc	2'-deoxythymidine 5'-diphosphate glucose
dTMP	2'-deoxythymidine 5'-monophosphate
dTTP	2'-deoxythymidine 5'-triphosphate
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunoassay
ESI-MS	electrospray ionization mass spectrometry
FMOC	fluorenylmethoxycarbonyl
FT-IR	Fourier transform-infrared
Gal	galactose
GDP	guanosine 5'-diphosphate
GDP-Glc	guanosine 5'-diphosphate glucose
GDP-Man	guanosine 5'-diphosphate mannose
Glc	glucose
Glc-1-P	α-D-glucose-1-phosphate
Glc-6-P	α-D-glucose-6-phosphate
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
DMTCl	dimethyl(methylthio)chloride
DMTST	dimethyl(methylthio)sulfonium triflate
FITC	fluorescein isothiocyanate
GlcNAc	*N*-acetylglucosamine

Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	high pressure liquid chromatography
IBCG	β-iosobutyl-C-galactoside
IDCP	iodonium dicollidine perchlorate
(iPr)$_2$NP(OBn)$_2$	dibenzyl N,N-diisopropyl phosphoramidite
ITC	isothermal titration calorimetry
IPP	inorganic pyrophosphatase
IPTG	β-isopropyl thiogalactoside
MALDI-TOF-MS	matrix assisted laser desorption time-of-flight mass spectrometry
Man	mannose
MAS	magic angle spinning
MAS-CH	magic angle spinning carbon proton
mCPBA	3-chloroperbenzoic acid
MeOTf	methoxymethanesulfonic acid
m/z	mass/charge ratio
NAD$^+$	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide, reduced form
NBS	N-bromosuccinimide
NDP	nucleotide diphosphate
NIS	N-iodosuccinimide
NTP	nucleotide triphosphate
PEG	polyethylene glycol
PCR	polymerase chain reaction
P$_i$	phosphate
PP$_i$	pyrophosphate
RCM	ring-closing metathesis
SDS	sodium dodecylsulfate
SIM	selected ion monitoring
TBDPS	$tert$-butyldiphenylsilyl
TEA	triethylamine
TFA	trifluoroacetic acid
TfOH	trifluormethanesulfonic acid
TIBAL	triisobutylaluminum
TMSOTf	trimethylsilylmethanesulfonic acid
Tris	tris(hydroxymethyl)aminomethane
U	uridine
UDP	uridine 5'-diphosphate

UDP-Gal	uridine 5'-diphosphate galactose
UDP-Glc	uridine 5'-diphosphate glucose
UMP	uridine 5'-monophosphate
UTP	uridine 5'-triphosphate

ABSTRACT

This dissertation describes 1) strategies for the rapid synthesis and arraying of carbohydrates and 2) the synthesis and application of stable carbon-based glycomimetics. An alternative fluorous tag-assisted solution-phase strategy described herein has these advantages of solid-phase synthesis, but requires substantially lower amounts of donor building blocks at each coupling cycle. The requisite fluorous tag is designed not only to aid in saccharide purification but is demonstrated to also allow direct formation of carbohydrate microarrays on fluorous-derivatized glass slides for biological screening with lectins, such as concanavalin A. The noncovalent interactions in the fluorous-based array are even strong enough to withstand the detergents used in assays with the *Erythrina crystagalli* lectin. This work is the first demonstration of surface patterning of compounds based on noncovalent fluorous-fluorous interactions.

The second part of this dissertation describes the chemoenzymatic synthesis of deoxysugar nucleotides and a stable carbasugar nucleotide and the synthesis and use of a stable mimic of isopropylthiogalactoside. The carbaglucose-1-phosphate and deoxyglucose-1-phosphate studies present evidence that sugar nucleotidyltransferases can exercise kinetic discrimination in choosing carbohydrates of comparable binding affinity for catalytic turnover. Synthetic 6-deoxy-, 4-deoxy-, and 3-deoxy-glucose-1-

phosphate and the natural substrate glucose-1-phosphate were tested with a representative prokaryotic glucose-1-phosphate uridylyltransferase from *Escherichia coli* and the analogous eukaryotic enzyme from yeast. The bacterial enzyme shows greater substrate promiscuity than the yeast enzyme for the synthesis of deoxygenated glucose-1-phosphate analogs. The first synthesis of the carbocyclic version of glucose-1-phosphate and its evaluation with these bacterial and yeast sugar nucleotidyltransferases also showed the bacterial enzyme to be more promiscuous. In contrast to results with the eukaryotic enzyme, carbaglucose-1-phosphate serves as a substrate for the bacterial enzyme to provide the carbocyclic uridinediphosphoglucose. This result demonstrates the first chemoenzymatic strategy to this class of glycosyltransferase inhibitors. Replacement of the glycosidic oxygen rather than the ring oxygen imparts stability that can be exploited in the induction of protein expression based on *lac* promoters. To this end, the first synthesis of isobutyl-*C*-galactoside (IBCG) as an isopropylthiogalactoside (IPTG) substitute is reported herein. The more stable *C*-glycoside analogue, IBCG, can serve as a surrogate for the commonly used inducer of protein expression IPTG and shows advantages over IPTG at long protein induction times.

CHAPTER 1

General introduction

1. Dissertation organization

This dissertation is divided into seven chapters. The first chapter is a general literature review that is related to the chemistry discussed in chapters 2 through 6. Chapter 1 discusses a general review and the fluorous-phase versus solid-phase synthesis of bacterial oligoglucosamines with a new method for colorimetric monitoring of the reaction cycles.[1] Solid phase oligoglucosamine synthesis requires excess donor building blocks for high yields, but has advantages over conventional solution-phase synthesis in the ease of intermediate purification and the ability for synthesis automation. An alternative fluorous tag-assisted solution-phase strategy described herein has the advantages of solution-phase synthesis, but requires significantly lower donor building block amounts at each coupling cycle. Chapter 2 was published in the *Journal of American Chemical Society* in 2005.[2] This chapter demonstrates a new simple concept in microarray formation based on noncovalent fluorous-based interactions. A fluorous tail is designed not only to aid in saccharide purification but also to allow direct formation of carbohydrate microarrays on fluorous-derivatized glass slides for biological screening with lectins. Firoz Jaipuri performed the synthesis of derivatized galactose and fucose compounds (described elsewhere) and helped to perform microarray assays. Chapter 3 is published in *The Journal of Organic Chemistry* in 2005.[3] This chapter discusses strategies for the chemoenzymatic synthesis of

deoxysugar nucleotides. Synthetic 6-deoxy, 4-deoxy, and 3-deoxyglucose-1-phosphate and the natural substrate glucose-1-phosphate were tested with a representative prokaryotic glucose-1-phosphate uridylyltransferase from *Escherichia coli*, which is also known to accept thymidine triphosphate, and the comparable eukaryotic enzyme from yeast. I present evidence that this class of enzymes can exercise kinetic discrimination in choosing carbohydrates of comparable binding affinity for catalytic turnover. This section contains an expanded version of the experimental section of the synthetic part from the original paper. Corbin Zea helped with the enzymatic reactions and provided the data of the kinetic performance in this paper. Chapter 4 is published in the *Journal of the American Chemical Society* in 2004.[4] This chapter discusses the surprising bacterial nucleotidyltransferase selectivity in the conversion of carbaglucose-1-phosphate as a substrate for a bacterial enzyme and marks the first chemoenzymatic strategy to this class of glycosyltransferase inhibitors. Corbin Zea also helped with enzymatic reactions and provided the data of the kinetic performance in this chapter. Chapter 5 describes a mass-differentiated library strategy for functional proteomic identification of sugar nucleotidyltransferase activities in cell lysates.[5] Dr. Rahman Mizanur helped with the enzymatic reactions and provided the data of an enzymatic reaction in this chapter. Chapter 6 is published in *Organic Letters* in 2003.[6] This chapter describes the synthesis of isobutyl-*C*-galactoside (IBCG) as an isopropylthiogalactoside (IPTG) substitute for increased induction of protein expression. I show that the more stable *C*-glycoside analogue, IBCG, serves as a surrogate for the commonly used inducer of protein expression IPTG and shows advantages over IPTG at long protein

induction times. Jerrid Kruse contributed some initial experimental strategies for the synthesis of IBCG. Chapter 7 provides general conclusions for the entire dissertation.

2. Importance of carbohydrates

Carbohydrates are the most abundant class of organic compounds on earth.[7] These compounds play important biological roles in cell-surface receptors, intercellular recognition, cell-cell communication, immunological recognition, and protein folding.[8, 9, 10] Oligosaccharides are involved in many cell-cell processes including immune system interactions between saccharides and their receptors.[11] The formation of specific oligosaccharides correlates with a host of diseases from infection to ovarian cancer, cystic fibrosis, respiratory capacity diseases, and diabetes.[12, 13] Carbohydrates are used as therapeutics, for example heparin is an important anticoagulation therapeutic and bacterial surface carbohydrates form glycoconjugate vaccines against meningitis.[14] However, the difficulty in obtaining pure oligosaccharides remains a challenge that limits the study and application of this class of compounds.

3. Iterative chemical oligosaccharide synthesis

To match the achievements of commercial automated solid-phase synthesis of other biopolymers such as nucleic acids and peptides, many scientists have tried to develop simple methods for the iterative synthesis of oligosaccharides. The first report of the solid-phase synthesis of oligosaccharides was described by Frechet and coworkers with the production of a disaccharide and trisaccharide.[15, 16] Solid-phase

synthesis can provide high yields by use of excess donors and has the advantages of ease of purification and synthesis automation when compared to conventional solution-phase methods. After a lull in activity for decades, more recently solid-phase synthesis of oligosaccharides has been automated by conversion of a standard peptide-synthesizer.[17] However, there are still unsolved matters in solid-phase synthesis of oligosaccharides that prevent its widespread use. Monitoring of reaction progress on solid-phase in the context of automation has not been demonstrated and a way to overcome the need for great excesses of amount donor group is needed for practicality.

There are, in principle, four important variables to consider when using an alternative phase for the synthesis of oligosaccharides: 1) the glycosylating agents (donors), 2) linker design, 3) the supporting group, and 4) general analysis of reaction progress (Figure 1.1).
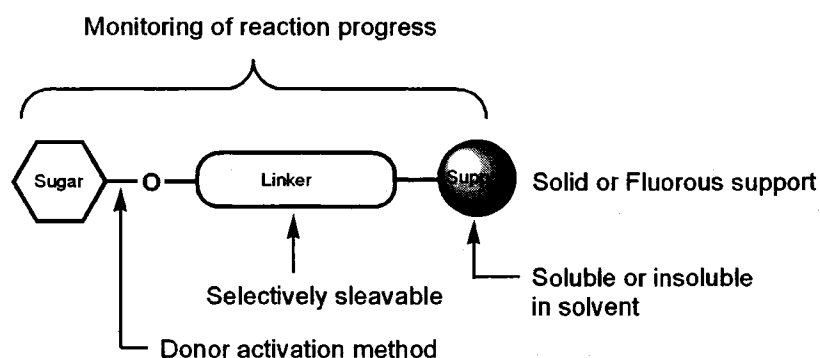

Figure 1.1. General strategy for the synthesis of oligosaccharides on an alternate phase.

3.1 Glycosylating agents (donors)

The generation of an anomeric leaving group for use in glycosyl donor building blocks has been accomplished in a variety of ways (Table 1.1). Glycosyl trichloroacetimidates as developed by Schmidt have emerged as the most commonly used donors due to their easy preparation from O-substituted reducing sugars.[18] Glycosyl trichloroacetimidates can be activated with mild Lewis acids such as BF_3-etherate and TMSOTf.[19] These agents can be introduced without harm to ester, silyl (for example, TBDPS), and any alkyl ether linkages. Other anomeric activating methods are less commonly used. According to Ikegami,[20] glycosyl phosphates can be activated in the presence of an acceptor –OH with TMSOTf. Seeberger and coworkers have recently reported that glycosyl phosphates are activated by catalytic amounts of TMSOTf to react with hindered glycosyl acceptors in high yield.[21] Glycosyl fluorides[22] are used as glycosyl donors and can be activated by strong Lewis acids, such as BF_3 and SiF_4, or organometallic reagents.[23] Thioglycosides[24] are activated by NBS, MeOTf, dimethyl(methylthio)sulfonium triflate (DMTST), iodonium dicollidine perchlorate (IDCP), and NIS. According to Kahne,[25] anomeric glycosyl sulfoxides are highly reactive glycosylating agents upon activation with Lewis acidic promoters such as triflic anhydride. This method has been useful for the glycosylation of unreactive hydroxy groups in acceptors.

Table 1.1. Glycosyl donors with activated reagents.
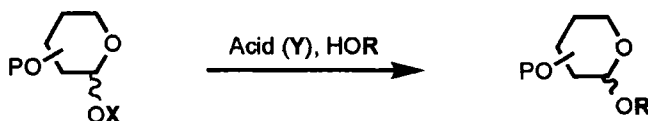
Glycosyl Donors-Leaving Groups (X)	Activated Reagents (Y)	References
Trichloroacetamidates	Lewis acids- BF_3, TMSOTf	19
Phosphates	TMSOTf, BF_4	20, 21
Phosphites	TMSOTf, BF_4	26
Thioglycosides	Thiophilic reagents- NBS, MeOTf, PhHgOTf, NIS, $TrClO_4$, Dimethyl-(methylthio)perchlorate(IDCP), Single electrontransfer reagent	24, 27
Fluorides	Strong Lewis acids- $AgClO_4/SnCl_2$, BF_3, $Cp_2HfCl_2/AgClO_4$	22
Iodides	BF_4, SiF_4	28
n-pentenyl	NIS;TfOH, NIS;TMSOTf	29
Glycals	Electrophilic reangets-Oxocarbenium ion	30
Sulfoxides	Triflic anhydride	25
Glycosyl 2-propenyl carbonates	TMSOTf	31

3.2 Linker design

The linkers that attach the first sugar to the support are crucial for success of solid-phase and related approaches to oligosaccharide synthesis. They should allow easy

attachment of the starting material to the support and be stable under the required reaction conditions. These linkers must enable selective cleavage at the end of the oligosaccharide synthesis without any damage or modification to the product. A variety of linkers have been developed to use many conflicting conditions, a few of which are outlined in Figure 1.2. Silyl ethers can be cleaved under mild acidic conditions.[32] Takahashi and coworkers have reported the compatibility of the silane with a range of different glycosylation agents such as thioglycosides, trichloroacetimidates, sulfoxides, and anomeric fluorides using various acidic activators.[32] For the cleavage of olefins from a support by metathesis the linker is stable under mild reaction conditions which efficiently catalyze olefin metathesis and which are compatible with a range of functional groups. Ring-closing metathesis (RCM) with Grubbs' catalyst was introduced by Schmidt *et al.*[33] This olefin linker has been utilized in the automated chemical synthesis of oligosaccharides on solid-phase by Seeberger and coworkers.[34] Cleavage conditions for alkyl benzyl ethers have been reported by treatment of the resin with triphenylmethylborontetrafluoride (TrBF$_4$).[35] Thiols can be cleaved by NBS in the presence of di-*tert*-butyl pyridine (DTBP).[36] According to Fraser-Reid, a secondary *o*-nitrobenzyl ether linkage can be cleaved from the resin by photolytic cleavage.[37]

(TFA, AcOH)   (TrBF₄ or DDQ)   (NBS, DTBP, MeOH)

(Olefin Metathesis)   (mCPBA)   (NaOMe, MeOH)   (hv)

(PdCl₂, NaOAc, AcOH)   ( ) Linkers can be cleaved by reagents
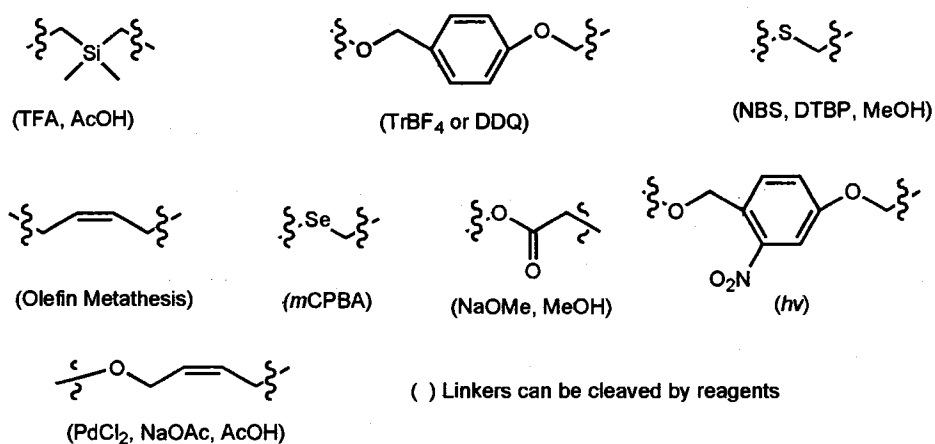
Figure 1.2. Several functional groups used as linkers for attaching sugars to supports such as solid-phase resins.

3.3 Supporting groups

The two types of supports for oligosaccharide synthesis to allow facile mechanical separation of the intermediates from reactants and solvents are 1) insoluble material or 2) soluble materials that can be phase-switched.

Copolymers of styrene with divinylbenzene are commonly used as the support for solid-phase synthesis because these resins are commercially available as beads with a broad choice of functional groups and linkers. Merrifield's resin (polystyrene)[38] has been used the most for solid-phase oligosaccharide synthesis because the required solvents can penetrate into the cross-linked polystyrene. Support swelling behavior is very important for reaction success and minimization of building block usage. Danishefsky and coworkers have reported solid-phase oligosaccharide synthesis using a divinylbenzene polystyrene copolymer as the supporting gourp.[39] Merrifield's resin (1% cross-linked polystyrene) and polystyrene-based Argopore have been used for the only

reported automated solid-phase synthesis of oligosaccharides.[40] Poly (ethylene glycol) (PEG)-polystyrene graft polymers allow increased mobility of intermediates linked to this Tentagel and have more desirable swelling properties.[41] Boons and co-worker have used the glycine-derivatized Tentagel hydroxyl resin for the solid-phase synthesis of trisaccharide libraries.[42] Kahne and coworker also have used the Tentagel resin for parallel synthesis and screening of a solid phase carbohydrate library.[43, 44] Unfortunately, all the solid-phase approaches require large excesses of building block amounts at each coupling cycle and therefore have found limited use.

Supports that allow solution-phase reaction kinetics but that can be switched to another phase for ease of purification can circumvent some of the problems of solid-phase approaches. Janda and coworkers have reported soluble polymer-supported small organic molecule synthesis using poly(ethylene glycol).[45] They mention that this soluble polymer can be easily removed from the unreacted components and be used in automation equipment, although automation has not yet been demonstrated. However, the method is limited by difficulties in reliable and quantitative phase-switching. Curran and co-workers have developed fluorous synthesis as an alternative to solid-phase synthesis for use in combinatorial chemistry and parallel synthesis.[46] Inazu and coworkers have reported a trisaccharide synthesis on a fluorous support in the liquid phase using liquid-liquid extraction to isolate the supported compound from other reagents.[47] Unfortunately, the large fluorous tags required often are not compatible with the range of solvents required in organic synthesis. More recently, solid-phase extraction using modified silica supports have proven useful as they require smaller,

more readily soluble supports. Manzoni and coworker have applied this fluorous-phase strategy to the synthesis of carbohydrates and have recently reported an anomeric silyl fluorous tag for the synthesis of a trisaccharide.[48, 49, 50] This approach has been used with fluorous tags with fluorous silica gel[51] and alkyl tags with C-18 supports.[52]

3.4 Analytical tools for reaction monitoring

Solution-phase supports have the advantage that reactions can be monitored using all the common techniques used in solution-phase organic synthesis. The use of solid-phase supports makes tracking reaction progress much more difficult. Current efforts to solve this problem include the use of FT-IR and FT-Raman spectrometry,[53, 54] solid-state and gel-phase ^{13}C NMR spectrometry, as well as ^1H and ^{13}C correlation NMR spectrometry,[55] high-resolution ^1H MAS (magic angle spinning) and MAS-CH correlation NMR spectrometry,[56, 57, 58, 59] ^{19}F NMR spectroscopy,[60, 61, 62, 63, 64] matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS),[65] and gel-phase ^{19}F NMR spectrometry[66] have been developed as a simple and versatile method for monitoring solid-phase synthesis. Commercial automated DNA and peptide synthesizers rely on the release of spectrophotometric tags such as FMOC and DMT during each deprotection cycle that allows the machines to simply gauge reaction success.[67] Although FMOC has been used in oligosaccharide synthesis,[68] this group has not been incorporated into an automated sequence with reaction monitoring using this tag. Also, unlike peptides and most nucleic acids, oligosaccharides are often branched and therefore a second group that can be used with FMOC is needed to apply

spectrophotometric reaction monitoring successfully to the carbohydrate synthesis problem.

4. Carbohydrate Microarray Formation

Given the difficulty of carbohydrate synthesis, the development of methods to array small quantities of sugars for bioactivity testing has been very active in recent years. Such biochip microarrays have focused on the important roles of sugars in many biological processes, including events of molecular recognition at fertilization, differentiation, and tumor-cell metastasis through carbohydrate-protein interactions.[69-80] Feizi and co-worker developed the first sugar microarray for high-throughput detection and specificity assignments of carbohydrate-protein interactions in 1985.[81] Mrksich and coworkers have reported an example of a carbohydrate chip made by Diels-Alder mediated immobilization of carbohydrates to a self-assembled monolayer presenting benzoquinone and penta(ethylene glycol) groups.[72] Many other methods for preparing carbohydrate microarrays have become available now that rely on either covalent or noncovalent attachment of sugars onto an array support (Figure 1.3).
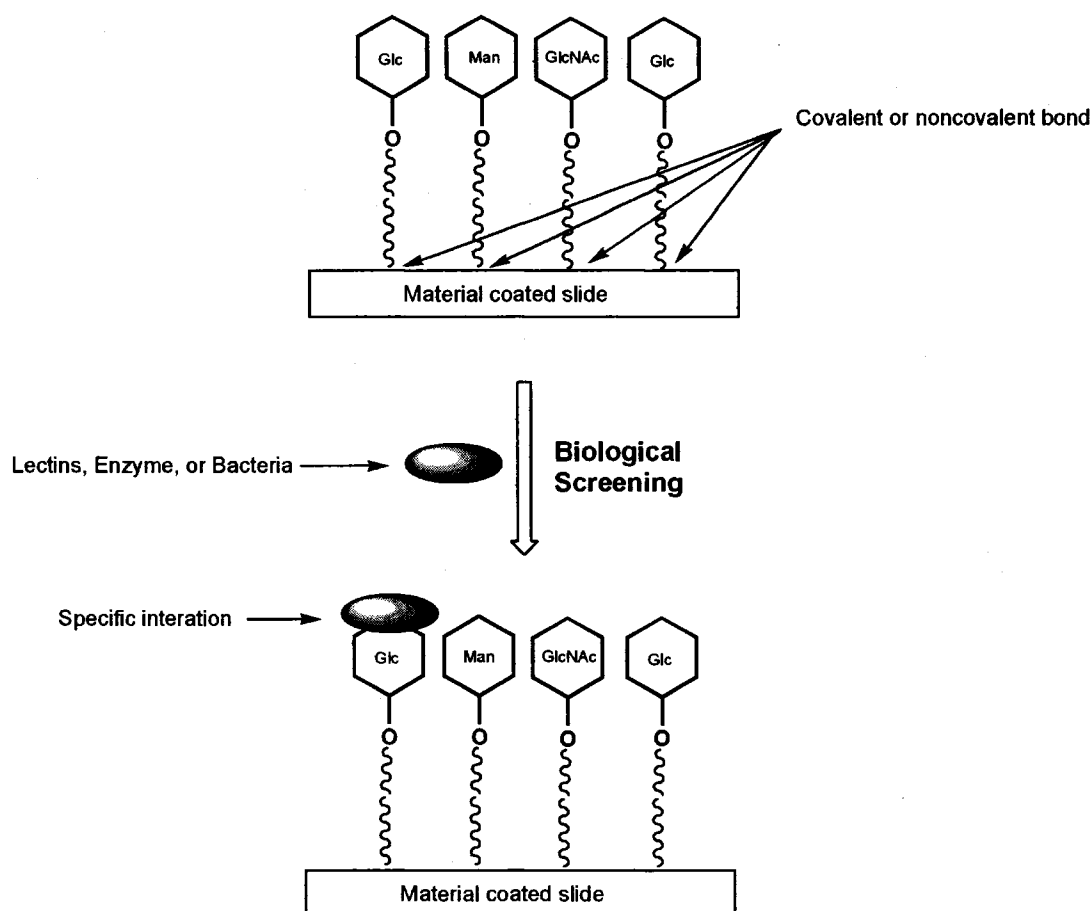
Figure 1.3. General strategy of carbohydrate microarrays.

A noncovalent carbohydrate microarray was described by attachment of relatively large glycoproteins and polysaccharides onto nitrocellulose-coated glass slides by Wang et al.[73] Wong and coworkers have described a different noncovalent attachment strategy for use with monosaccharides in microtiter plates.[75, 80] They have shown that galactosyl lipids containing hydrophobic groups at the anomeric position allow noncovalent binding to microtiter plates. The limitation of this approach is that commonly-used detergents found in assay buffers have to be avoided as they will rinse

away the noncovalently-bound sugars from the plates. Feizi and co-workers have further developed their microarrays of oligosaccharide as neoglycolipids.[82, 83]

Multiple groups have used glass slides modified by thiol ether groups for covalent binding of sugars to solid supports.[84, 85] They have described a method for constructing carbohydrate microarrays that relied on covalent immobilization of carbohydrates on glass slides via chemoselective ligation between maleimide-linked monosaccharides and thiol ether to modified glass surface. Mrksich and coworkers have reported another approach to a microarray of ten monosaccharides on gold-coated glass slides.[72] They have developed self-assembled monolayers of alkanethiolates on gold-coated glass slides in a mixture of alkanethiol and penta(ethylene glycol) groups for immobilizing an array of carbohydrates. One limitation of this work is the many required step to make the penta(ethylene glycol) linkage. To date, no method to form carbohydrate methods has emerged as the clear best solution.

Monitoring of protein binding to the arrayed carbohydrates, however, is almost always carried out by modification of the protein either directly by a dye or indirectly through an antibody-mediated process. For instance, FITC-labeled lectins (carbohydrate-binding proteins) can be monitored using a fluorescent laser scanner.[73, 77, 84] Alternatively, the protein can be detected by standard enzyme-linked immunoassay (ELISA) methods.[75, 85]

5. Enzymatic synthesis of carbohydrates

Enzymes can also be used to make carbohydrate structures and thereby sometimes avoid some of the problems associated with chemical oligosaccharide synthesis. There are three main types of enzymes in carbohydrate chemistry: sugar nucleotidyltransferases, glycosyltransferases, and glycosidases. Although genome sequencing projects have uncovered a range of sequences that could code for proteins involved in carbohydrate biosynthesis, a variety of methods to understand the chemical and biological functions of these carbohydrate-active enzymes and to exploit them for organic synthesis are largely still lacking.[86]
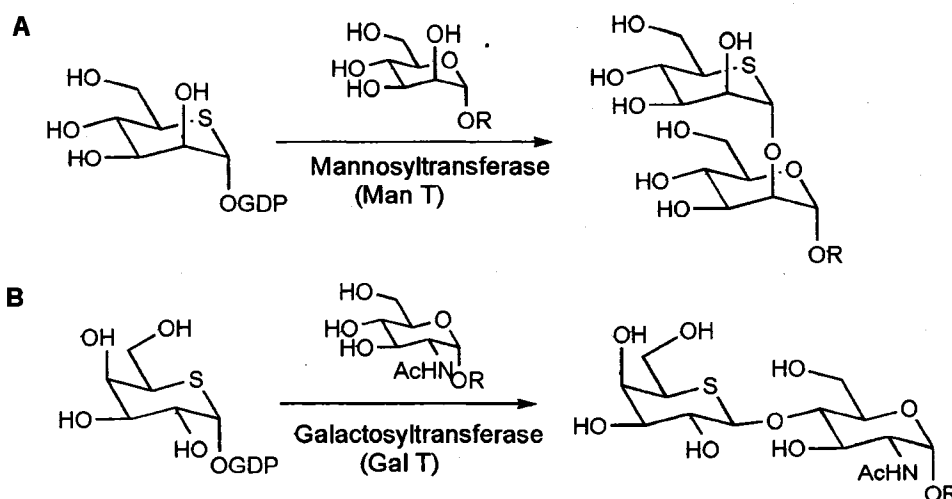
5.1 Sugar nucleotidyltransferases

Sugar nucleotidyltransferases can catalyze the formation of a nucleotide diphosphate (NDP)-sugar from sugar-1-phosphate and nucleotide triphosphates (NTPs) with suitable buffer, metal, temperature, and pH conditions. These activated forms of NDP-sugars produced by these sugar nucleotidyltransferases then serve as glycosyl donors in glycosyltransferase reactions. Sugar nucleotidyltransferases, enzymes expressed in prokaryotic and eukaryotic organisms, catalyze both the forward and reverse reactions shown in Scheme 1.[87]
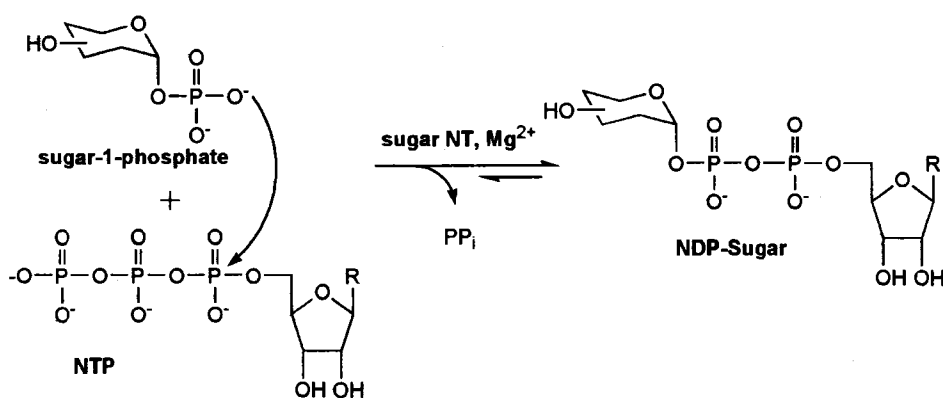
Scheme 1. Sugar nucleotidyltransferases catalyze the synthesis of NDP-sugars from sugar-1-phosphates and NTPs.

5.2 Glycosyltransferases

Glycosyltransferases catalyze the formation of glycosidic linkages in oligosaccharide biosynthesis from activated sugar nucleophiles. Palcic and coworker have shown that synthetic thio-analogs, with sulfur replacing a pyranose oxygen, of GDP-sugars such as GDP-5-thio-mannose (Scheme 1.2, A), GDP-5-thio-galactose (Scheme 1.2, B) and GDP-5-thio-L-fucose are accepted as substrates for glycosyltransferases.[88, 89] These analogs serve as carbohydrate antigens, such as the sialyl-Lex tetrasaccharide, that can be enzymatically prepared and used in studies to develop carbohydrate-based drugs that are more stable to glycosidases, enzymes that cleave glycosidic linkages. Interestingly, the same group chemically prepared an activated galactose substrate with carbon rather than sulfur replacing the ring oxygen.[88] This carbasugar analog served as an inhibitor rather than substrate for the galactosyltransferase.

Scheme 1.2. Biosynthetic method to obtain 5-thiosugar-mimetics with glycosyltransferases.[88, 89]

6. Strategies for the synthesis of sugar nucleotides

Because sugar nucleotides are important as inhibitors or substrates of glycosyltransferases, chemical and enzymatic methods for their synthesis are needed. Several naturally-occurring monosaccharide-1-phosphates are commercially available. However, modified and less common sugar-1-phosphates are not commercially available. To this end, Thorson and coworkers have reported the synthesis of various activated deoxysugars by a bacterial sugar nucleotidyltransferase.[90, 91, 92] Several natural and nonnatural sugar nucleotides have also been synthesized using archaeal enzymes.[93, 94] Although the chemical synthesis of a few NDP-sugars have been reported by some groups,[95] the methods were complicated by the many steps involving protecting and deprotecting groups to make the target molecule. For example, Wong and coworker have reported the chemical synthesis of various NDP-sugars with 1H-tetrazole as

catalyst in phosphomorpholidate coupling reactions, but the reactions required 2-3 days.[96]

7. Glycomimetics: Enhancing carbohydrate stability

The resistance of a glycosidic linkage to enzymatic hydrolysis as well as the search for more chemically and biochemically stable sugar derivatives and carbohydrate-based therapeutics has led to the synthesis of a variety of analogs that replace the glycosidic oxygen with sulfur, nitrogen and carbon. These analogs serve as components of biologically important compounds which can inhibit the growth of malignant or pathogenic cells.[97] There are two important parts to define non-hydrolysable sugar mimetics: 1) replacement of the glycosidic oxygen with S, NH, or CH_2 and 2) replacement of the ring oxygen with one of these groups.

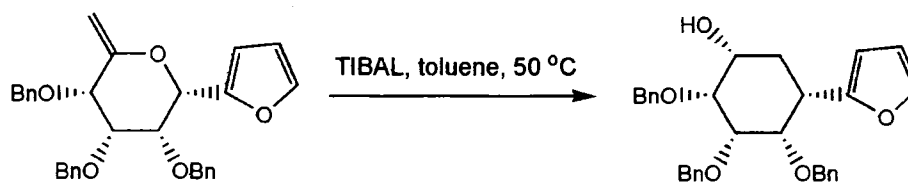## 7.1 Strategies for the synthesis of carbasugars

Sugars whose ring oxygen is replaced with a carbon are known as carbasugars.[98] A few synthetic strategies to carbasugars have been based on the Diels-Alder reaction of a 1,4-disubstituted 1,3-butadiene with a dienophile,[99] radical cyclization,[100] and a Ferrier rearrangement.[101] Ogawa and his coworker have developed the synthesis of racemic carbasugars based on cyclohexane derivatives from the Diels–Alder cycloaddition of furan and acrylic acid.[99] Gomez and coworker have described two synthetic tools based on radical cyclizations as the key step in the formation of the carbasugar cyclohexane

ring and on a stereodivergent strategy for the preparation of carbasugars from a single carbocyclic intermediate as shown in Scheme 1.3.[100, 101]

A

B
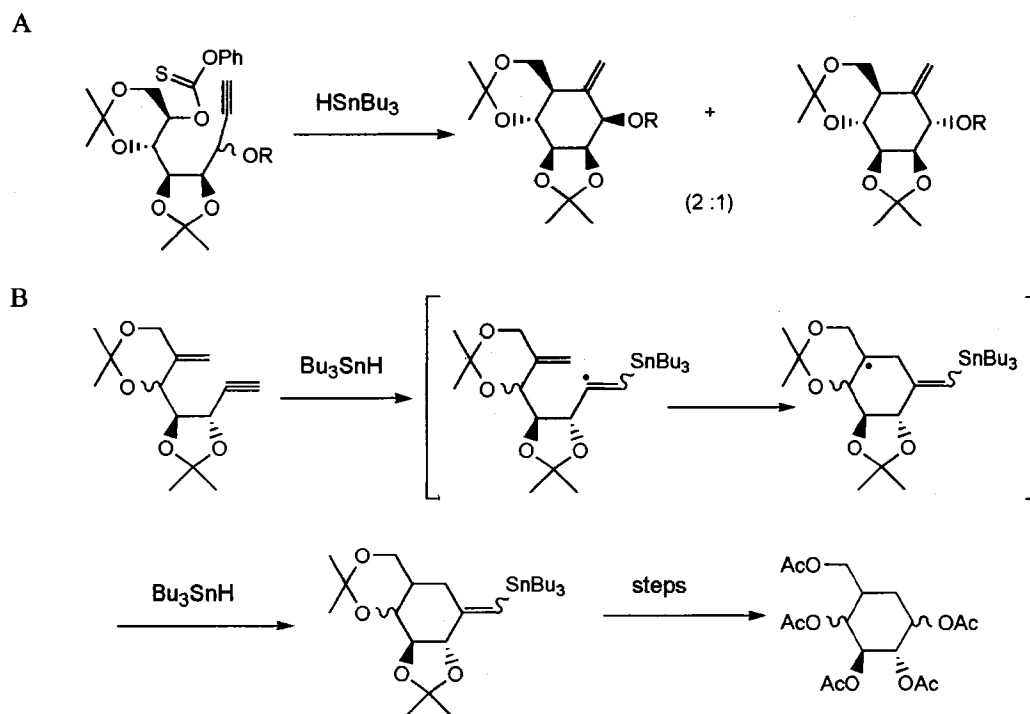
Scheme 1.3. Synthetic strategies based on radical reactions for the production of carbasugars from monosaccharides, A[100]and B[101].

Sinay and coworker have reported the development of the Ferrier rearragement by using the triisobutylaluminum-(TIBAL) promoted conversion of carbohydrates as shown in Scheme 1.4.[102]

Scheme 1.4. Ferrier rearrangement to produce carbasugars.[102]

7.2 Strategies for the synthesis of *C*-glycosides

Cyclic *C*-glycosides can serve as enzyme inhibitors,[103] antimicrobial antibiotics,[104] and antitumor antibiotics.[105] This class of glycomimetics has attracted more interest and therefore more methods have been developed for C-glycosides than for carbasugars.[106]

For example, most recently Fairbanks and co-worker have reported the stereoselective synthesis of *C*-glycosides via Tebbe methylenation and Claisen rearrangement to produce compounds for biological screening purposes.[107] Bertozzi and coworker have described a stereoselective C-C bond formation at the anomeric center to make metabolically stable glycopeptide mimetics.[108] The range of synthetic methods to make *C*-glycosides has now made the discovery of new applications of these glycomimetics a key direction.

8. References

1. Ko, K. S.; Yu, Y.; Pohl, N. L., unpublished results.

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CHAPTER 2

Fluorous-Based Carbohydrate Microarrays

A paper published in *The Journal of the American Chemical Society*[1]

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Abstract

The success of microarrays, such as DNA chips, for biosample screening with minimal sample usage has led to a variety of technologies for assays on glass slides. Unfortunately, for small molecules, such as carbohydrates, these methods usually rely on covalent bond formation, which requires unique functional handles and multiple chemical steps. A new simpler concept in microarray formation is based on noncovalent fluorous-based interactions. A fluorous tail is designed not only to aid in saccharide purification but also to allow direct formation of carbohydrate microarrays on fluorous-derivatized glass slides for biological screening with lectins, such as concanavalin A. The noncovalent interactions in the fluorous-based array are even strong enough to withstand the detergents used in assays with the *Erythrina crystagalli* lectin. Additionally, the utility of benzyl carbonate protecting groups on fucose building blocks for the formation of α-linkages is demonstrated.

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Introduction

The success of microarrays, such as DNA chips, for biosample screening with minimal sample usage has led to a variety of technologies for assays on glass slides.[1] Unfortunately, for small molecules, such as sugars, these methods usually rely on covalent bond formation, which requires unique functional handles and multiple chemical steps.[2] Herein, we present a new simpler concept in microarray formation that is based on noncovalent fluorous-based interactions and demonstrate the strength of these interactions in the direct formation of carbohydrate microarrays for biological screening.

Unlike comparable lipid tails interacting with hydrophobic solid phases,[3] a single C_8F_{17} tail is sufficient to bind biomolecules, such as peptides, to fluorinated solid supports.[4] The use of such fluorinated solid supports for affinity chromatography can substantially simplify the purification of synthetic carbohydrate intermediates in a process amenable to automation (Figure 1).[5] An additional potential advantage of a fluorous-based approach is the direct formation of microarrays; the production of carbohydrate microarrays from compounds made on solid-phase still requires multiple solution-phase deprotection and derivatization steps.[2c]
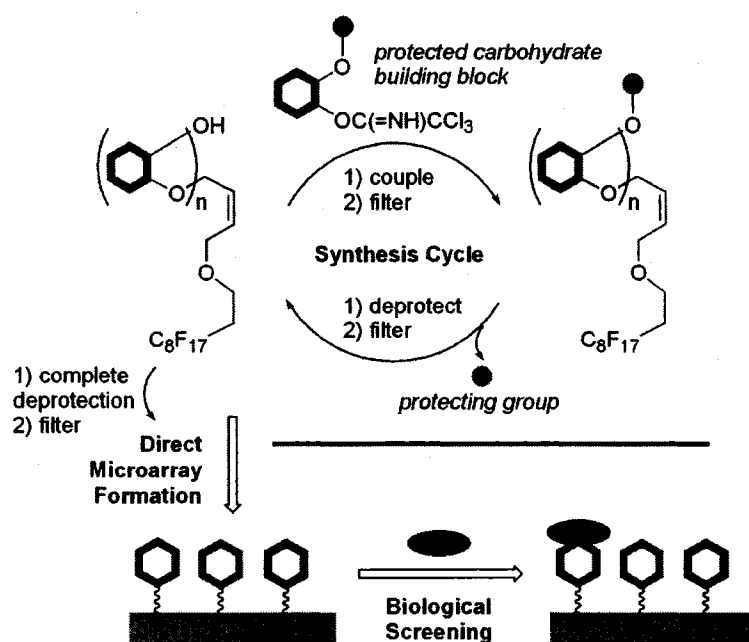
Figure 1 Strategy for the synthesis of carbohydrates and direct formation of fluorous-based carbohydrate microarrays.

Results and discussion

We began our studies with the design of a suitable fluorous tag for carbohydrate synthesis that could survive the necessary sequential reaction conditions and also be removed if desired. An allyl group would be orthogonal to the trichloroacetimidate coupling conditions and other deprotection conditions. Therefore, to create a fluorous allyl protecting group as well as potential anchor for microarray formation, a fluorous tail was synthesized in one step by reaction of cis-1,4-butenediol with substoichiometric amounts of $1H,1H,2H,2H$-perfluorodecyl iodide to produce an alcohol for use in glycosylations. Sugars commonly found in plants were chosen as initial targets to test the feasibility of a noncovalent fluorous-based array for protein-binding studies (Scheme 1). Genome sequencing projects have revealed that the number of genes related to carbohydrate

metabolism is far greater in plants, such as *Arabidopsis thaliana*, than in animals or fungi.[6]

In addition to providing an understanding of plant biology, the study of carbohydrate-protein interactions in plants could lead to the discovery of new sugar-binding lectins for use as glycobiology tools.[7] The known trichloroacetimidates of peracylated mannose,[8] galactose,[9] and *N*-acetylglucosamine[10] were reacted with the fluorous-tagged allyl alcohol, and subsequent deacylation and hydrogenation of the double bond yielded the initial array components 1-3.

1 Man   2 GlcNAc   3 Gal   4 Fuc
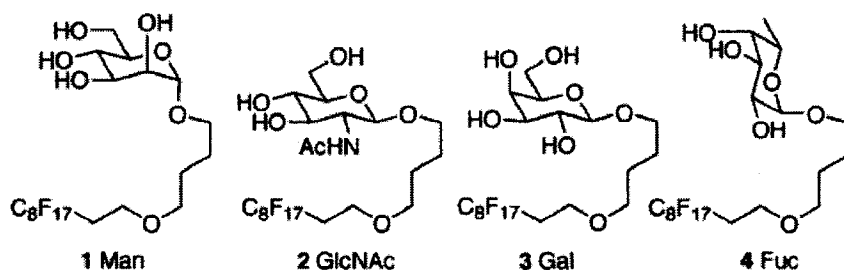
Scheme 1

Although neighboring-group participation could be used to control the stereochemistry of glycosylation for mannose, galactose, and *N*-acetylglucosamine, the synthesis of α-linked fucose required a different approach. Fucose glycosyl donors are usually built with nonparticipating benzyl protecting groups to provide predominantly the α-configured glycosylation products. Unfortunately, such electron-donating groups on a 6-deoxysugar also serve to make the resulting glycosidic linkage more acid sensitive. Several strategies alleviate this problem, for example, the use of more electron-withdrawing halobenzyl groups[11] or replacing the 3- and 4-OH protecting groups with an ester[2] that can potentially serve as a distant participating group. The latter approach is appealing, but

requires an additional basic deprotection step. To avoid this extra step, we decided to test if a benzyl carbonate protecting group could serve the same purpose with the advantage of removal during the hydrogenation step. The requisite glycosyl donor was synthesized from the known allylated compound[13] 5 in three steps to provide the desired trichloroacetimidate 6 (Scheme 2). This donor was glycosylated with the fluorous-tagged alcohol using trimethylsilyl triflate to yield only the axially linked product in 90% yield. Indeed, the benzyl carbonate could serve to direct formation of the α-anomer and be removed by hydrogenation.
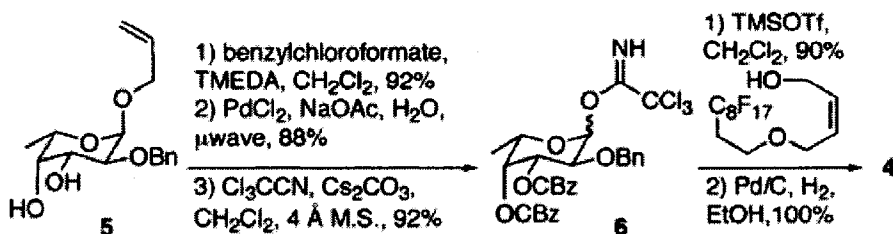
Scheme 2

After synthesis of the requisite fluorous-tagged sugars, the next challenge was to find a suitable fluorinated surface. Solutions of each sugar were spotted onto a commercially available glass microscope slide coated with a Teflon/epoxy mixture employing a standard robot used for DNA arraying. The spots were dried, incubated with a solution of the fluorescein isothiocyanate-labeled jack bean lectin concanavalin A (FITC-ConA) for 20 min, rinsed repeatedly with assay buffer and distilled water, and then scanned with a standard fluorescent slide scanner. The scan clearly showed binding of

FITC-ConA only to the mannose-containing spots. The anomeric position could be distinguished as the β-linked GlcNAc (2) was not recognized.

This lectin experiment demonstrated the ability of the C_8F_{17} tail to anchor the carbohydrates to the slide surface even after repeated washes. However, the slide was also intrinsically and unevenly fluorescent at 488 nM, a wavelength that is commonly used to detect labeled analytes. Clearly, a new approach was necessary to obtain an optically and fluorescently clear surface for the formation of compound microarrays. To this end, a glass microscope slide was reacted[14] with a fluoroalkylsilane to provide a clear coating on which water forms beads.

With the new microarray substrate in hand, we next probed the scope of a fluorous-based microarray approach for compound screening. Fluorous-tagged sugars were spotted on the coated slide using an arraying robot, and then the slides were incubated with FITC-labeled lectins (Figure 2). To test the reproducibility of the process, the same concentration of sugar was spotted repeatedly. In addition, several different concentrations of sugars were spotted. To test the ability of the array to withstand detergents often included in biological screens, the labeled plant lectin from the bush *Erythrina crystagalli* (FITC-ECA) was used to probe the microarrays with Tween-20. The array withstood the 20 min incubation time and repeated rinsing with this detergent-containing buffer.

Man (1)   GlcNAc (2)   Gal (3)   Fuc (4)
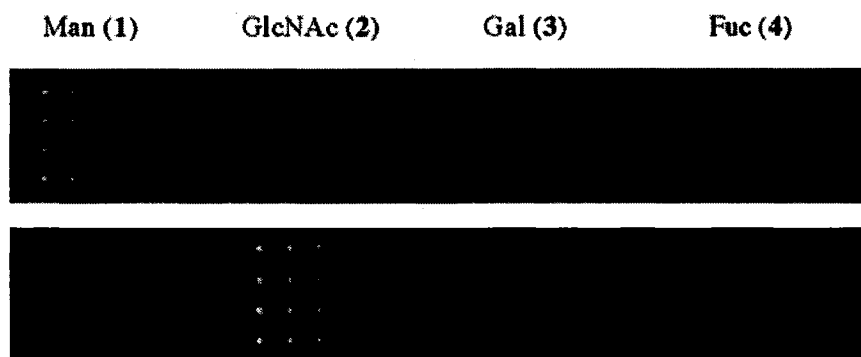
Figure 2 Fluorescence images of arrayed carbohydrates probed with FITC-labeled lectins.

Columns of 4 spots each of 2, 1, 0.5, and 0.1 mM carbohydrates were incubated for 20 min

with FITC-ConA (top) or FITC-ECA with 1%

Conclusion

A fluorous-based microarray method allows the facile formation of a range of

carbohydrate chips for the plant and other sciences using synthetic sugars produced with

the aid of fluorous-tagged synthesis. Efforts are underway to automate portions of the

solution-phase fluorous-based synthetic process and to incorporate enzymatic steps to

expand the scope of carbohydrates that can be easily arrayed for biological screening.

Although not limited to carbohydrates, the approach should be especially valuable for the

production of arrays containing compounds, such as glycosaminoglycan fragments, that

contain nucleophiles that complicate current defined covalent attachment strategies.

Experimental section

General methods

Reaction solvents were distilled from calcium hydride for dichloromethane and from sodium metal and benzophenone for diethyl ether. Amberlyst 15 ion-exchange resin was washed repeatedly with methanol before use. All other commercial reagents and solvents were used as received without further purification. The reactions were monitored and the R_f values determined using analytical thin layer chromatography (tlc) with 0.25 mm EM Science silica gel plates (60F-254). The developed tlc plates were visualized by immersion in p-anisaldehyde solution followed by heating on a hot plate. Flash chromatography was performed with Selecto Scientific silica gel, 32-63 μm particle size. All moisture-sensitive reactions were performed in flame- or oven-dried glassware under a nitrogen atmosphere. Bath temperatures were used to record the reaction temperature in all cases run without microwave irradiation. All reactions were stirred magnetically at ambient temperature unless otherwise indicated. ^{1}H NMR, ^{13}C NMR, and [17]F spectra were obtained with a Bruker DRX400 at 400 MHz, 100 MHz, and 162 MHz respectively. ^{1}H NMR spectra were reported in parts per million (δ) relative to $CDCl_3$ (7.27 ppm) as an internal reference. ^{13}C NMR spectra were reported in parts per million (δ) relative to $CDCl_3$ (77.23 ppm) or CD_3OD (49.15 ppm).

Cleaning of glass slide[15]: Glass slides were cleaned by immersion for 2 h in 60% ethanol containing NaOH 10 g/100 mL. The slides were washed twice in distilled water and then submerged in 95% ethanol.

Microarray Preparation and Screening.

Formation of fluorous-derivatized glass slides. Cleaned slides were immersed three times in a solution of Rf8-ethyl-SiCl$_3$ (3% solution 100 mL in methanol) and then dried for a few minutes and repeated two times more. The slides were washed twice by immersion in MilliQ-filtered water and then dried in a dessicator at room temperature.

Formation of carbohydrate microarrays. Flurous-tagged carbohydrate compounds were dissolved in 60% methanol in water and spotted on the fluorinated glass slide using robot (Cartesian PixSys 5500 Arrayerand arrayer) at 30% humidity. The glass slide was dried in a humidifying chamber at 30% humidity for 2 h.

Detection of protein-carbohydrate binding[16,17]. For Con A; FITC-labeled Con A in HEPES buffer (pH = 7.5, 10 mM) 1 mM CaCl$_2$, 1 mM MnCl$_2$, 100 mM NaCl, 1% BSA (w/v) or without BSA, and for ECA; FITC-labeled ECA in PBS buffer (pH = 6.8), 1.0% TWEEN 20, ECA (25 ug mL-1; EY laboratoris) were used the detection of protein-carbohydrate interactions. For protein incubation, 0.5 mL of protein solution was applied to the printed glass slide. The arrays was incubated by using a PC500 CoverWell incubation chamber and gently shaken to every 5 min for 30 min. It was then washed three times with the same buffer followed by three washes with distilled water. It was then dried for 30 min in a dark humidity chamber. Glass slide was scanned using fluorescence scanner.

Synthetic Procedures.
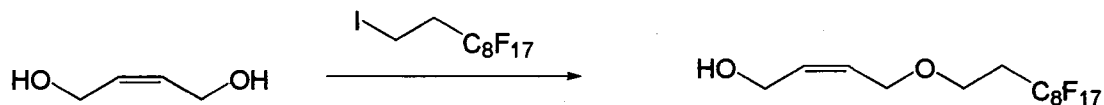
1H,1H,2H,2H-perfluorodecyloxybutenyl alcohol[18].

1,4-butenediol (140 mg, 1.59 mmol) was dissolved with 20 mL of DMF. TBAB (153 mg, 48.0 mmol and 1H, 1H, 2H, 2H-perfluorodecyliodide (1.8 g, 3.18 mmol) were added into a solution. The reaction mixture was heated at 50 °C for 10 min and then KOH (267 mg, 4.77 mmol) was added into the mixture. The reaction mixture was heated at 70 °C for 30 min. The reaction mixture was poured into ice water. The organic layer was extracted with ethyl ether (100 mL x 2) and dried with over $MgSO_4$, filtered, and the solvent removed under reduced pressure. The crude product was purified by solid-phase extraction[5] using fluoro flash column. Non-fluorous compounds were eluted with 80% MeOH/water and the desired product was eluted by 100% MeOH (1H,1H,2H,2H-perfluorodecyloxybutenyl alcohol as a colorless oil, 1.12 g, 2.1 mmol, 67% as a clear oil.

Rf.= 0.46 (ethyl acetate/hexane/2/3).

^{1}H NMR (CDCl$_3$, 400 MHz): δ (ppm) 5.75 (m, 1H), 5.62 (m, 1H), 4.26 (m, 2H), 4.14 (m, 4H), 2.36 (m, 2H).

^{13}C NMR (CDCl$_3$, 160 MHz): δ (ppm) 132.8, 127.5, 66.2, 61.9, 61.8, 58.7, 46.8.

^{17}F NMR (CDCl$_3$, 400 MHz): δ (ppm) -81.3(m), -118.2(4), -112.5(m), -123.3(m), -126.6(m), -127.1(m).

MS (ESI) m/z = 535 [M+H]$^+$
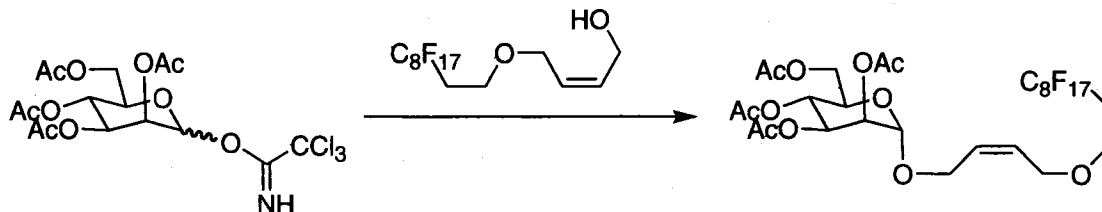
1H,1H,2H,2H-perfluorodecyloxybutenyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside.

To a solution of 2,3,4,6-tetra-O-acetyl-α/β-D-mannopyranoside trichloroacetimidate[6] (165.0 mg, 0.34 mmol) and 1H,1H,2H,2H-perfluorodecyloxybutenyl alcohol (140.0 mg, 0.25 mmol) in dichloromethane (5 mL) were added powdered 4Å molecular sieves (10 mg) and the mixture was cooled down to -15 °C. TMSOTf (40 µL, 0.17 mmol) was added and the reaction mixture was stirred at –15 °C for 30 min. The reaction was quenched with triethylamine (0.1 mL) and concentrated. The crude product was purified by solid-phase extraction using fluoro flash column. Non-fluorous compounds were eluted with 80% MeOH/water and the desired product was eluted by 100% MeOH (1H,1H,2H,2H-perfluorodecyloxybutenyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside as a colorless oil, 207.1 mg, 24.0 mmol, 93%).

Rf = 0.26 (ethyl acetate/hexane/2/3).

^{1}H NMR (CDCl$_3$, 400 MHz): δ (ppm) 5.69 – 5.74 (m, 2H), 5.32 (dd, 1H, J = 10.0, 3.2 Hz), 5.26 (t, 1H, J = 9.6 Hz), 5.20 (m, 1H), 4.81 (m, 1H), 4.26 – 4.18 (m, 3H), 4.10 – 4.0.2 (m, 5H), 3.96 (m, 1H), 2.14 (s, 3H), 2.07 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H).

^{13}C NMR (CDCl$_3$, 160 MHz): δ (ppm) 171.4, 170.9, 170.2, 170.0, 129.7, 128.9, 98.5, 71.6, 69.3, 68.5, 66.3, 66.2, 63.1, 62.4, 62.0, 21.0, 20.9(2), 20.8.

MS (ESI) m/z = 887 [M + Na]
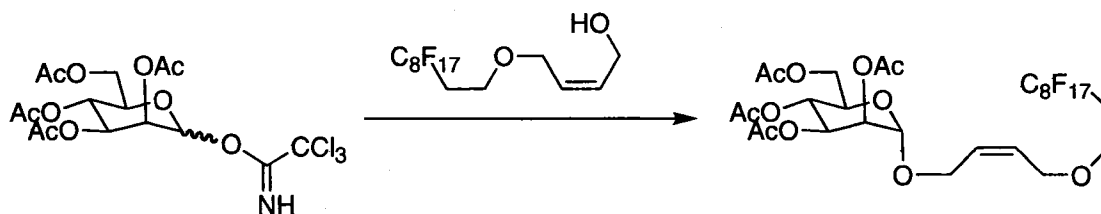
1H,1H,2H,2H-perfluorodecyloxybutanyl-α-D-mannopyranoside.

1H,1H,2H,2H-perfluorodecyloxybutenyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (184.0 mg, 0.21 mmol) was dissolved in MeOH (5 mL) and 5% Pd/C (10 mg) was added. The reaction mixture was put under hydrogen atmosphere and stirred for 1 h. It was then filtered over Celite and ethanol was removed under reduced pressure to give the hydrogenated product in quantitative yield. To a solution of the resulting compound (184.0 mg, 21.3 mmol) in methanol (10 mL) was added K$_2$CO$_3$ (117.0 mg, 0.85 mmol) and the reaction mixture was stirred for 2 h. The mixture was then neutralized using Amberlyst-15 ion-exchange resin and filtered. The solvent was removed under reduced pressure and 1H,1H,2H,2H-perfluorodecyloxybutanyl-α-D-mannopyranoside was obtained as white solid (146.0 mg, 0.21mmol, 100%).

Rf = 0.36 (methanol/ethyl acetate/hexane/2/2/3).

^{1}H NMR (MeOD, 400 MHz): δ (ppm) 4.71 (br, 1H), 3.80 (dd, 1H, J = 12.0, 2.4 Hz), 3.75 (m, 2H), 3.70 (d, 1H, J = 5.6 Hz), 3.65 (m, 1H), 3.60 (m, 2H), 3.55 (m,2H), 3.48 (m, 2H), 3.40 (m, 1H), 2.03 (m, 2H), 1.64 (m, 4H).

^{13}C NMR (MeOD, 160 MHz): δ (ppm) 100.2, 73.3, 71.3, 70.9, 70.5, 67.2, 66.9, 61.4, 61.3, 27.4 (2), 26.1(2).

MS (ESI) m/z = 721 [M + Na]$^+$
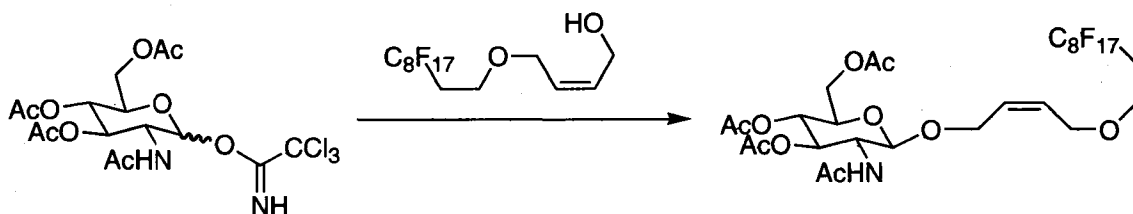
1H,1H,2H,2H-perfluorodecyloxybutenyl-3,4,6-tri-*O*-acetyl-2-deoxy-2-aceylamino-β-D-glucopyranoside.

To a solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2-acetylamino-α/β-D-mannopyranoside trichloroacetimidate[7] (148.8 mg, 0.30 mmol) and 1H,1H,2H,2H-perfluorodecyloxybutenyl alcohol (110.0 mg, 0.25 mmol) in dichloromethane (5 mL) were added powdered 4Å molecular sieves (10 mg) and the mixture was cooled down to -15 °C. TMSOTf (34 μL, 0.15 mmol) was added and the reaction mixture was stirred at −15 °C for 30 min. The reaction was quenched with triethylamine (0.1 mL) and concentrated. The crude product was purified by solid-phase extraction using fluoro flash column. Non-fluorous compounds were eluted with 80% MeOH/water and the desired product was eluted by 100% MeOH (1H,1H,2H,2H-perfluorodecyloxybutenyl-3,4,6-tri-*O*-acetyl-2-deoxy-2-acetylamino-β-D-glucopyranoside as a colorless oil, 150.3 mg, 0.17 mmol, 86%).

Rf = 0.26 (ethyl acetate/hexane/2/3).

^{1}H NMR (CDCl$_3$, 400 MHz): δ (ppm) 5.82 − 5.63 (m, 2H), 5.25 (dd, 1H, J = 10.0, 9.6 Hz), 5.03 (t, 1H, *J* = 10.0 Hz), 4.68 (d, 1H, J = 8.4 Hz), 4.32 (m, 1H), 4.19 (m, 4H), 4.06

(m, 1H), 3.99 (m, 2H), 3.80 (m, 1H), 3.65 (m, 1H), 2.04 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.90 (s, 3H).

^{13}C NMR (CDCl$_3$, 160 MHz): δ (ppm) 170.9, 170.7, 170.3, 169.4, 129.3, 128.9, 99.6, 72.3, 71.9, 68.6, 66.4, 64.4, 62.1, 61.9(2), 54.7, 23.3, 20.7(2), 20.6.

MS (ESI) m/z = 886 [M+ Na]$^+$

2 GlcNAc
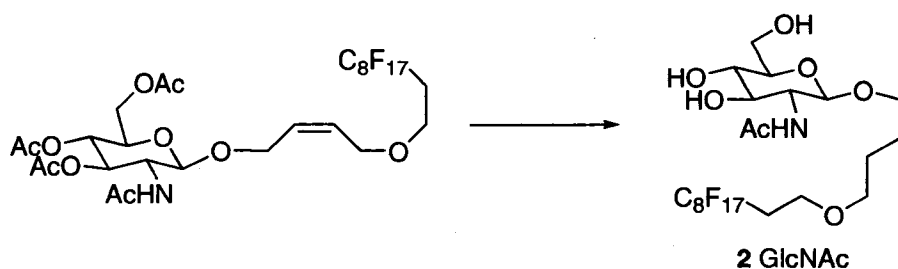
1H,1H,2H,2H-perfluorodecyloxybutanyl-2-deoxy-2-acetylamino-β-D-glucopyranoside.

1H,1H,2H,2H-perfluorodecyloxybutenyl-3,4,6-tri-*O*-acetyl-2-deoxy-2-acetylamino-β-D-glucopyranoside (138.0 mg, 0.16 mmol) was dissolved in MeOH (5 mL) and 5% Pd/C (10 mg) was added. The reaction mixture was put under hydrogen atmosphere and stirred for 1 h. It was then filtered over Celite and ethanol was removed under reduced pressure to give the hydrogenated product in quantitative yield. To a solution of the resulting compound (138.0 mg, 0.16 mmol) in methanol (10 mL) was added K$_2$CO$_3$ (66.0 mg, 0.48 mmol) and the reaction mixture was stirred for 2 h. The mixture was then neutralized using Amberlyst-15 ion-exchange resin and filtered. The solvent was removed under reduced pressure and 1H,1H,2H,2H-perfluorodecyloxybutanyl-2-deoxy-2-acetylamino-β-D-gluco-pyranoside was obtained as white solid (118.0 mg, 0.16mmol, 100%).

Rf = 0.23 (methanol/ethyl acetate/hexane/2/2/3).

^{1}H NMR (MeOD, 400 MHz): δ (ppm) 4.41 (d, 1H, J = 8.4 Hz), 3.86 (m, 2H), 3.84 (dd, 1H, J = 12.4, 1.6 Hz), 3.63 (m, 2H), 3.46 (m, 5H), 3.21 (m, 2H), 2.02 (m, 2H), 1.95 (s), 3H), 1.59 (m, 4H).

^{13}C NMR (MeOD, 160 MHz): δ (ppm) 172.3, 101.4, 78.2, 77.9, 77.6, 74.7, 70.7, 64.2, 61.4, 56.0, 25.9, 25.8, 21.6(2).

MS (ESI) m/z = 762 [M + Na]$^+$

Additional Information: Experimental details, including copies of ^{1}H NMR spectra, for the synthesis and production of the carbohydrate microarrays and the complete ref 2h, and competition experiment plots are found in the Appendix at the end of this chapter.

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CHAPTER 3

Strategies for the Chemoenzymatic Synthesis of Deoxysugar Nucleotides: Substrate Binding versus Catalysis

Kwang-Seuk Ko, Corbin J. Zea, and Nicola L. Pohl*

Abstract

Sugar nucleotidyltransferases, also known as sugar pyrophosphorylases, catalyze the formation of a phosphate linkage to produce sugars activated for use by Leloir pathway glycosyltransferases and are subjects of protein engineering for chemoenzymatic synthesis strategies. Herein we present evidence that this class of enzymes can exercise kinetic discrimination in choosing carbohydrates of comparable binding affinity for catalytic turnover. Synthetic 6-deoxy, 4-deoxy, and 3-deoxyglucose-1-phosphate and the natural substrate glucose-1-phosphate were tested with a representative prokaryotic glucose-1-phosphate uridylyltransferase [EC 2.7.7.9] from *Escherichia coli*, which is also known to accept thymidine triphosphate, and the comparable eukaryotic enzyme from yeast. Percent conversion studies show that product yields, as determined by an electrospray ionization mass spectrometry-based assay, were much lower after 30 min for the nonnatural substrates than for the natural substrate. Surprisingly, kinetic analysis demonstrates that differences in substrate binding affinity do not primarily account for these contrasts in

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product yields. This kinetic analysis is the first evidence that sugar nucleotidyltransferases can make use of not only differences in substrate binding affinity, but also kinetic differences in discriminating among carbohydrate substrates, and has implications for the in vivo and in vitro function and use of these enzymes.

Introduction

Numerous studies have begun to delineate the binding determinants of sugars to receptors such as lectins and antibodies,[1] but relatively little is understood about how binding relates to catalysis by the many enzymes involved in carbohydrate biosynthesis. These enzymes, particularly sugar nucleotidyltransferases and glycosyltransferases, are used extensively for the chemoenzymatic synthesis and in vivo biosynthesis of carbohydrate natural products and their analogues.[2] The desire to expand the substrate range of these enzymes has also sparked protein engineering efforts.[3] However, syntheses using nonnatural substrates, even those with conservative modifications, often fail for reasons that are not clearly understood. Unfortunately, the usual percent conversion studies do not shed light on whether the fault lies in low substrate affinity for the enzyme, low turnover rates, or both, and therefore, reaction optimization must be empirical. Herein we present evidence from the first kinetics analyses of sugar nucleotidyltransferases with deoxysugar substrates that these enzymes can exercise kinetic discrimination in choosing carbohydrates of comparable binding affinity for catalytic turnover. The consequences of this finding for using this class of enzymes for the synthesis of sugar nucleotides, as well as for understanding of their biological roles, will be discussed.

Sugar nucleotidyltransferases catalyze the formation of a phosphate linkage to produce sugars activated for use by Leloir pathway glycosyltransferases (Figure 1). The reaction is known to proceed via nucleophilic attack of the sugar-1-phosphate on the α-phosphate of the nucleotide triphosphate;[4] hence, the carbohydrate portion is somewhat removed from the reactive site during catalysis. Although the structures of only a few sugar nucleotidyltransferases are presently known [5,6] and no apparent sequence homology between enzymes of similar function from eukaryotes and prokaryotes is evident, the process is crucial for carbohydrate biosynthesis. Sugar nucleotidyltransferases have been proposed as potential antibiotic targets,[5] but a more profound knowledge of differences in carbohydrate substrate recognition between bacteria and humans is needed to design compounds with the necessary selectivity. Greater knowledge of these enzymes also will aid in their use for the synthesis of nonnatural sugar nucleotides as glycobiology tools and precursors for in vitro glycosylation strategies.
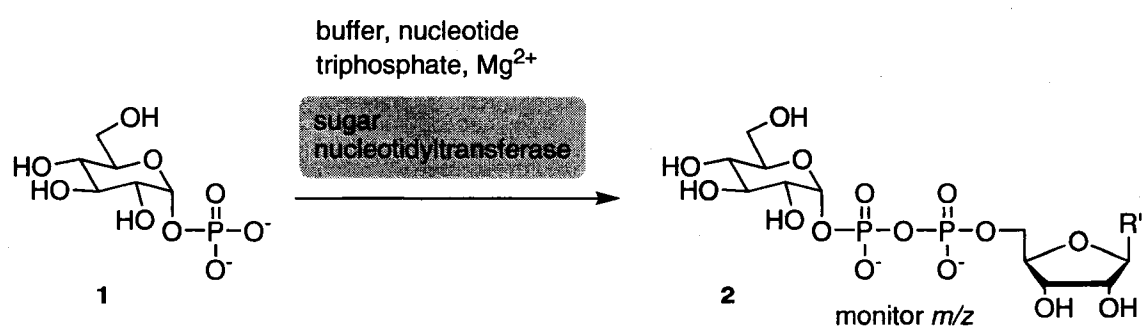
Figure 1 Sugar nucleotidyltransferases catalyze the synthesis of activated sugar nucleotides, such as uridinediphosphoglucose (2) when R' = uridine, from sugar-1-phosphates, such as glucose-1-phosphate (1) and nucleotide triphosphates. Analysis of

product formation by ESI-MS allows the facile kinetic analysis of the reaction independent of the structure of the carbohydrate portion of the sugar-1-phosphate.

A major impediment to studying sugar nucleotidyltransferases is the lack of an assay that can quickly determine kinetic parameters and inhibition constants for a range of compounds. The use of coupled assays[7] or radioactive assays[8] limits the use of nonnatural substrates. HPLC-based assays[9], require separation protocols for every new substrate and are so relatively time-consuming to probably have inhibited kinetics studies that require analysis of multiple reaction points in triplicate. Our recent development of an electrospray ionization mass spectrometry (ESI-MS)-based assay[10] enables the rapid determination of kinetic parameters for both natural and nonnatural substrates of this class of enzymes and prompted our investigation into the origins of substrate discrimination by sugar nucleotidyltransferases to optimize their use in synthesis.

Results and discussion

A sugar nucleotidyltransferase from yeast that activates the most common naturally occurring sugar, glucose, is commercially available, and therefore, we commenced our studies with probing the tolerance of this enzyme to conservative substrate modifications. The systematic removal of hydroxyl moieties should provide substrates that still fit into the enzyme active site; however, previous work with a bacterial enzyme has shown that even such minor changes can significantly impact product yields.[9b] We therefore first undertook

the synthesis of the required 6-, 4-, 3-, and 2-deoxyglucose-1-phosphates9b (dGlc-1-phosphate) (Figure 2) to probe the origin of these differences in product yields.
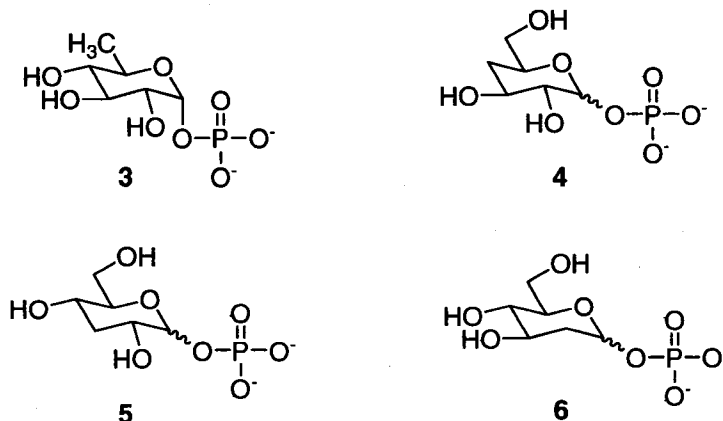
Figure 2 Synthetic substrates 6dGlc-1-phosphate (**3**), 4dGlc-1-phosphate (**4**), 3dGlc-1-phosphate (**5**), and 2dGlc-1-phosphate (**6**) used to test the substrate tolerance of UDP-glucose pyrophosphorylases from yeast and *E. coli*.

Substrate Synthesis. Commercially available methyl-glucoside **7**[11] was treated with dimethyl benzaldehyde acetal and camphorsulfonic acid to give benzylidene **8** in 76% yield. Benzylation of hydroxyl groups was accomplished by using benzyl bromide and sodium hydride. Reduction of benzylidene with lithium aluminum hydride gave compound **9** in 80% (Scheme 1) yield.[12] The primary hydroxyl group at C-6 was protected with tosyl chloride and reduced with LiAlH₄ to produce the desired deoxygenated sugar **10** in quantitative yield. Acidic hydrolysis of the anomeric methyl ether provided a free anomeric hydroxyl group. Although 1*H*-tetrazole is often used to aid phosphoramidite couplings,[13] the recently reported *N*-(phenyl)imidazolium triflate[14] proved superior in catalyzing the addition of dibenzyl diisopropylphosphoramidite. The desired phosphate

was produced after oxidation of the phosphite with mCPBA in 93% yield. Finally, hydrogenation of benzyl ether followed by treatment with acidic ion exchange resin provided **3** in 97% yield.

SCHEME 1. Preparation of 6-deoxyglucose-1-phosphate.[a]
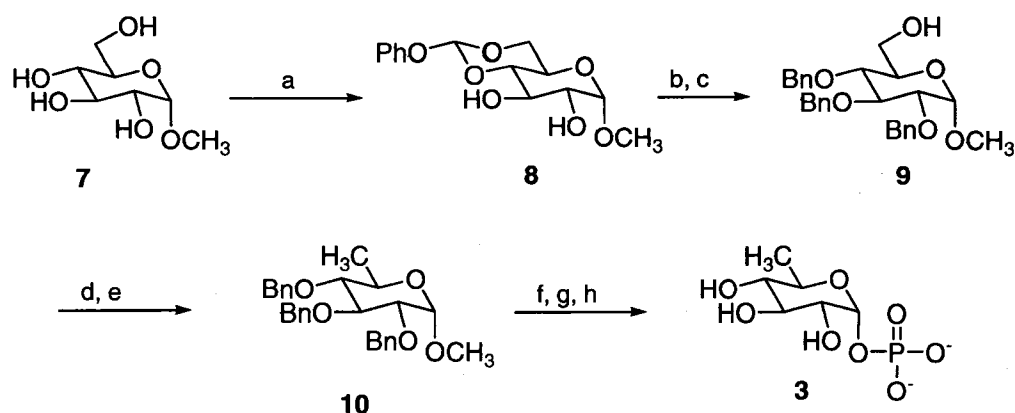
[a] Reagents and conditions: a) PhCH$_2$(OCH$_3$)$_2$, DMF, CSA, 76%; b) BnBr, NaH, DMF, 84%; c) LAH/AlCl$_3$, 1:1 Et$_2$O/CH$_2$Cl$_2$, 80%; d) TsCl, pyridine, rt, quantiative; e) LAH, Et$_2$O, rt, quantitative yield; f) CH$_3$CO$_2$H, 3.0 M H$_2$SO$_4$, 85 °C, 73%; g) (iPr)$_2$NP(OBn)$_2$, CH$_3$CN, 4 Å MS, N-(phenyl)imidazolium triflate; mCPBA, 93%; h) Pd/C, H$_2$, CH$_3$OH, 97%.

For the synthesis of 2-deoxyglucose-1-phosphate (**6**), commercially available acetylglucal **11** was converted to compound **12**[15] in 3 steps as previously reported. Compound **12** was subjected to phosphorylation condition followed by hydrogenolysis as described above for synthesis of compound **6** (Scheme 2).

SCHEME 2. Preparation of 2-deoxyglucose-1-phosphate.[a]
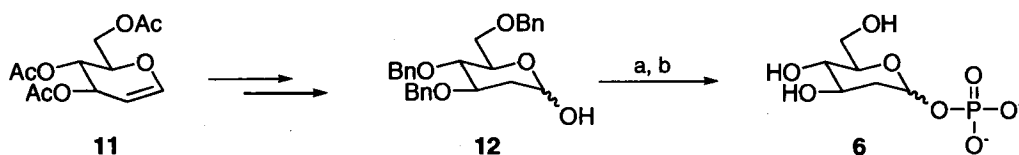
^{a} Reagents and conditions: a) $(i\text{Pr})_2\text{NP(OBn)}_2$, CH_3CN, 4 Å MS, N-(phenyl)imidazolium triflate; $m\text{CPBA}$, 84%; b) Pd/C, H_2, CH_3OH. 89%.

Compound **8** was selectively benzylated at C-2 position to give compound **13** in 84% yield (Scheme 3). The secondary hydroxyl group in compound **13** was converted to Barton's ester[16] **14** by using 1,1'-thiocarbonyldiimidazole. Reduction of Barton's ester with Bu_3SnH produced 3-deoxyglucose derivative **15**. Acidic cleavage of benzylidene resulted in the formation of diol which was protected with benzyl bromide to give compound **16**. Removal of anomeric methyl group followed by phosphorylation and hydrogenolysis produced compound **5**.

SCHEME 3. Preparation of 3-deoxyglucose-1-phosphate.^{a}
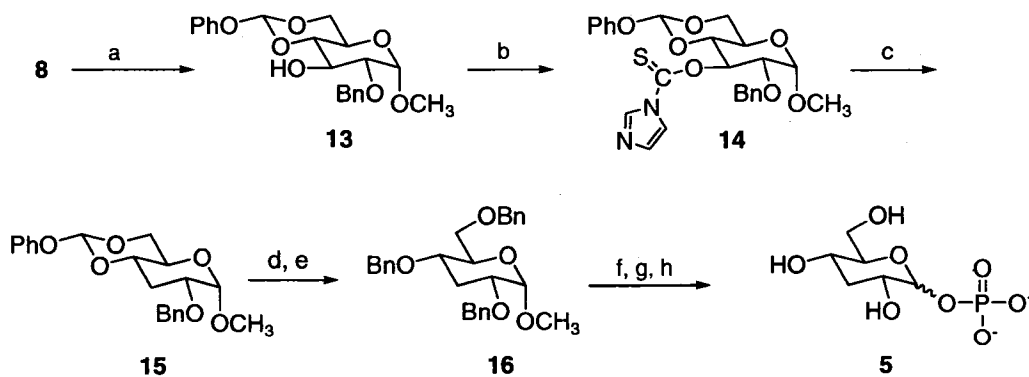
^{a} Reagents and conditions: a) BnBr, NaH, DMF, 84%; b) 1,1 thiocarbonyl diimidazole, THF, reflux, 91 %; c) Bu_3SnH, Toluene, reflux, 86 %; d) 2 N H_2SO_4, MeOH, 89 %; e) BnBr, NaH, DMF, 89 %; f) $\text{H}_3\text{CO}_2\text{H}$, 3.0 M H_2SO_4, 85 °C, 73%; g) $(i\text{Pr})_2\text{NP(OBn)}_2$,

CH$_3$CN, 4 Å MS, *N*-(phenyl)imidazolium triflate; *m*CPBA, 93%; h) Pd/C, H$_2$, CH$_3$OH, 97%.

The 4,6-O-benzylidene **8** was selectively opened with TES and TFA to produce the secondary alcohol **17**[17] in 96 % yield (Scheme 4). The secondary alcohol **17** was converted to Barton's ester followed by reduction with Bu$_3$SnH to give 4-deoxy compound **18**. Acidic hydrolysis of the anomeric methyl ether followed by phosphorylation with dibenzyl diisopropylphosphoramidite and catalytic amount of *N*-(phenyl)imidazolium triflate and hydrogenolysis of benzyl ether provided compound **4**.

SCHEME 4. Preparation of 4-deoxyglucose-1-phosphate.[a]
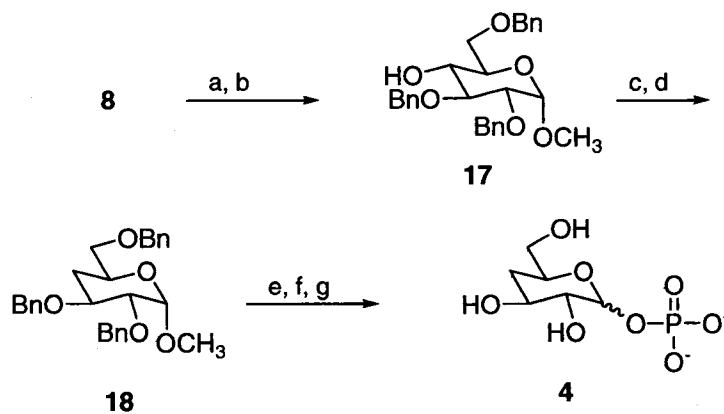
[a] Reagents and conditions: a) BnBr, NaH, DMF, 84%; b) TES, TFA, CH$_2$Cl$_2$, 96 %; c)1,1'- thiocarbonyl diimidazole, THF, reflux, 96 %; d) Bu$_3$SnH, Toluene, reflux, 79 %; e) H$_3$CCO$_2$H, 3.0 M H$_2$SO$_4$, 85 °C, 73%; f) (*i*Pr)$_2$NP(OBn)$_2$, CH$_3$CN, 4 Å MS, *N*-(phenyl)imidazolium triflate; *m*CPBA, 93%; g) Pd/C, H$_2$, CH$_3$OH, 97%.

Relative substrate acceptance of UDP-glucose pyrophosphorylases. With the four required deoxyglucose-1-phosphates in hand, we could test the ability of the commercially available

yeast enzyme, a representative eukaryotic enzyme, to accept these nonnatural substraes. The acceptance of the natural substrate (**1**) and various deoxyglucose-1-phosphates (**3**, **4**, **5**, and **6**) by the yeast UDP-glucose pyrophosphorylase enzyme was tested in the presence of 0.02 U of enzyme, 10 mM uridine 5'-triphosphate (UTP) or 2'-deoxy-thymidine 5'-triphosphate (dTTP), and 10 mM Mg^{2+}. The relative acceptance rate is defined as the ratio of nonnatural substrate (**3**, **4**, **5**, and **6**) to the natural substrate (**1**) converted in the corresponding nucleotide diphosphate sugar. As shown in Figure 3B, the yeast UDP-glucose pyrophosphorylase enzyme only accepted the 6-deoxysubstrate **3** in the presence of UTP. No product formation was observed in the absence of the enzyme, Mg^{2+}, the nucleotide triphosphate, or the α-D-hexopyranosyl-1-phosphate. Unfortunately, the commercial enzyme was rather selective and therefore did not show promise for chemoenzymatic synthesis of activated deoxyglucose analogs. Therefore, a recombinant bacterial enzyme was investigated next. Fortunately, the relative acceptance of the UDP-glucose pyrophosphorylase from *Escherichia* coli[10] of the nonnatural substrates in the presence of UTP and dTTP was much better than the yeast enzyme (Figure 3A). Only the 2-deoxysubstrate **6** failed to generate product in the presence of UTP. However, the percent conversions were significantly lower for the deoxysubstrates than the natural substrate, despite no obvious negative steric factors.
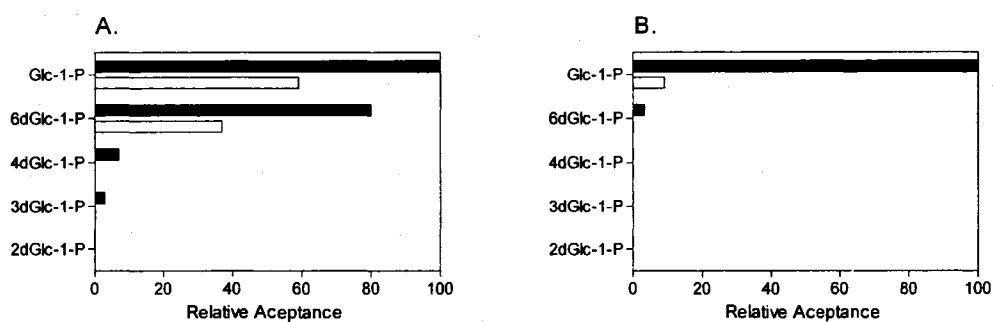
FIGURE 3. Relative substrate acceptance of UDP-glucose pyrophosphorylase from *E. coli* (A.) and yeast (B.) in the presence of UTP (solid bar) and dTTP (open bar) after 30 min.

Kinetic analysis. To ascertain whether the differences in product yields were the result of decreased substrate binding or decreased turnover efficiencies, a kinetic analysis of the reactions was necessary. We therefore determined the kinetic parameters of the substrate – enzyme systems in which turnover was noted in the relative acceptance study. The kinetic parameters were measured by varying the concentration of the modified substrates **3, 4,** and **5** (2-100 μM) at a constant UTP or dTTP concentration (300 μM) in the presence of 10 mM Mg^{+2} and 0.02 U of UDP-glucose pyrophosphorylase from *E. coli* or yeast. The reactions were quenched after 5 min and the initial reaction rate was determined by the change in concentration of UDP or dTDP-dGlc-1-phosphate measured via ESI-MS. The kinetic values K_m and V_{max}, Table 1, were determined from nonlinear regression of the Michealis-Menten plots (See Supporting Information).

Table 1. Kinetic analysis of glucose-1-phosphate uridylyltransferases.

source	substrates	K_M (μM)	V_{max} (μM/min)	k_{cat}/K_M (s^{-1} μM^{-1})
E. coli	**1** + UTP[a]	12 ± 2	1.15 ± 0.06	1.45
	1 + dTTP[a]	13 ± 2	0.90 ± 1.05	1.05
	3 + UTP	15 ± 3	0.57 ± 0.03	0.58
	3 + dTTP	17 ± 3	0.39 ± 0.03	0.35
	4 + UTP	17 ± 3	0.47 ± 0.03	0.42
	5 + UTP	18 ± 3	0.27 ± 0.01	0.23
yeast	**1** + UTP[a]	7 ± 1	3.8 ± 0.1	10.9
	1 + dTTP	11 ± 2	1.84 ± 0.09	3.4

[a] Reference 24.

Surprisingly, independent of the enzyme source, all of the substrates **1, 3, 4,** and **5** had essentially identical K_m values for the *E. coli* enzyme. However, V_{max} values decreased from the natural substrate (**1**) to the nonnatural substrates (**3, 4,** and **5**) thereby explaining the difference in product yields that were seen in the relative acceptance study. The yeast enzyme exhibited a slightly lower affinity for **2** and the V_{max} value dropped two-fold replicating what was noted for the *E. coli* enzyme.

Clearly, differences in substrate affinity do not explain the differences in product yields for these two UDP-glucose pyrophosphorylases. It has been proposed that a glycosyltransferase involved in blood group determinant biosynthesis can distinguish between UDP-galactose and UDP-*N*-acetylgalactosamine based on kinetics rather than binding affinity;[18] however, this reaction involves both bond-making and bond-breaking directly on the sugar ring. That sugar nucleotidyltransferases can use this same strategy to select among carbohydrate substrates even though the sugar is three atoms from the site of bond formation is a surprising observation. However, the consequence is that use of higher enzyme concentrations rather than higher substrate concentrations can provide optimal yields of sugar nucleotides.

Conclusion

In addition to the implications for using these enzymes as catalysts for the synthesis of sugar nucleotides, this kinetic analysis is the first evidence that sugar nucleotidyltransferases can make use of not only differences in substrate binding affinity,

but also kinetic differences in discriminating among carbohydrate substrates. This finding has several implications. Sugar nucleotidyltransferase substrates, such as **3**, that are turned over preferentially by prokaryotic enzymes when compared to eukaryotic enzymes could serve as prodrugs if the resulting products block glycosyltransferase activity. Protein engineering efforts cannot expect mutations that allow steric access of the sugar to the binding pocket to necessarily result in efficient turnover of the modified substrate. Chemoenzymatic reactions may benefit from an increase in enzyme concentration rather than substrate concentration to increase product yields. The same may be true for the turnover of nonnatural substrates in vivo. Upregulation of sugar nucleotidyltransferases also might result in the undesired turnover of alternate substrates that could lead to negative phenotypes. Finally, since the level of active enzyme is hard to quantify until that activity is known, newly isolated proteins believed to have sugar nucleotidyltransferase activity should be screened against a library of possible substrates to accurately determine the biological function of the enzyme.

Experimental Section

General methods

Reaction solvents were distilled from calcium hydride for dichloromethane and from sodium metal and benzophenone for diethyl ether. Amberlyst 15 ion-exchange resin was washed repeatedly with methanol before use. All other commercial reagents and solvents were used as received without further purification. The reactions were monitored and the R_f values determined using analytical thin layer chromatography (tlc) with 0.25 mm EM

Science silica gel plates (60F-254). The developed tlc plates were visualized by immersion in *p*-anisaldehyde solution followed by heating on a hot plate. Flash chromatography was performed with Selecto Scientific silica gel, 32-63 μm particle size. All moisture-sensitive reactions were performed in flame- or oven-dried glassware under a nitrogen atmosphere. Bath temperatures were used to record the reaction temperature in all cases run without microwave irradiation. All reactions were stirred magnetically at ambient temperature unless otherwise indicated. ^{1}H NMR, ^{13}C NMR, and ^{31}P spectra were obtained with a Bruker DRX400 at 400 MHz, 100 MHz, and 162 MHz respectively. ^{1}H NMR spectra were reported in parts per million (δ) relative to CDCl$_3$ (7.27 ppm) as an internal reference. ^{13}C NMR spectra were reported in parts per million (δ) relative to CDCl$_3$ (77.23 ppm) or CD$_3$OD (49.15 ppm). ^{31}P NMR spectra were reported in δ relative to H$_3$PO$_4$ (0.00 ppm) as an internal reference.

Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (8):

Methyl α-D-glucopyranoside (19.4 g, 100 mmol) in DMF (250 mL) and comphorsulfanic acid (6.9 g, 30 mmol) was treated with benzaldehyde dimethyl acetal(15.0 g, 100 mmol) and stirred at 65 °C for 16 h under aspirator. The reaction was cooled to room temperature, quenched with triethylamine (30 mL), diluted with ethyl acetate (300 mL), and washed with distilled water(2 x 50 mL), saturated aqueous sodium bicarbonate(3 x 50 mL) and distilled water(2 x 50 mL). The solvent was removed under reduced pressure. The product was separated by flash chromatography, which was white solid (22.8 g, 80.0 mmol, 76%). ^{1}H NMR and ^{13}C NMR spectra match those previously reported.[25]; methyl 2,3,4-tri-*O*

benzyl-6-*O*-tosyl-α-D-glucopyranoside: p-Toluene sulfonyl chloride (1.10g, 5.77 mmol) was added to a solution of methyl 2,3,4-tri-O-benzyl glucopyranoside (2.22 g, 4.79 mmol) in pyridine (45 mL) at room temperature for 3 h. The mixture was concentrated down under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 1/1, v/v) to afford methyl 2,3,4-tri-O-benzyl-6-*O*-tosyl-α-D-gluco-pyranoside as a yellowish syrup (2.90 g, 4.68 mmol, quantitative yiel);

R$_f$ 0.81 (ethyl acetate/hexane, 1/1, v/v);

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.99-7.16 (m, 19 H, 3 Ar, 1 tosyl), 5.00 (d , 1 H, *J* = 10.8 Hz), 4.86 (d, 1 H, *J* = 10.8 Hz), 4.83 (dd, 2 H, *J*$_1$ = 6.3, *J*$_2$ = 11.1 Hz), 4.69 (d, 1 H, *J* = 12.0 Hz), 4.56(d, 1 H, *J* = 2.7), 4.46 (d, 1 H, *J* = 10.8 Hz), 4.17 (m, 1 H), 4.01 (t, 1 H, *J* = 12.4 Hz), 3.82 (m, 1 H), 3.52 (dd, 1 H, *J*$_1$ = 3.6, *J* = 9.6 Hz), 3.50 (m, 1 H), 3.30 (s, 3 H, -OCH$_3$), 2.40 (s, 3 H, tosyl-CH$_3$);

^{13}C NMR (100 MHz, CDCl$_3$) δ 145.1-127.3 (aromatics), 98.3, 82.1, 80.0, 76.0, 75.2, 73.7, 68.8, 68.7, 55.6, 21.9.

MS (ESI) m/z = 641 [M+Na]$^+$;

Methyl 2,3,4-tri-*O*-benzyl-6-deoxy-α-D-glucopyranoside (10):

To a solution of methyl 2,3,4-tri-*O*-benzyl-6-*O*-tosyl-α-D-glucopyranoside (2.32 g, 3.75 mmol) in Et$_2$O (45 mL) was added a solution of lithium aluminum hydride (0.043 g, 1.13 mmol) in Et$_2$O (35 mL) at room temperature for 2 h. The mixture was diluted with Et$_2$O (2 × 50 mL), acidified with 2 N HCl (2 × 45 mL), and washed with water (2 × 50 mL). The

solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 1/1, v/v) to afford **10** as a yellowish syrup (1.65 g, 3.68 mmol, 98%);

R_f 0.47 (ethyl acetate/hexane, 2/3, v/v);

¹H NMR (400 MHz, CDCl₃) δ 7.42-7.26 (m, 10 H, Ar), 5.01 (d, 1 H, J = 10.8 Hz), 4.92 (d, 1 H, J = 10.8 Hz), 4.88 (dd, 2 H, J_1 = 12.0, J_2 = 13.6 Hz), 4.69 (d, 1 H, J = 12.0 Hz), 4.65 (d, 1H, J = 10.8 Hz), 4.56 (d, 1 H, J = 3.6 Hz), 3.99 (t, 1 H, J = 9.2 Hz), 3.76 (m, 1 H), 3.54 (dd, 1 H, J_1 = 3.6, J_2 = 9.6 Hz), 3.40 (s, 3 H, -OCH₃), 3.15 (t, 1 H, J = 9.2 Hz), 1.27 (d, 3 H, J = 6.4 Hz);

¹³C NMR (100 MHz, CDCl₃) δ 139.0-127.9 (aromatic), 98.2, 84.0, 82.1, 80.5, 76.0, 75.6, 73.6, 66.8, 55.3, 18.2.

MS (ESI) m/z = 471 [M+Na]⁺;

2,3,4-tri-*O*-benzyl-6-deoxy-α-D-glucopyranose:

Compound **10** (2.18 g, 4.86 mmol) was treated with glacial acetic acid (45 mL) and 3.0 M sulfuric acid (2.3 mL) at 85 °C for 3 h. The mixture was cooled to ambient temperature and then diluted with ice water (60 mL) extracted by toluene (80 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (3 × 45 mL), water (2 × 40 mL), and brine (50 mL). The solvent was removed under reduced pressure. The product was isolated by silica gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford

84% an yield of anomeric mixture as 56/44 α/β mixture 2,3,4-tri-*O*-benzyl-6-deoxy-α-D-glucopyranose as a white solid (0.99 g, 2.28 mmol, 56%) and β compound (0.78 g, 1.79 mmol, 44%);

R$_f$ 0.26 (ethyl acetate/hexane, 3/7, v/v);

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.41-7.29 (m, 30 H, Ar), 5.15-5.14 (d, 1 H, J_1 = 3), 4.97-4.95 (t, 1 H, J = 2.4 Hz), 4.93-4.87 (m, 4 H), 4.86-4.76 (m, 4 H), 4.73-4.62 (m, 4 H), 4.04-3.89 (m, 2 H), 3.67-3.54 (m, 2 H), 3.49-3.35 (m, 2 H), 3.25-3.11 (m, 2 H), 1.31 (d, 3 H, J = 6.4 Hz), 1.26 (d, 3 H, J = 6.4 Hz);

^{13}C NMR (100 MHz, CDCl$_3$) δ 138.9-125.6 (aromatics), 97.4, 91.3, 84.7, 83.9, 83.6, 83.6, 81.8, 80.7, 76.0, 75.9, 75.6, 75.1, 73.5, 71.6, 67.2, 18.2, 18.2.

MS (ESI) m/z = 434 [M]$^+$;

Dibenzyl-(2,3,4-tri-*O*-benzyl-6-deoxy-α-D-glucopyranosyl) phosphate:

A solution of 2,3,4-tri-*O*-benzyl-6-deoxy-α/β-D-glucopyranose (0.32 g, 0.74 mmol) in acetonitrile (6 mL) was added to a mixture of dibenzyl diisopropyl phosphoramidite (0.35 mL) and N-phenyl imidazolium triflate (0.33 g) in dry acetonitrile (8 mL). The TLC showed no starting material left (R$_f$ 0.75, ethyl acetate/hexane, 1/1, v/v). The solvent was evaporated under reduced pressure and then 3-chloroperoxybenzoic acid (0.13 g, 0.76 mmol) was added to a solution of the mixture in 15 mL dichloromethane at -50 °C; the resulting solution was stirred at 0 °C for 30 min. The mixture was diluted with CH$_2$Cl$_2$ (30 mL) and a 20% Na$_2$SO$_3$ solution (40 mL) was added and stirred at room temperature for 3

h. The organic layer was washed with saturated $NaHCO_3$ (3 × 30 mL), water (3 × 30 mL), brine (40 mL), and dried over magnesium sulfate. The product was isolated by silica gel column chromatography (chloroform/diethyl ether, 9/1, v/v) to afford 87% of dibenzyl-(2,3,4-tri-O-benzyl-6-deoxy-α-D-glucopyranosyl) phosphate as a white solid (0.23 g, 0.33 mmol, 52% for α compound, 0.22 g, 0.31 mmol, 48% for β compound);

R_f 0.34 (chloroform/diethyl ether, 9/1 v/v);

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31-7.24 (m, 25 H, 5 benzyl), 5.98 (dd, 1 H, J_1 = 3.2, J_2 = 8.8 Hz), 5.07 (m, 4H), 4.88 (dd, 2 H, J_1 = 10.8, J_2 = 11.2 Hz), 4.75 (dd, 2 H, J_1 = 6.0 Hz, J_2 = 11.2 Hz), 4.63 (dd, 2 H, J_1 = 11.2, J_2 = 19.2 Hz), 3.38 (m, 2 H), 3.57 (m, 1 H), 3.14 (t,1 H, J = 9.2 Hz), 1.26 (d, 3 H, J = 6.4 Hz);

^{13}C NMR (100 MHz, $CDCl_3$) δ 138.7, 138.6, 138.5, 138.3, 138.2, 129.9, 129.6, 128.6, 128.5, 128.5, 128.4, 128.2, 128.0, 127.9, 127.9, 127.9, 127.8, 127.7, 95.4, 83.0, 81.0, 79.7, 75.7, 75.4, 73.0, 69.3, 69.1, 17.8;

^{31}P NMR (162 MHz, $CDCl_3$) δ 2.92.

MS (ESI) m/z = 717 [M+Na]$^+$

Disodium 6-deoxy-α-D-glucopyranosyl phosphate (3):

A solution of dibenzyl-(2,3,4-tri-O-benzyl-6-deoxy-α-D-glucopyranosyl) phosphate (0.20 g, 0.28 mmol) in MeOH (15 mL) was stirred with 20 wt.% palladium hydroxide on carbon (0.08 g) with saturated sodium bicarbonate (3 mL) under atmospheric H_2 (gas) at ambient temperature for 12 h. The mixture was filtered over Celite and the filtrate was

concentrated under reduced pressure to afford **3** as a white solid (0.07 g, 0.29 mmol, 98%,

weighed after treating with an acidic ion-exchange resin to pH 4 resulting in the exchange

of one sodium ion. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra match those previously

reported.[9b];

R$_f$ 0.14 (methanol/ethylether/hexane, 5/1/1 v/v/v);

MS (ESI) m/z = 243 [M-H]$^-$;

3,4,6-tri-O-benzyl-2-deoxy-α/β-D-glucopyranose (12)[16]:

The product was isolated by silica gel column chromatography (ethyl acetate/hexane, 2/3,

v/v) to afford **12** as the white solid (0.43 g, 1.0 mmol, 51% for α compound, 0.39 g, 0.89

mmol, 49% for β compound);

R$_f$ 0.47 (ethyl acetate/hexane, 2/3, v/v);

^{1}H NMR (CDC$_{l3}$, 300 MHz) δ 7.37-7.23 (m, 15 H, Ar), 5.41 (d, 1 H, J = 2.4 Hz), 4.91 (d,

1 H, J = 10.8 Hz), 4.67 (dd, 2 H, J_1 = 9.0, J_2 = 12.3 Hz), 4.54 (m, 4 H), 4.05 (m, 1 H),

3.67 (dd, 2 H, J_1 = 7.8, J_2 = 11.4 Hz), 3.51 (m, 1 H), 2.30 (m, 1 H), 1.68 (m, 1H);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 138.8, 138.6, 138.5, 138.5, 138.2, 138.2, 128.7, 128.7,

128.7, 128.6, 128.6, 128.6, 128.3, 128.2, 128.0, 127.9, 127.9, 127.9, 127.9, 127.8, 94.4,

92.4, 79.4, 78.8, 78.0 75.2, 75.1, 73.7, 73.7, 72.0, 71.7, 71.0, 69.5, 69.5, 38.2, 35.7;

MS (ESI) m/z = 434 [M]$^+$;

Dibenzyl-(3,4,6-tri-*O*-benzyl-2-deoxy-α-D-glucopyranosyl) phosphate:

A solution of 3,4,6-tri-*O*-benzyl-2-deoxy-α/β-D-glucopyranose (0.32 g, 0.74 mmol) in acetonitrile (6 mL) was added to a mixture of dibenzyl diisopropyl phosphoramidite (0.35 mL) and N-phenyl imidazolium triflate (0.33 g) in dry acetonitrile (8 mL). The TLC showed no starting material left (R_f 0.75, ethyl acetate/hexane, 1/1, v/v). The solvent was evaporated under reduced pressure and then 3-chloroperoxybenzoic acid (0.13 g, 0.76 mmol) was added to a solution of the mixture in 15 mL dichloromethane at -50 °C; the resulting solution was stirred at 0 °C for 30 min. The mixture was diluted with CH_2Cl_2 (30 mL) and a 20% Na_2SO_3 solution (40 mL) was added and stirred at room temperature for 3 h. The organic layer was washed with saturated $NaHCO_3$ (3 × 30 mL), water (3 × 30 mL), brine (40 mL), and dried over magnesium sulfate. The product was isolated by silica gel column chromatography (chloroform/diethyl ether, 9/1, v/v) to afford 87% of dibenzyl-(3,4,6-tri-*O*-benzyl-2-deoxy-α-D-glucopyranosyl) phosphate as a white solid (0.23 g, 0.33 mmol, 52% for α compound, 0.22 g, 0.31 mmol, 48% for β compound);

R_f 0.47 (ethyl acetate/hexane, 2/3, v/v);

^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.37-7.23 (m, 50 H, Ar), 4.61 (m, 2 H), 4.50 (m, 4 H), 4.09 (m, 2 H), 3.67 (m, 3 H), 3.50 (m, 2 H), 2.30 (m, 1 H), 1.71 (m, 1 H);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 138.9, 138.8, 138.6, 138.3, 135.8, 135.7, 128.9, 128.9, 128.8, 128.8, 128.8, 128.7, 128.7, 128.7, 128.6, 128.6, 128.6, 128.6, 128.3, 128.3, 128.3, 128.3, 128.2, 128.2, 128.2, 128.2, 127.9, 127.9, 127.9, 127.8, 127.8, 127.8, 94.4, 92.3, 79.6, 78.8, 77.8, 75.1, 73.7, 72.0, 71.7, 70.9, 69.6, 69.5, 67.446.4, 46.3, 35.9;

MS (ESI) m/z = 717 [M+Na]$^+$;

Disodium 2-deoxy-α-D-glucopyranosyl phosphate (6):

A solution of dibenzyl-(3,4,6-tri-O-benzyl-2-deoxy-α-D-glucopyranosyl) phosphate (0.15 g, 0.22 mmol) in MeOH (10 mL) was stirred with 20 wt.% palladium hydroxide on carbon (0.01 g) with saturated sodium bicarbonate (1.0 mL) under atmospheric H$_2$ (gas) at ambient temperature for 12 h. The mixture was filtered over Celite and the filtrate was concentrated under reduced pressure to afford **6** as a white solid (0.05 g, 0.21 mmol, 97%, weighed after treating with an acidic ion-exchange resin to pH 4 resulting in the exchange of one sodium ion.

MS (ESI) m/z = 243 [M-H]$^-$; ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra match those previously reported.[9b]

Methyl 2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (13):

A compound of methyl-4,6-O-benzylidene-α-D-glucopyranoside (4.5 g, 14.3 mmol) was refluxed in dry toluene (250 mL) at 95 °C for 8 h with a Dean-Stark apparatus. The dimutyltin dimethoxide (3.95 mL) was added to the solution of methyl 4,6-O-benzylidene-α-D-glucopyanoside at room temperature under nitrogen gas. The mixture was refluxed for 3h and then added benzylbromide (2.04 mL, 16.7 mmol) and tetrabutyl ammonium iodide (7.2 g, 19.5 mmol). The mixture was stirred at 92 °C under N$_2$. After 2.5 days, TLC was completely converted to products. The mixture was concentrated under reduced

pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 1/1, v/v) to afford **13** as the white solid (4.57 g, 11.3 mmol, 79 %).

R$_f$ 0.60 (ethyl acetate/hexane, 1/1, v/v);

MS (ESI) m/z = 411 [M+Na]$^+$;

^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.53-7.33 (m, 10 H, Ar), 5.53 (s, 1 H), 4.80 (d, 1 H, J = 12.3), 4.80 (d, 1 H, J = 12.3), 4.71 (d, 1 H, J = 12.3), 4.63 (d, 1 H, J = 3.6), 4.28 (dd, 1 H, J = 4.5), 4.17 (t, 1 H, J = 9.3), 3.83 (ddd, 1 H, J = 4.5), 3.71 (t, 1 H, J = 9.9), 3.50 (t, 1 H, J = 9.9), 3.47 (t, 1 H, J = 9.3), 3.39 (s, 3 H, -OCH$_3$);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 138.5-126.6, 102.3, 98.6, 77.2, 74.3, 71.6, 69.9, 64.5, 55.6, 30.6, 16.9;

Methyl 3-(1,1'-thiocarbonyl imidazole)-4,6-*O*-benzylidene-α-D-glucopyranoside (14):

1,1'-thiocarbonyl diimidazole (1.69 g, 9.5 mmol) was added to a solution of a compound of **13** (2.55 g, 6.3 mmol) in THF (40 mL) at 25 °C and then the mixture was stirred at 70 °C under N$_2$ for 4 h. The solution was concentrated in reduced pressure. The product was isolated by silica gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford **14** as an yellowish syrup (2.95 g, 5.7 mmol, 91 %);

R$_f$ 0.29 (ethyl acetate/hexane, 2/3, v/v);

MS (ESI) m/z = 521 [M+Na]$^+$;

^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.20 (t, 1 H, J = 1.2 Hz), 7.30 (m, 10 H), 7.04 (dd, 1 H, J_1

= 0.6, J_2 = 1.5 Hz), 6.38 (t, 1 H, J = 9.6 Hz), 5.47 (s, 1 H), 4.82 (d, 1 H, J = 3.6 Hz), 4.65

(m, 2 H), 4.54 (d, 1 H, J = 12.6 Hz), 4.31 (dd, 1 H, J_1 = 4.8, J_2 = 10.2 Hz), 3.98 (dd, 1 H,

J_1 = 4.5, J_2 = 9.6 Hz), 3.74 (m, 3 H), 3.47 (s, 3 H);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 183.7, 137.3, 137.1, 136.8, 136.8, 130.8, 129.4, 129.4,

128.8, 128.8, 128.6, 128.5, 128.4, 128.2, 118.6, 101.8, 98.6, 81.6, 79.6, 79.4, 77.2, 73.6,

69.1, 62.5, 55.8;

Methyl 2-O-benzyl-4,6-O-benzylidene-3-deoxy-α-D-glucopyranoside (15):

A compound of **14** (1.89 g, 3.7 mmol) in dry toluene (5 mL) was added dropwise 45 min.

to a stirred of refluxing toluene (20 mL) and tributyltin hydride (2.1 g, 7.4 mmol, 2 eq.)

under nitrogen. The solution was cooled to room temperature after the TLC was

completely migrated to product, and then concentrated. The mixture was extracted with hot

acetonitrile (60 mL) and washed with hexane (2 x 30 mL). The solvent was removed under

reduced pressure. The product was isolated by silica gel column chromatography (ethyl

acetate/hexane, 2/3, v/v) to afford **15** as an yellowish syrup (1.3 g, 3.2 mmol, 86 %);

R$_f$ 0.71 (ethyl acetate/hexane, 2/3, v/v);

MS (ESI) m/z = 395 [M+Na]$^+$;

^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.51-730 (m, 10 H, Ar), 5.51 (s, 1 H), 4.70 (d, 1 H, J = 3.6

Hz), 4.69 (d, 1 H, J = 12.3 Hz), 4.59 (d, 1 H, J = 12.3 Hz), 4.26 (dd, 1 H, J_1 = 4.2, J_2 =

9.9 Hz), 3.77 (m, 1 H), 3.74 (d, 1 H, J = 10.2 Hz), 3.65 (m, 1 H), 3.55 (m, 1 H), 3.46 (s, 3 H), 2.31 (m, 1 H), 2.06 (dd, 1 H, J_1 = 11.4, J_2 = 23.1 Hz);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 138.1, 137.6, 129.4, 129.3, 128.7, 128.6, 128.2, 128.1, 126.4, 102.0, 98.2, 76.9, 74.1, 71.3, 69.6, 64.2, 55.4, 30.3;

Methyl 2-O-benzyl-3-deoxy-α-D-glucopyranoside:

The 2 % sulfuric acid (0.22 g, 2.1 eq.) was added to the solution of methyl 2-O-benzyl-4,6-O-benzylidene-3-deoxy-α-D-glucopyranoside (1.02 g, 2.63 mmol) in methanol (30 mL) at room temperature for 3 h. The solution was concentrated under reduced pressure and extracted with ethyl acetate (40 mL) and washed with water. The solvent was removed under reduced pressure. The product was isolated by silica gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford methyl 2-O-benzyl-3-deoxy-α-D-glucopyranoside as the white solid (0.63 g, 2.3 mmol, 89 %);

R_f 0.23 (methanol/ethyl acetate/hexane, 0.5/2/3, v/v);

MS (ESI) m/z = 291 [M+Na]$^+$;

^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.35-7.28 (m, 5 H, Ar), 4.63 (d, 1 H, J = 3.3 Hz), 4.59 (dd, 2 H, J_1 = 12.6, J_2 = 24.0 Hz), 3.77 (d, 1 H, J = 4.2 Hz), 3.59 (m, 1 H), 3.49 (t, 1 H, J = 4.2 Hz), 3.46 (m, 1 H), 3.40 (s, 3 H), 2.60 (s, 2 H, 2 -OH), 2.20(m, 1 H), 1.85 (dd, 1 H, J_1 = 11.4, J_2 = 23.1 Hz);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 138.2, 128.7, 128.1, 128.1, 97.4, 73.8, 72.2, 71.3, 66.2, 62.8, 55.2, 33.3;

Methyl 2,4,6-tri-*O*-benzyl-3-deoxy-α-D-glucopyranoside (16):

Sodium hydride (0.21 g, 50 % of oil) was slowly added into a solution of methyl 2-*O*-benzyl-3-deoxy-α-D-glucopyranoside (0.61 g, 2.28 mmol) in DMF (20 mL) at room temperature under nitrogen. Benzylbromide (0.67 mL, 5.5 mmol) was added to the solution. The mixture was stirred at room temperature for 4 h under N_2 and then diluted with ethyl acetate (70 mL) and washed with water (40 mL x 3), aq. saturated sodium bicarbonate (15 mL x 2). The solution was dried with magnesium sulfate. The solvent was removed under reduced pressure. The product was isolated by silica gel column chromatography (ethyl acetate/hexane, 7/3, v/v) to afford **16** as the white solid (0.90g, 2.0 mmol, 89%);

R_f 0.81 (ethyl acetate/hexane, 5/5, v/v);

MS (ESI) m/z = 471 $[M+Na]^+$;

^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.37-7.18 (m, 15 H, Ar), 4.70 (d, 1 H, J = 3.3 Hz), 4.62 (dd, 2 H, J_1 = 2.1, J_2 = 12.3 Hz), 4.59(dd, 2 H, J_1 = 2.1, J_2 = 12.3 Hz), 4.49 (d, 1 H, J = 12.0 Hz), 4.36 (d, 1 H, J = 11.4 Hz), 3.72 (m, 3 H), 3.54 (m, 2 H), 3.41 (s, 3 H), 2.33 (m, 1 H), 1.86 (dd, 1 H, J_1 = 11.4, J_2 = 23.1 Hz);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 138.4, 138.3, 138.3, 128.7, 128.6, 128.6, 128.6, 128.6, 128.3, 128.1, 128.1, 127.9, 127.9, 127.9, 127.8, 97.4, 73.8, 73.7, 72.0, 71.3, 71.0, 70.7, 68.9, 55.1, 30.3, 16.6;

2,4,6-tri-*O*-benzyl-3-deoxy-α/β-D-glucopyranose:

Compound **16** (0.84 g, 1.9 mmol) was treated with glacial acetic acid (25 mL) and 3.0 M

sulfuric acid (1.3 mL) at 85 °C for 3 h. The mixture was cooled to ambient temperature

and then diluted with ice water (40 mL) extracted by toluene (80 mL). The organic layer

was washed with saturated aqueous sodium bicarbonate (3 × 45 mL), water (2 × 40 mL),

and brine (50 mL). The solvent was removed under reduced pressure. The product was

isolated by silica gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford

84% an yield of anomeric mixture as 75/25 α/β mixture 2,4,6-tri-O-benzyl-3-deoxy-α/β-D-

glucopyranose as a white solid (0.51 g, 1.2 mmol, 75%) and β compound (0.17 g, 0.39

mmol, 25%);

R$_f$ 0.85 (ethyl acetate/hexane, 2/3, v/v);

MS (ESI) m/z = 434 [M]$^+$;

^{1}H NMR (CDC$_{l3}$, 300 MHz) δ 7.36-7.17 (m, 30 H, Ar), 5.26 (d, 1 H, J = 3.3 Hz), 4.82 (d,

1 H, J = 12.0 Hz), 4.68 (m,2 H), 4.58 (m, 6 H), 4.53 (m, 2 H), 4.38 (m,2 H), 4.00 (m, 1 H),

3.74 (m, 2 H), 3.66 (m, 2 H), 3.56 (m, 3 H), 3.50 (m, 2 H), 3.29 (m, 1 H), 2.54 (m,1 H),

2.37 (m, 1 H), 1.84 (dd, 1 H, J_1 = 11.4, J_2 = 23.1 Hz), 1.50 (dd, 1 H, J_1 = 11.7, J_2 = 23.3

Hz);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 138.8, 138.6, 138.6, 138.6, 138.6, 138.6, 138.5, 138.5,

138.5, 138.5, 138.5, 138.4, 138.4, 128.6, 128.6, 128.6, 128.4, 128.4, 128.2, 128.2, 128.2,

128.2, 128.1, 128.1, 128.1, 128.0, 128.0, 128.0, 127.9, 127.9, 127.9, 127.9, 99.2, 90.4,

78.0, 76.3, 73.9, 73.3, 73.7, 72.5, 72.0, 71.5, 71.1, 71.0, 70.7, 69.4, 69.0, 34.8, 29.7;

Dibenzyl-(2,4,6-tri-*O*-benzyl-3-deoxy-α/β-D-glucopyranosyl) phosphate:

A solution of 2,4,6-tri-*O*-benzyl-3-deoxy-α/β-D-glucopyranose (0.28 g, 0.65 mmol) in acetonitrile (6 mL) was added to a mixture of dibenzyl diisopropyl phosphoramidite (0.32 mL) and *N*-phenyl imidazolium triflate (0.30 g) in dry acetonitrile (8 mL). The TLC showed no starting material left (R_f 0.75, ethyl acetate/hexane, 1/1, v/v). The solvent was evaporated under reduced pressure and then 3-chloroperoxybenzoic acid (0.13 g, 0.76 mmol) was added to a solution of the mixture in 15 mL dichloromethane at -50 °C; the resulting solution was stirred at 0 °C for 30 min. The mixture was diluted with CH_2Cl_2 (30 mL) and a 20% Na_2SO_3 solution (40 mL) was added and stirred at room temperature for 3 h. The organic layer was washed with saturated $NaHCO_3$ (3 × 30 mL), water (3 × 30 mL), brine (40 mL), and dried over magnesium sulfate. The product was isolated by silica gel column chromatography (chloroform/diethyl ether, 9/1, v/v) to afford 83% of dibenzyl-(2,4,6-tri-*O*-benzyl-3-deoxy-α/β-D-glucopyranosyl) phosphate as a white solid (0.28 g, 0.40 mmol, 75% for α compound, 0.10 g, 0.13 mmol, 25% for β compound);

MS (ESI) m/z = 717 [M+Na]$^+$;

^{31}**P NMR** (D_2O, 400 MHz) δ 1.9;

^{1}**H NMR** (CDCl$_3$, 300 MHz) δ 7.37-7.23 (m, 50 H, Ar), 5.80 (dd, 1 H, J_1 = 3.3, J_2 = 8.4 Hz), 5.02 (m, 9 H), 4.77 (m, 5 H), 4.60 (m, 4 H), 3.75 (m, 4 H), 3.56 (m, 3 H), 2.26 (m, 2 H), 1.65 (m, 2 H);

^{13}**C NMR** (CDCl$_3$, 300 MHz) δ138.9, 138.9, 138.9, 138.8, 138.8, 138.7, 138.6, 138.6, 138.6, 138.6, 138.5, 138. 5, 128.6, 128.6, 128.6, 128.6, 128.6, 128.6, 128.6, 128.6, 128.3,

128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.9, 127.9, 127.8, 97.7, 92.4, 83.9, 80.6,

78.5, 75.3, 75.2, 73.8, 73.6,73.5, 72.6, 72.4, 72.4, 71.2, 71.1 67.0, 69.9, 32.3, 32.3;

Disodium 3-deoxy-α/β-D-glucopyranosyl phosphate (5):

A solution of dibenzyl-(2,4,6-tri-O-benzyl-3-deoxy-α/β-D-glucopyranosyl) phosphate

(0.32 g, 0.46 mmol) in MeOH (15 mL) was stirred with 20 wt.% palladium hydroxide on

carbon (0.08 g) with saturated sodium bicarbonate (3 mL) under atmospheric H_2 (gas) at

ambient temperature for 12 h. The mixture was filtered over Celite and the filtrate was

concentrated under reduced pressure to afford **5** as a white solid (0.11 g, quantitative yield,

weighed after treating with an acidic ion-exchange resin to pH 4 resulting in the exchange

of one sodium ion.

MS (ESI) m/z = 243 [M-H]$^-$; ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra match those

previously reported.[22] ;

Methyl 2,3,6-tri-O-benzyl-4-(1,1'-thiocarbonyl imidazole)-α-D-glucopyranoside:

1,1'-thiocarbonyl diimidazole (1.1 g, 6.3 mmol), was added to a solution of a compound of

17 (1.94 g, 4.2 mmol) in THF (40 mL) at 25 °C and then the mixture was stirred at 70 °C

under N_2 for 4 h. The solution was concentrated in reduced pressure. The product was

isolated by silica gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford

methyl 2,3,6-tri-O-benzyl-4-(1,1'-thiocarbonyl imidazole)-α-D-glucopyranoside as an

yellowish syrup (2.3 g, 4.0 mmol, 96 %);

R$_f$ 0.47 (ethyl acetate/hexane, 2/3, v/v);

MS (ESI) m/z = 597 [M+Na]$^+$;

^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.05 (t, 1 H, J = 1.2 Hz), 7.41-7.14 (m,15 H), 6.97 (m,1

H), 5.87 (dd, 1 H, J_1 = 9.3, J_2 = 9.9 Hz), 4.87 (dd, 2 H, J_1 = 6.6, J_2 = 11.7 Hz), 4.68 (d, 1

H, J = 12.0 Hz), 4.64(d, 1 H, J = 3.6 Hz), 4.58 (d, 1 H, J = 11.7 Hz), 4.43(dd, 2 H, J_1 =

11.7, J_2 = 13.5 Hz), 4.08 (t, 1 H, J = 9.3 Hz), 3.97 (m, 1 H), 3.70 (dd, 1 H, J_1 = 3.6, J_2 =

9.3 Hz), 3.58 (dd, 1 H, J_1 = 3.6, J_2 = 10.8 Hz), 3.45 (dd, 1 H, J_1 = 4.5, J_2 = 10.5 Hz), 3.44

(s, 3 H);

^{13}C NMR (CDCl$_3$, 300 MHz) δ183.5, 138.0, 137.9, 137.6, 136.9, 130.9, 128.8, 128.5,

128.5, 128.5, 128.5, 128.5, 128.4, 128.4, 128.4, 128.3, 128.3, 128.2, 128.0, 127.9, 127.9,

118.2, 98.4, 80.0, 79.2, 78.6, 75.6, 74.0, 73.9, 69.0, 68.3, 55.9;

Methyl 2,3,6-tri-*O*-benzyl-4-deoxy-α-D-glucopyranoside (18):

A compound of methyl 2,3,6-tri-*O*-benzyl-4-(1,1'-thiocarbonyl imidazole)-α-D-gluco-

pyranoside (2.04 g, 3.6 mmol) in dry toluene (6 mL) was added dropwise 45 min. to a

stirred of refluxing toluene (40 mL) and tributyltin hydride (1.26mL, 7.1 mmol, 2 eq.)

under nitrogen. The solution was cooled to room temperature after the TLC was

completely migrated to product, and then concentrated. The mixture was extracted with hot

acetonitrile (60 mL) and washed with hexane (40 mL x 2). The solvent was removed under

reduced pressure. The product was isolated by silica gel column chromatography (ethyl

acetate/hexane, 2/3, v/v) to afford **18** as an yellowish syrup (1.26 g, 2.8 mmol, 79 %);

MS (ESI) m/z = 471 [M+Na]$^+$;

^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.41-7.26 (m, 15 H, Ar), 4.87 (d, 1 H, J = 12.3 Hz), 4.78

(dd, 2 H, J_1 = 11.7, J_2 = 18.3 Hz), 4.74 (m, 2 H), 4.58 (s, 2 H), 3.96 (m, 2 H), 3.51 (m, 3

H) 3.41 (s, 3 H), 2.10 (m, 1 H), 1.54 (dd, 1 H, J_1 = 12.0 J_2 = 23.2 Hz);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 139.2, 138.8, 138.8, 128.6, 128.3, 127.9, 127.9, 127.8,

127.4, 99.3, 80.7, 75.6, 73.7, 73.7, 72.4, 72.7, 66.9, 55.4;

2,3,6-tri-*O*-benzyl-4-deoxy-αβ-D-glucopyranose:

Compound **18** (1.08 g, 2.4 mmol) was treated with glacial acetic acid (45 mL) and 3.0 M

sulfuric acid (2.3 mL) at 85 °C for 3 h. The mixture was cooled to ambient temperature

and then diluted with ice water (60 mL) extracted by toluene (80 mL). The organic layer

was washed with saturated aqueous sodium bicarbonate (3 × 45 mL), water (2 × 40 mL),

and brine (50 mL). The solvent was removed under reduced pressure. The product was

isolated by silica gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford 65

% an yield of anomeric mixture as 55/45 α/β mixture 2,3,6-tri-*O*-benzyl-4-deoxy-α-D-

glucopyranose as a white solid (0.37 g, 0.86 mmol, 55%) and β compound (0.31 g, 0.71

mmol, 45%);

R$_f$ 0.47 (ethyl acetate/hexane, 2/3, v/v);

MS (ESI) m/z = 434 [M]$^+$;

¹H NMR (CDCl₃, 300 MHz) δ 7.47-7.26 (m, 30 H, Ar), 5.30 (d, 1 H, J = 2.1 Hz), 4.96 (d, 1 H, J = 11.1 Hz), 4.83 (dd, 2 H, J_1 = 4.8, J_2 = 11.7 Hz), 4.70 (m, 7 H), 4.56 (m, 4 H), 4.23 (m, 1 H), 3.96 (m, 2 H), 3.60 (m, 4 H), 3.46 (m, 4 H), 3.30 (m, 2 H), 2.08 (m, 2 H), 1.49 (m, 2 H);

¹³C NMR (CDCl₃, 300 MHz) δ 138.7, 138.7, 138.6, 138.6, 138.6, 136.5, 128.6, 128.6, 128.6, 128.6, 128.6, 128.6, 128.6, 128.6, 128.3, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.9, 127.9, 127.8, 97.7, 92.4, 83.9, 80.6, 78.5, 75.3, 75.2, 73.8, 73.6, 73.5, 72.6, 72.4, 72.4, 71.2, 67.2, 33.8;

Dibenzyl-(2,3,6-tri-*O*-benzyl-4-deoxy-α-D-glucopyranosyl) phosphate:

A solution of 2,3,6-tri-*O*-benzyl-4-deoxy-α-D-glucopyranose (0.38 g, 0.88 mmol) in acetonitrile (6 mL) was added to a mixture of dibenzyl diisopropyl phosphoramidite (0.35 mL) and *N*-phenyl imidazolium triflate (0.33 g) in dry acetonitrile (8 mL). The TLC showed no starting material left (R_f 0.75, ethyl acetate/hexane, 1/1, v/v). The solvent was evaporated under reduced pressure and then 3-chloroperoxybenzoic acid (0.13 g, 0.76 mmol) was added to a solution of the mixture in 15 mL dichloromethane at -50 °C; the resulting solution was stirred at 0 °C for 30 min. The mixture was diluted with CH₂Cl₂ (30 mL) and a 20% Na₂SO₃ solution (40 mL) was added and stirred at room temperature for 3 h. The organic layer was washed with saturated NaHCO₃ (3 × 30 mL), water (3 × 30 mL), brine (40 mL), and dried over magnesium sulfate. The product was isolated by silica gel column chromatography (chloroform/diethyl ether, 9/1, v/v) to afford 75% of dibenzyl-

(2,3,6-tri-*O*-benzyl-4-deoxy-α-D-glucopyranosyl) phosphate as a white solid (0.25 g, 0.36

mmol, 55% for α compound, 0.20 g, 0.30 mmol, 45% for β compound);

R_f 0.47 (ethyl acetate/hexane, 2/3, v/v);

MS (ESI) m/z = 717 [M+Na]$^+$;

^{31}P NMR (CDCl$_3$, 400 MHz) δ 0.89;

^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.41-7.24 (m, 25 H, Ar), 6.23 (dd, 1 H, J_1 = 2.4, J_2 = 5.1

Hz), 5.08 (m, 6 H), 4.80 (m, 2 H), 4.70 (m, 3 H), 4.52 (dd, 2 H, J_1 = 9.0, J_2 = 11.4 Hz),

4.15 (m, 1 H), 3.92 (m, 1 H), 3.59 (m, 1 H), 3.45 (d, 2 H, J = 3.6 Hz), 2.41 (m, 1 H), 1.65

(dd, 1 H, J_1 = 9.3, J_2 = 18.3 Hz);

^{13}C NMR (CDCl$_3$, 300 MHz) δ 138.4, 138.4, 138.4, 138.3, 138.3, 128.7, 128.7, 128.6,

128.6, 128.6, 128.6, 128.3, 128.3, 128.3, 128.3, 128.2, 128.2, 128.2, 128.2, 127.9, 127.9,

127.8, 127.8, 127.8, 97.7, 92.4, 83.9, 80.6, 78.5, 75.3, 75.2, 73.8, 73.6, 73.5, 72.6, 72.4,

72.4, 71.2, 67.2, 33.5;

Disodium 4-deoxy-α-D-glucopyranosyl phosphate (4):

A solution of dibenzyl-(2,3,6-tri-*O*-benzyl-4-deoxy-α-D-glucopyranosyl) phosphate (0.25

g, 0.36 mmol) in MeOH (15 mL) was stirred with 20 wt.% palladium hydroxide on carbon

(0.08 g) with saturated sodium bicarbonate (3 mL) under atmospheric H$_2$ (gas) at ambient

temperature for 12 h. The mixture was filtered over Celite and the filtrate was

concentrated under reduced pressure to afford **4** as a white solid (0.08 g, quantitative yield,

weighed after treating with an acidic ion-exchange resin to pH 4 resulting in the exchange of one sodium ion.

MS (ESI) m/z = 243 [M-H]$^-$; ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra match those previously reported.[9b]

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CHAPTER 4

Surprising Bacterial Nucleotidyltransferase Selectivity in the Conversion of Carbaglucose-1-phosphate

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Abstract

The drive to understand the molecular determinants of carbohydrate binding as well as the search for more chemically and biochemically stable sugar derivatives and carbohydrate-based therapeutics has led to the synthesis of a variety of analogues that replace the glycosidic oxygen with sulfur or carbon. In contrast, the effect of substitution of the ring oxygen on the conformations and enzymatic tolerance of sugars has been largely neglected, in part because of the difficulty in obtaining these analogues. Herein we report the first synthesis of the carbocyclic version of the most common naturally occurring sugar-1-phosphate, glucose-1-phosphate, and its evaluation with bacterial and eukaryotic sugar nucleotidyltransferases. In contrast to results with the eukaryotic enzyme, the carbaglucose-1-phosphate serves as a substrate for the bacterial enzyme to provide the carbocyclic uridinediphosphoglucose. This result demonstrates the first chemoenzymatic strategy to this class of glycosyltransferase inhibitors and stable activated sugar mimics for cocrystallization with glycosyltransferases and their glycosyl acceptors. This difference in

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turnover between enzymes also suggests the possibility of using sugar nucleo-tidyltransferases in vivo to convert prodrug forms of glycosyltransferase inhibitors. In addition, we report several microwave-assisted reactions, including a five minute Ferrier rearrangement with palladium, that accelerate the synthesis of carbocyclic sugars for further studies.

Introduction

Herein we report the first synthesis of the carbocyclic version of the most common naturally occurring sugar-1-phosphate, glucose-1-phosphate, and its evaluation with a bacterial and a eukaryotic sugar nucleotidyltransferase. In contrast to results with the eukaryotic enzyme, the carbocyclic glucose-1-phosphate serves as a substrate for the bacterial enzyme to provide the carbocyclic uridinediphosphoglucose. This result demonstrates not only the first chemoenzymatic strategy to this class of glycosyltransferase inhibitors but also the possibility of using sugar nucleotidyltransferases in vivo to convert prodrug forms of glycosyltransferase inhibitors. In addition, we report several general microwave-assisted reactions that serve to accelerate the synthesis of carbasugars for further studies.

Sugars mediate a large variety of protein-protein and cell-cell interactions implicated in disease states, thereby making carbohydrate-based therapeutics attractive.[1] The drive to understand the molecular determinants of these carbohydrate binding interactions as well as the search for more chemically and biochemically stable sugar derivatives has led to the synthesis of a variety of analogues that replace the glycosidic

oxygen with sulfur or carbon.[2] In contrast, the effect of substitution of the ring oxygen on

the conformations and biological activity of pyranose sugars has been largely neglected, in

part because of the difficulty in obtaining these analogues. The small amount of existing

biological data outside glycosidase inhibitors has shown sulfur versions of activated

nucleotidediphosphosugars to be poor substrates for glycosyltransferases,[3] whereas the

carbocyclic versions serve as inhibitors of these enzymes.[4] These substrates are difficult to

synthesize chemically but led us to consider strategies to form such analogues biologically.

However, no data are available for the tolerance of sugar nucleotidyltransferases to ring

oxygen substitutions.

The ubiquitous Leloir pathway glycosyltransferases require activated sugars that are

produced by sugar nucleotidyltransferases (Figure 1). The latter enzymes have been

proposed as possible antibiotic targets,[5a,5g,6] but facile screening assays and knowledge of

differences in carbohydrate substrate recognition between bacteria and humans are needed

to design compounds with the necessary selectivity. Presently, only a few structures of

sugar nucleotidyltransferases are known.[5] No sequence homology between enzymes of

similar function from eukaryotes and prokaryotes is apparent; therefore, differences in

substrate recognition and turnover could be expected for exploitation in antibiotic design.
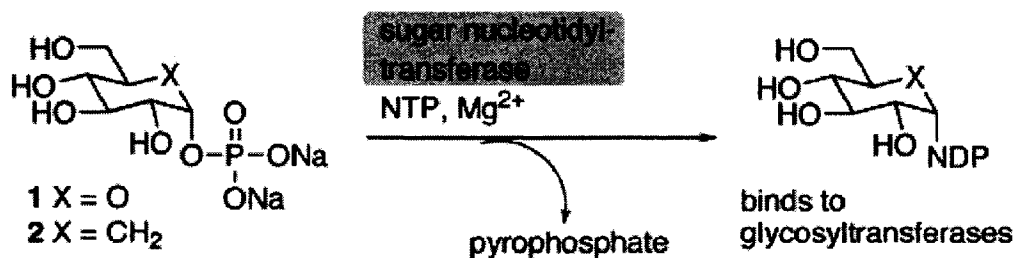
Figure 1 Sugar nucleotidyltransferases catalyze the formation of activated nucleotide diphosphate sugars that are substrates for, when X = O, but inhibitors of, when X = CH$_2$, Leloir pathway glycosyltransferases.

Results and discussion

Discovery of the tolerance of sugar nucleotidyltransferases to carbocyclic sugar substrates first required the synthesis of carbasugar-1-phosphates. Several strategies have been applied to the synthesis of carbasugars, including radical cyclizations, conversion of quinic acid or bacterial metabolites, zirconium-mediated ring contractions, ring-closing metathesis, Cope rearrangements, and anionic or transition metal-mediated cyclizations.[7,4b] The Ferrier rearrangement is the most common approach to carbasugars, but mercury is often used and the rearrangements require hours. Many reaction times, especially for transition metal-mediated reactions, can be significantly shortened with microwave assistance.[8] Indeed, we found that not only could the Ferrier rearrangement be carried out in less time and with higher yields in the presence of palladium dichloride but that the synthesis of the necessary precursor 5 could also be hastened by the application of microwave-assisted reactions rather than conventional heating (Scheme 1 and Supporting Information). Iodination[9] of the selectively protected glucose precursor 3 took place in a minute; the subsequent elimination reaction took place in a half hour under microwave irradiation without any competing side reactions. The development of this series of microwave-assisted reactions significantly shortened the time to form the core carbocyclic

structure from a protected D-glucose, thereby providing a route that should be applicable to a variety of other sugars for studies with sugar nucleotidyltransferases.
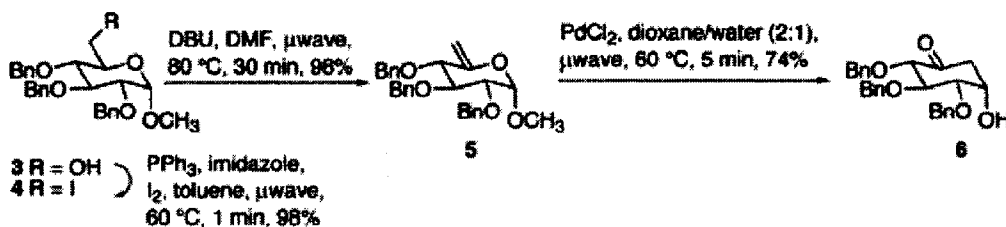
Scheme 1

To complete the synthesis of the desired carbaglucose-1-phosphate (2), the free hydroxyl group of the Ferrier product 6 was silylated and methylation with Tebbe's reagent yielded the corresponding exo-methylene derivative 7 (Scheme 2). Hydroboration/oxidation of alkene 7 resulted in alcohol 8 with the desired equatorial configuration in 87% overall yield. Alcohol protection by benzylation followed by silyl protecting group removal produced free hydroxyl 9. Treatment of 9 with dibenzyl diisopropyl phosphoramidite in the presence of a catalytic amount of N-(phenyl)imidazolium triflate[10] produced a phosphite intermediate that was oxidized in situ to the phosphate. Global debenzylation afforded the desired carbasugar 2.
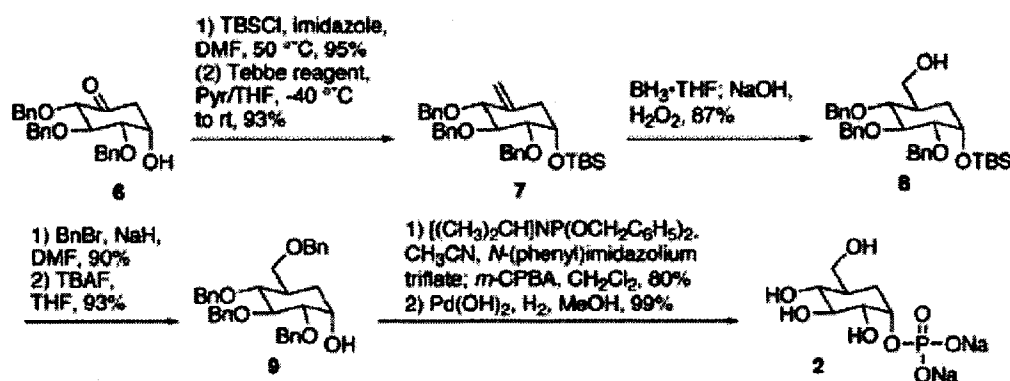
Scheme 2

With the desired carbocyclic analogue in hand, we next compared its interactions

with representative bacterial and eukaryotic sugar nucleotidyltransferases. A major

obstacle in testing nonnatural substrates and inhibitors with this class of enzymes has been

the lack of a rapid assay to determine kinetic parameters for a variety of compounds;

however, the recent development of an electrospray ionization mass spectrometry (ESI-

MS)-based assay[11] circumvents these difficulties. The carbasugar 2 was first incubated

with a glucose-1-phosphate uridylyltransferase from *Escherichia coli*, which is known to

also accept thymidine triphosphate and is homologous to a range of bacterial sugar

nucleotidyltransferases.[12] Surprisingly, the analogue was turned over to produce the

carbocyclic version of UDP-glucose. In fact, carbasugar 2 exhibited K_m values (17 ± 2 μM)

similar to those of the natural substrate 1 (12 ± 2 μM). However, a lower turnover rate

meant that k_{cat}/K_m values (s^{-1} μM^{-1}) were 0.0020 for the analogue compared to 1.45 for the

natural substrate. In contrast, the corresponding sugar nucleotidyltransferase from yeast,

which is also homologous to the human enzyme, showed no evidence for carbocyclic

UDP-glucose formation even with 5-fold higher enzyme concentrations.

These data provide the first evidence that carbocyclic sugar analogues can serve to inhibit the class of enzymes that provide sugar nucleotide donors to glycosyltransferases, which make compounds such as the cyclic glucans that render some bacteria resistant to standard antibiotics.[13] The relatively weak inherent affinity of glycosyltransferase substrates has been a large hurdle in the design of potent and, most importantly, selective inhibitors for this class of enzymes. This difficulty stems in part from the fact that a large portion of the protein binding energy of these charged sugar substrates comes from the phosphates and not from the carbohydrate itself. However, the incorporation of a catalytic step in addition to a binding step can create a more prominent distinction between prokaryotes and eukaryotes. Differences in substrate turnover that have been exploited in the design of cancer drugs (cancer cells often upregulate enzymes that convert prodrugs)[14] also can serve as a potential strategy to increase the selectivity of drugs targeted for carbohydrate biosynthetic pathways. Compounds can be designed to make use of not only the inherent differences in bacterial versus eukaryotic substrate binding pockets but also the differences in substrate turnover.

Conclusion

We have shown that sugar nucleotidyltransferases provide means for the facile chemoenzymatic synthesis of carbocyclic versions of activated sugars for further studies of the effects of this substitution on the conformations and properties of carbasugars and for cocrystallization studies with glycosyltransferases and their respective glycosyl acceptors.

Experimental Section

General methods

Trizma base and trizma hydrochloride were purchased from Fischer Scientific Company (Hanover Park, IL). Thermostable inorganic pyrophosphatase (IPP) from *Thermococcus litoralis* (EC 3.6.1.1, M0296S) was purchased from New England Biolabs. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted and used without further purification. Yeast UDP-glucose pyrophosphorylase (EC 2.7.7.9, 9026-22-6) was desalted by buffer exchange using a Microcon Centrifugal Filter Device, MWCO 10 kDa (Millipore, Billerica, MA). *E. coli* UDP-glucose pyrophosphorylase was prepared as previously reported.[1] Nanopure water (18.1 MHz) prepared from a Barnstead E-pure water purification system was employed throughout. Reaction solvents were distilled from calcium hydride for dichloromethane and from sodium metal and benzophenone for diethyl ether. Amberlyst 15 ion-exchange resin was washed repeatedly with methanol before use. All other commercial reagents and solvents were used as received without further purification. The reactions were monitored and the R_f values determined using analytical thin layer chromatography (tlc) with 0.25 mm EM Science silica gel plates (60F-254). The developed tlc plates were visualized by immersion in *p*-anisaldehyde solution followed by heating on a hot plate. Flash chromatography was performed with Selecto Scientific silica gel, 32-63 μm particle size. All moisture-sensitive reactions were performed in flame- or oven-dried glassware under a nitrogen atmosphere. Bath temperatures were used to record the reaction temperature in all cases run without microwave irradiation. All reactions were stirred magnetically at ambient temperature

unless otherwise indicated. ^{1}H NMR, ^{13}C NMR, and ^{31}P spectra were obtained with a Bruker DRX400 at 400 MHz, 100 MHz, and 162 MHz respectively. ^{1}H NMR spectra were reported in parts per million (δ) relative to CDCl$_3$ (7.27 ppm) as an internal reference. ^{13}C NMR spectra were reported in parts per million (δ) relative to CDCl$_3$ (77.23 ppm) or CD$_3$OD (49.15 ppm). ^{31}P NMR spectra were reported in δ relative to H$_3$PO$_4$ (0.00 ppm) as an internal reference. Microwave-assisted reactions were performed in a CEM (Matthews, NC) Model Discover microwave.

MS Conditions. The instrument calibration conditions were as previously reported.[15]

Enzyme Activity Determination. The enzymatic activity determination conditions were also as previously reported.[15] One unit of enzyme is defined as one micromole glucose-1-phosphate consumed per minute.

Standard Curves. Calibration curves of UDP-carbocyclic-glucose and dTDP-carbocyclic-glucose were created by allowing the *E. coli* UDP-glucose pyrophosphorylase enzyme to react with carbocyclic-glucose-1-phosphate (2-25 μM) in the presence of UTP and TTP (300 μM) in a reaction mixture containing Tris buffer (25 mM, pH 7.8), IPP (0.2 U), and UDP-glucose pyrophosphorylase (0.1 U). Reactions were carried out at 37 °C and after 1 h, as determined by the complete disappearance of carbocyclic-glucose-1-phosphate by ESI-MS, 30 μL of the reaction mixture was quenched by addition to 30 μL of 70% methanol/water containing AMP (6 μM) as an internal standard. The quenched solutions were centrifuged 10 minutes at 10,000 × g to precipitate the protein. Aliquots (15 μL) of the reaction mixtures were diluted with 135 μL of acetonitrile/water/triethylamine

(35/65/0.2). These samples (30 μL) were subjected to analysis via ESI-MS to determine the amount of UDP-carbocyclic-glucose (m/z = 563) and dTDP-carbocyclic-6-deoxyglucose (m/z = 561) produced as compared to the internal standard, AMP (m/z = 346). The relative intensity of the product was then plotted against the concentration of carbocyclic-glucose-1-phosphate.

Kinetic analysis.

Measurement of K_M and V_{max} of carbocyclic glucose-1-phosphate. The values of K_M and V_{max} for the *E. coli* UDP-glucose pyrophosphorylase were derived from enzymatic reactions run in triplicate and determined from the initial rates of UDP/dTDP-carbocyclic-glucose formation by monitoring the rate of UDP/dTDP-carbocyclic-glucose formation using ESI-MS. The reaction conditions were as previously reported.[1] The enzymatic reaction was initiated by the addition of carbocyclic-glucose-1-phosphate (2 – 200 μM) to a reaction mixture containing Tris buffer (25 mM, pH 7.6), IPP (0.2 U), UDP-glucose pyrophosphorylase (0.01 U), and UTP or dTTP (300 μM). Reactions were carried out at 37 °C and after 5 minutes 30 μL of the reaction mixture was quenched by addition to 30 μL of 70% methanol/water containing AMP (6 μM) as an internal standard. The quenched solutions were centrifuged 10 minutes at 10,000 × g to precipitate the protein. Aliquots (15 μL) of the reaction mixtures were diluted with 135 μL of acetonitrile/water/triethylamine (35/65/0.2). These samples (30 μL) were subjected to analysis via ESI-MS to determine the amount of UDP/dTDP-carbocyclic-glucose formed.

Activity determination of yeast UDP-glucose pyrophosphorylase. The activity of yeast UDP-glucose pyrophosphorylase was determined by monitoring the appearance of UDP-carbocyclic-glucose. The enzymatic reaction was initiated by the addition of carbocyclic-glucose-1-phosphate (5 mM) to a reaction mixture containing Tris buffer (25 mM, pH 7.6), IPP (0.2 U), UDP-glucose pyrophosphorylase (0.05 U), and UTP (10 mM). Reactions were carried out at 37 °C and after 30 and 90 minutes 15 µL of the reaction mixture was quenched by addition to 30 µL of 70% methanol/water containing AMP (3 mM) as an internal standard. The quenched solutions were centrifuged 10 minutes at 10,000 × g to precipitate the protein. Aliquots (15 µL) of the reaction mixtures were diluted with 135 µL of acetonitrile/water/triethylamine (35/65/0.2). These samples (3 µL) were subjected to analysis via ESI-MS to determine the amount of UDP -carbocyclic-glucose formed.
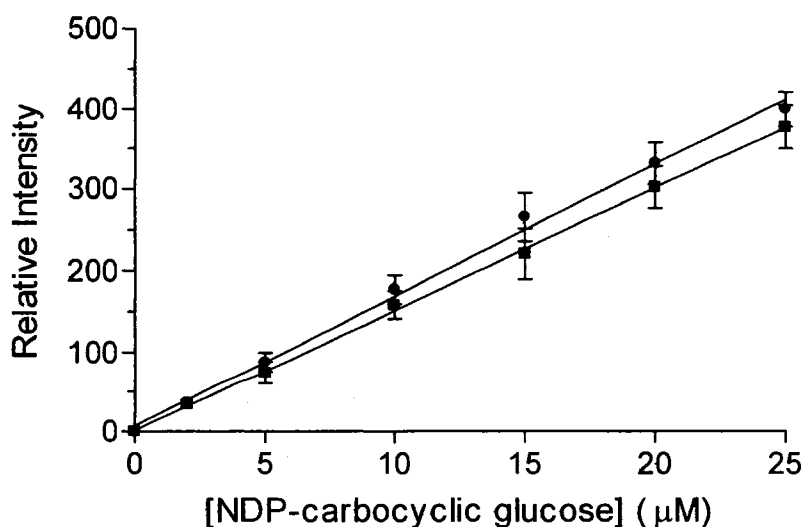
Figure S1. Calibration curve for UDP-carbocyclic-glucose (■) and dTDP-carbocyclic-glucose (●).
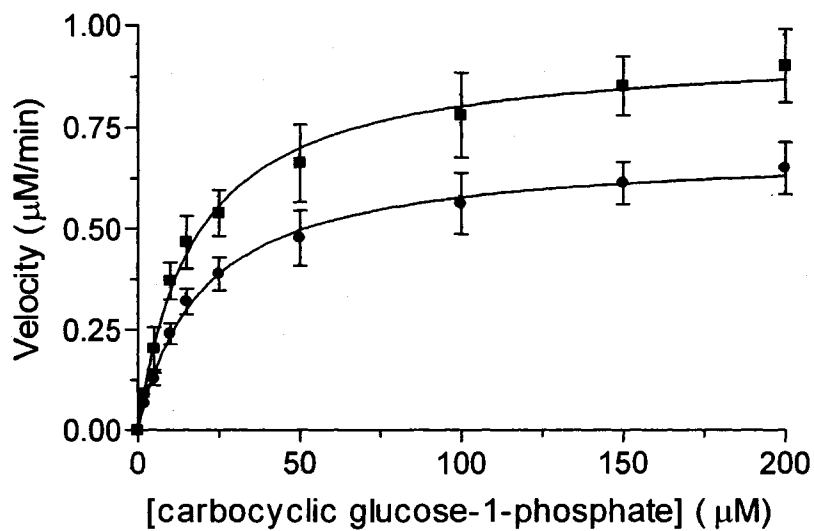
Figure S2. Michealis-Menten plots in the direction of UDP-glucose synthesis for *E. coli*

UDP-glucose pyrophosphorylase and carbocyclic-glucose-1-phosphate in the presence of

UTP (■) and dTTP (●).

Table S1. Kinetic analysis of UDP-glucose pyrophosphorylase. Error bars represent the

standard error in the non-linear regression of three independent trials.

Source	Substrates	K_m (μM)	V_{max} (μM/min)	k_{cat}/K_m (s^{-1} μM^{-1})
E. coli	1 + UTP[a]	12 ± 2	1.15 ± 0.06	1.45
	1 + dTTP[a]	13 ± 2	0.90 ± 0.05	1.05
	carbo + UTP	17 ± 2	0.94 ± 0.03	0.0021
	carbo + dTTP	20 ± 2	0.69 ± 0.02	0.0013
yeast	1 + UTP[a]	7 ± 1	3.8 ± 0.1	10.9
	1 + ATP[a]	7 ± 1	1.67 ± 0.07	4.8
	carbo + UTP	N/A		
	carbo + ATP	N/A		

[a] Zea, C. J.; Pohl, N. L. *Anal. Biochem.* **2004**, *328*, 196-202.

N/A – no activity

Synthetic procedures.

4
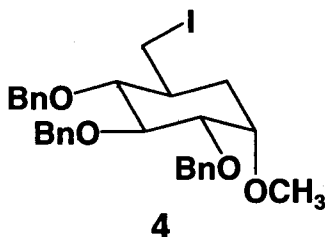
Methyl-6-deoxy-6-iodo-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside.

To a solution of methyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside[2] (4.2 g, 9.0 mmol) in toluene (60 mL) containing triphenylphosphine (4.7 g, 18.0 mmol) and imidazole (3.1 g, 45 mmol) was added iodine (4.6 g, 18 mmol). The mixture was stirred at 60 °C with 300 W power in the microwave for 5 min. The mixture was concentrated under reduced pressure, and the resulting solid was removed by filtration and rinsed with ethyl acetate (80 mL). The organic filtrate was concentrated under reduced pressure. The product was purified by column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford **4** as a white solid (5.1 g, 8.9 mmol, 98%).

R_f 0.78 (ethyl acetate/hexane, 2/3 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.36-7.30 (m, 15 H), 5.00 (dd, 2 H, J_1 = 10.8 Hz, J_2 = 22.0 Hz), 4.83 (dd, 2 H, J_1 = 7.2, J_2 = 5.6 Hz), 4.69 (t, 2 H, J = 10.8 Hz), 4.64 (d, 1 H, J = 3.2 Hz), 4.05 (t, 1 H, J = 9.2 Hz), 3.58 (dd, 1 H, J_1 = 3.6, J_2 = 9.6 Hz), 3.48 (m, 2 H), 3.44 (s, 3H), 3.37 (d, 1 H, J = 9.2 Hz), 3.33 (m, 1 H).

^{13}C NMR (100 MHz, CDCl$_3$) δ 138.6, 138.1, 138.1, 128.6, 128.6, 128.6, 128.2, 128.0, 127.8, 98.2, 81.7, 81.6, 80.2, 75.9, 75.5, 73.5, 69.4, 55.6, 7.8.

MS (ESI) *m/z* = 597 [M+Na]$^+$.

BnO—⟍⫝̸⟍—O
BnO—⟍⟍
BnO⟍OCH₃

5
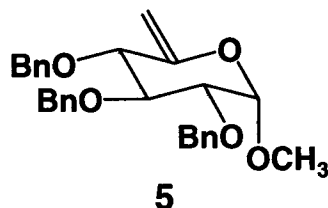
Methyl-6-deoxy-2,3,4-tri-*O*-benzyl-α-D-hex-5-enopyranoside.

To a solution of the methyl-6-deoxy-6-iodo-2,3,4-tri-O-benzyl-α-D-glucopyranoside (3.8 g, 6.6 mmol) in DMF (70 mL), 1,8-dizabicyclo[5.4.0]undec-7-ene (1.1 mL, 7.2 mmol) was added at room temperature. The mixture was stirred at 80 °C with 300 W power in the microwave for 30 min. The mixture was diluted with ethyl acetate (120 mL), washed with saturated aqueous $NaHCO_3$ (3 x 60 mL), and washed with water (2 x 60 mL). The organic solvent was removed under reduced pressure. The product was purified by column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford **5** as a white solid (2.9 g, 6.5 mmol, 98%).

R_f 0.75 (ethyl acetate/hexane, 2/3 v/v).

¹H NMR (400 MHz, CDCl₃) δ 7.38-7.31 (m, 15 H), 4.96-4.69 (m, 8 H), 4.65 (d, 1 H, J = 3.2 Hz), 4.00 (t, 1 H, J = 9.2 Hz), 3.94 (m, 1 H), 3.63 (dd, 1 H, J_1 = 3.2, J_2 = 9.2 Hz), 3.45 (s, 3 H).

¹³C NMR (100 MHz, CDCl₃) δ 153.9, 138.9, 138.3, 138.2, 128.8, 128.7, 128.7, 128.6, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 99.3, 97.1, 81.5, 79.8, 79.5, 76.0, 74.8, 73.9, 55.7.

MS (ESI) m/z = 447 [M+H]⁺.

6
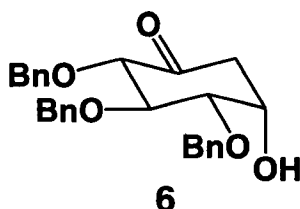
2,3,4-tri-*O*-benzyl-α-D-cyclohexanone.

To a solution of methyl-6-deoxy-2,3,4-tri-O-benzyl-α-D-hex-5-enopyranose (2.3 g, 5.2 mmol) in 1,4-dioxane (20 mL) and water (10 mL), PdCl$_2$ (0.37 g, 2.0 mmol) was added at room temperature. The mixture was stirred at 60 °C with 300 W power in the microwave for 5 min. The mixture was diluted with ethyl acetate (60 mL), washed with saturated aqueous NaHCO$_3$ (2 x 50 mL), and washed with water (2 x 50 mL). The organic solvent was removed under reduced pressure. The product was purified by silica gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford a 93% yield of the mixture of α/β compounds, **6** as a white solid (1.7 g, 3.8 mmol, 80% of the mixture) and the β compound (0.41 g, 0.95 mmol, 20% of the mixture).

R$_f$ 0.27 (ethyl acetate/hexane, 3/7 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.38-7.25 (m, 15 H), 4.94 (dd, 2 H, J_1 = 10.8, J_2 = 11.2 Hz), 4.78 (dd, 2 H, J_1 = 8.0, J_2 = 11.6 Hz), 4.69 (d, 1 H, J = 11.6 Hz), 4.53 (d, 1 H, J = 11.6 Hz), 4.22 (m, 1 H), 4.01 (m, 2 H), 3.76 (m, 1 H), 2.64 (dd, 1 H, J_1 = 3.6, J_2 = 14.0 Hz), 2.41 (s, 1 H), 2.40 (m, 1 H).

^{13}C NMR (100 MHz, CDCl$_3$) δ 202.3, 138.4, 137.7, 137.7, 128.6, 128.4, 128.4, 128.1, 127.9, 127.8, 127.8, 85.3, 81.8, 81.6, 76.0, 73.5, 73.3, 66.7, 42.7.

MS (ESI) *m/z* = 455 [M+Na]$^+$.

6a
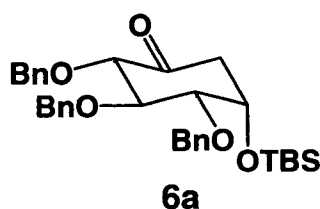
TBS-2,3,4-tri-*O*-benzyl-α-D-*gluco*-cyclohexanone.

To a solution of the 2,3,4-tri-O-benzyl-α-D-cyclohexanone (1.5 g, 3.5 mmol) in DMF (30 mL), *tert*-butyldimethylsilyl (TBS) chloride (1.1 g, 7.0 mmol) and imidazole (0.5 g, 7.0 mmol) were added at room temperature. The mixture was stirred at 50 °C. The solid byproduct was removed by filteration and rinsed with ethyl acetate (40 mL). The resulting filterate was diluted with ethyl acetate (60 mL), washed with saturated aqueous NaHCO$_3$ (3 x 50 mL), and washed with water (2 x 50 mL). The organic layer was concentrated under reduced pressure. The product was isolated by silica gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford **6a** as a solid (1.8 g, 3.3 mmol, 95%).

R$_f$ 0.63 (ethyl acetate/hexane, 3/7 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.40-7.30 (m, 15 H), 4.97 (d, 1 H, J = 12.0 Hz), 4.88 (dd, 2 H, J_1 = 10.8, J_2 = 20.4 Hz), 4.78 (s, 2 H), 4.62 (d, 1 H, J = 11.6 Hz), 4.32 (m, 1 H), 4.11 (t, 1 H, J = 8.8 Hz), 4.04 (d, 1 H, J = 9.2 Hz), 3.71 (dd, 1 H, J_1 = 2.0, J_2 = 8.8 Hz), 2.52 (m, 1 H), 2.47 (m, 1 H), 0.95 (s, 9 H), 0.10 (d, 6 H, J = 4.4 Hz).

^{13}C NMR (100 MHz, CDCl$_3$) δ 204.2, 138.6, 138.3, 137.9, 128.4, 128.3, 128.2, 128.1, 127.8, 127.6, 127.5, 127.2, 85.8, 82.3, 81.9, 75.7, 73.5, 73.1, 67.9, 45.2, 25.8, 18.2, -4.3.

MS (ESI) *m/z* = 570 [M+Na]$^+$.

7
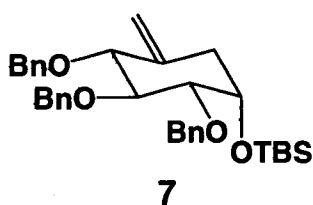
TBS-2,3,4-tri-*O*-benzyl-α-D-*gluco*-hex-5-enopyranoside

A 0.5 M solution of Tebbe reagent in toluene (1.2 mL, 2.1 mmol) was added dropwise to a solution of TBS-2,3,4-tri-O-benzyl-α-D-gluco-cyclohexanone (0.48 g, 0.9 mmol) and pyridine (2 mL) in THF (8 mL) at -40 °C. The mixture was stirred at room temperature for 1 h and then the mixture was cooled to -40 °C, quenched dropwise with a saturated NaHCO$_3$ (15 drops), stirred at room temperature for 30 min, filtered through a short column of Celite, and concentrated. The product was purified by column chromatography (ethyl acetate/hexane, 2/3, v/v) to yield **7** as a white solid (0.4 g, 0.8 mmol, 93%)

R_f 0.67 (ethyl acetate/hexane, 3/7 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.48-7.31 (m, 15 H), 5.36 (br s, 1 H), 4.97 (br s, 1 H), 4.92 (d, 2 H, J = 2.8 Hz), 4.79 (d, 2 H, J = 6.8 Hz), 4.74 (d, 2 H, J = 3.2 Hz), 4.31 (dd, 1 H, J_1 = 5.2, J_1 = 7.6 Hz), 4.19 (m, 1 H), 3.89 (m, 2 H), 3.47 (m, 1 H), 2.46 (dd, 1 H, J_1 = 5.6, J_2 = 18.42 Hz), 2.12 (m, 1 H), 0.95 (s, 9 H), 0.13 (d, 6 H, J = 4.4 Hz).

^{13}C NMR (100 MHz, CDCl$_3$) δ 167.8, 139.3, 139.0, 138.8, 128.5, 128.4, 128.3, 128.2, 128.2, 128.0, 128.0, 127.7, 127.7, 127.7, 127.5, 127.4, 110.9, 83.5, 83.4, 83.3, 75.7, 73.9, 72.8, 68.3, 68.2, 38.9, 28.8, 18.4, -4.7, -4.7.

MS (ESI) *m/z* = 568 [M+H]$^+$.

8
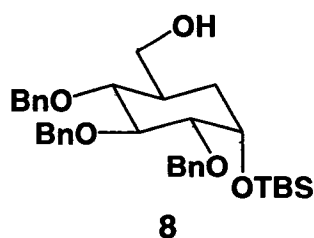
Carbocyclic TBS-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside

To a solution of BH_3·THF (1.0 M a solution of THF, 2.2 mL, 3 eq.) in anhydrous THF at 0

°C was added, dropwise, a solution of TBS-2,3,4-tri-O-benzyl-α-D-*gluco*-hex-5-

enopyranoside (0.39 g, 0.72 mmol) in anhydrous THF (4 mL). The mixture was stirred at

room temperature for 2 h and then 3 N NaOH (2 mL) and 30 % H_2O_2 (2 mL) were added

dropwise. The mixture was stirred for 0.5 h and then diluted with CH_2Cl_2 (40 mL). The

mixture was washed with saturated aqueous $NaHCO_3$ (3 x 30 mL), water (3 x 30 mL), and

brine (40 mL) and then dried over magnesium sulfate. The product was isolated by silica

gel column chromatography (chloroform/ethyl ether, 9/1, v/v) to afford **8** as a white solid

(0.35 g, 0.62 mmol, 87 %).

R_f 0.42 (ethyl acetate/hexane, 3/7 v/v).

¹H NMR **¹H NMR** (400 MHz, CDCl₃) δ 7.31-7.24 (m, 15 H), 4.78-4.54 (m, 6 H), 4.08 (m,

1 H), 4.00 (m, 2 H), 3.62 (m, 2 H), 3.36 (m, 1 H), 2.24 (m, 1 H), 1.90 (m, 1 H), 1.50 (m, 1

H), 0.90 (s, 9 H), 0.06 (d, 6 H, J = 5.6 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 138.9, 138.7, 138.5, 128.5, 128.4, 128.2, 128.1, 128.0,

127.9, 127.8, 127.7, 127.6, 127.4, 82.4, 82.0, 81.9, 73.7, 69.7, 65.6, 39.4, 25.9, 18.2, -4.5.

MS (ESI) m/z = 586 [M+Na]⁺.
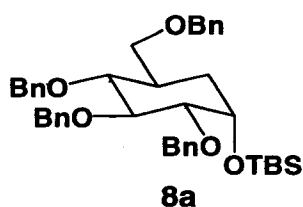
8a

Carbocyclic TBS-2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranoside.

Sodium hydride (0.04 g, 1.8 mmol) was slowly added into a solution of compound **8** (0.723 g, 1.56 mmol) in DMF (30 mL) at room temperature. Benzylbromide (0.22 mL, 1.8 mmol) was added to the solution. The mixture was stirred at room temperature for 4 h under N_2, diluted with ethyl acetate (60 mL), washed with water (40 mL x 3), and aqueous saturated sodium bicarbonate (35 mL). The solution was dried over magnesium sulfate. The solvent was removed under reduced pressure. The product was purified by silica gel column chromatography (ethyl acetate/hexane, 7/3, v/v) to afford **8a** as a solid (0.78 g, 1.40 mmol, 90%).

R_f 0.64 (ethyl acetate/hexane, 3/7 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.39-7.23 (m, 20 H), 4.83-4.47 (m, 8 H), 4.10 (m, 1 H), 3.82 (m, 1 H), 3.72 (t, 1 H, J = 8.4 Hz), 3.57 (m, 1 H), 2.34 (m, 1 H), 2.06 (m, 1 H), 1.43 (m, 1 H), 0.94 (s, 9 H), 0.09 (s, 6 H).

^{13}C NMR (100 MHz, CDCl$_3$) δ 139.4, 139.0, 138.9, 138.6, 128.5, 128.4, 128.4, 128.3, 128.2, 128.2, 128.0, 127.9, 127.8, 127.8, 127.7, 127.7, 127.5, 127.4, 127.3, 79.9, 79.9, 79.8, 79.8, 73.8, 73.1, 72.1, 71.2, 70.5, 37.2, 26.1, 18.3, -4.5.

MS (ESI) *m/z* = 676 [M+Na]$^+$.

9
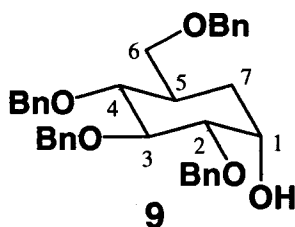
^{1}H-^{1}H COSY NMR to confirm the stereochemistry

Carbocyclic 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranose.

To a solution of carbocyclic TBS-2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside (0.72 g, 1.1 mmol) in THF (10 mL) was added dropwise a 1 M solution of tetrabutylammonium fluoride (2.2 mL, 5.5 mmol) in THF at 0 °C. The cooling bath was removed and the reaction mixture stirred for 1 h at room temperature and then diluted with ethyl acetate (40 mL). The mixture was washed with saturated aqueous NaHCO$_3$ (2 x 20 mL), water (3 x 20 mL), and brine (30 mL) and then dried over magnesium sulfate. The organic layer was washed with brine, dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure to give a crude product which was purified by column chromatography to obtain compound **9** (0.55 g, 1.0 mmol, 93%) as an oil.

R$_f$ 0.35 (ethyl acetate/hexane, 2/3 v/v).

^{1}**H NMR** (400 MHz, CDCl$_3$) δ 7.35-7.25 (m, 20 H), 4.68-4.50 (m, 8 H), 3.96 (m, 1 H, 1-H), 3.85 (t, 1 H, J = 5.2 Hz, 3-H), 3.67 (t, 1 H, J = 4.4 Hz, 4-H), 3.60 (m, 3 H, 2-H, 6-H), 2.71 (br s, 1 H, -OH), 2.30 (m, 1 H, 5-H), 1.88 (m, 1 H, 7-H), 1.56 (m, 1 H, 7-H).

^{13}**C NMR** (100 MHz, CDCl3) δ 138.7, 138.5, 138.5, 138.3, 128.5, 128.4, 128.4, 128.3, 128.3, 128.0, 127.9, 127.9, 127.8, 127.8, 127.7, 127.6, 79.9, 79.9, 79.8, 79.8, 73.6, 73.2, 72.6, 70.6, 67.7, 36.5, 28.5.

MS (ESI) $m/z = 455$ [M+Na]$^+$.
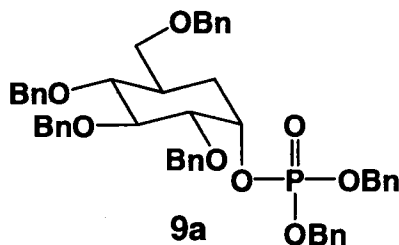
Carbocyclic Dibenzyl-(2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl) phosphate.

The compound 2,3,4,6-tetra-O-benzyl-6-deuterium-α-D-glucopyranose (0.32 g, 0.59 mmol) in acetonitrile (4 mL) was added to a mixture of dibenzyl diisopropyl phosphoramidite (0.35 mL) and N-phenyl immidazolium triflate (0.33 g) in anhydrous acetonitrile (8 mL). When the tlc showed no remaining starting material (R_f 0.75, ethyl acetate/hexane, 1/1, v/v), the solvent was removed under reduced pressure. Then 3-chloroperoxybenzoic acid (0.13 g, 0.76 mmol) was added to the solution of the mixture in 15 mL dichloromethane at −50 °C and the resulting solution was stirred at 0 °C for 30 min. The mixture was diluted with CH_2Cl_2 (30 mL). A 20 % aqueous Na_2SO_3 (40 mL) solution was added and the mixture was stirred at room temperature for 3 h. The organic layer was washed with saturated aqueous $NaHCO_3$ (3 x 30 mL), water (3 x 30 mL), and brine (40 mL) and then dried over magnesium sulfate. The product was isolated by silica gel column chromatography (chloroform/ethylether, 9/1, v/v) to afford **9a** as a white solid (0.38 g, 0.48 mmol, 80%).

R_f 0.34 (chloroform/ethylether, 2/3 v/v).

¹H NMR (400 MHz, CDCl₃) δ 7.31-7.17 (m, 30 H), 5.06-4.39 (m, 12 H), 3.78 (m, 1 H), 3.71 (t, 1 H, J = 4.4 Hz), 3.61 (m, 2 H), 3.43 (t, 1 H, J = 7.6 Hz), 2.27 (m, 1 H), 2.17 (m, 1 H), 1.60 (m, 1 H).

¹³C NMR (100 MHz, CDCl₃) δ 138.5, 138.5, 138.1, 137.87, 136.1, 136.1, 128.8, 128.6, 128.6, 128.6, 128.4, 128.4, 128.4, 128.3, 128.1, 127.9, 127.9, 127.9, 127.9, 127.8, 127.8, 127.7, 127.5, 127.5, 76.4, 76.4, 76.4, 76.3, 76.3, 76.3, 73.3, 73.0, 72.2, 69.2, 69.1, 36.6, 26.3.

³¹P NMR (162 MHz, CDCl₃) δ 2.79.

MS (ESI) m/z = 822 [M+Na]⁺.

2
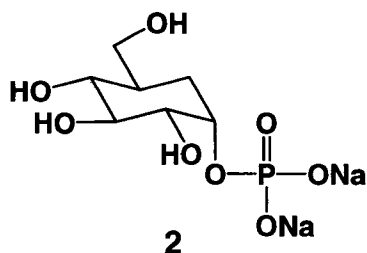
Carbocyclic disodium α-D-glucopyranosyl phosphate

A solution of **9a** (0.20 g, 0.25 mmol) in MeOH (15 mL) was stirred with 20 wt. % palladium hydroxide on carbon (0.08 g) with saturated sodium bicarbonate (3 mL) under atmospheric H₂ (gas) at ambient temperature overnight. The mixture was filtered over Celite and the filtrate was concentrated under reduced pressure to afford **2** as a white solid (0.065 g, 0.25 mmol, quantitative).

R$_f$ 0.14 (methanol/ethyl ether/hexane, 5/1/1 v/v/v).

¹H NMR (400 MHz, D₂O) δ 4.31 (br s, 1 H), 3.77 (br s, 1 H), 3.64 (m, 3 H), 3.41 (m, 1 H), 2.02 (m, 1 H), 1.84 (m, 1 H), 1.54 (m, 1 H).

¹³C NMR (100 MHz, CD₃OD) δ 75.0, 73.0, 70.9, 70.6, 63.2, 38.5, 24.5.

³¹P NMR (162 MHz, D₂O) δ 2.92.

MS (ESI) m/z = 257 [M+Na]⁺.

Additional Information: Experimental details, including copies of ¹H NMR spectra, for the synthesis and production of the carbohydrate microarrays and the complete ref 2h, and competition experiment plots are found in the Appendix at the end of this chapter.

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CHAPTER 5

A Mass-Differentiated Library Strategy for Functional Proteomic Identification of Sugar Nucleotidyltransferase Activities from Cell Lysates

A paper to be submitted to *The Journal of the American Chemical Society*

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Introduction

Sugar nucleotidyltransferases (NTs) are a ubiquitous class of enzymes that serve to activate sugars for most glycosyltransferases. These enzymes are central to primary and secondary metabolism and their activities have been correlated to disease states such as diabetes and to the state of virulence of pathogens such as *Giardia*.[1] Activity-dependent labeling of enzymes is a powerful method to identify proteins in a complex mixture with specific chemical functions.[2] However, this proteomics strategy is limited to enzymes such as proteases and glycosidases with key nucleophilic residues in their active sites and therefore cannot be readily applied to identifying the presence of sugar nucleotidyltransferases. Herein we report a rapid method to identify a range of sugar nucleotidyltransferase activities of purified proteins or in cell lysates using a library designed for mass spectrometry-based analysis.

Unlike the hydrolytic enzymes identified by activity-based labeling protocols, sugar nucleotidyltransferases require two substrates: a sugar-1-phosphate and a nucleotide triphosphate (NTP). This class of enzymes is often assayed by monitoring of the reverse

reaction—cleavage of a sugar nucleotide by pyrophosphate. However, a cell extract could contain other proteins that lead to the disappearance of sugar nucleotides. In addition, some sugar NTs can readily accept more than one sugar-1-phosphate or more than one NTP as substrates; therefore, conditions to monitor the reaction in the synthetic direction are necessary for the facile characterization of sugar NT activities. Several methods have been developed to monitor the reaction in the synthetic direction: HPLC-based assays,[3] radioactivity-based assays,[4] coupled assays that require various other enzymes,[5] and most recently mass-spectrometry-based assays.[6] Ideally, enzyme activity could be monitored quickly with a range of substrates in a limited number of reactions to conserve sample rather than in multiple parallel reactions.[7] Mass-spectrometry appears best-suited for such a proteomics-based approach.

Results and discussion

The basic strategy of a chemical proteomics approach to detecting sugar NT activities (Figure 1) relies on the differentiation of all possible substrates by mass to track their conversion in one-pot to sugar nucleotide products. However, although some of the naturally occurring sugar-1-phosphates and sialic acid itself do not share molecular weights (Figure 2), several potential library members cannot be distinguished using mass spectrometry. Also, the library cannot contain any inhibitors of sugar NT activity for the triumph of this one-pot approach.

To address the first problem, the mass redundancy of the three common sugar phosphates based on glucose, galactose and mannose was broken by introduction of

deuterium labels (Figure 3). Lithium aluminum deuteride reduction of the known

aldehyde[8] or carboxylic acid[9] produced monodeuterated galactose and dideuterated

glucose analogs. Protection of the resulting alcohols, followed by hydrolysis of the

anomeric methyl group, allowed the selective installation of an anomeric phosphate via the

phosphite.[10] Finally, the phosphorylated compounds were deprotected by hydrogenation

with $Pd(OH)_2$ and the resulting compounds were converted into their sodium salts using an

ion exchange resin to provide the new deuterium labeled sugar-1-phosphates **5** and **6**.
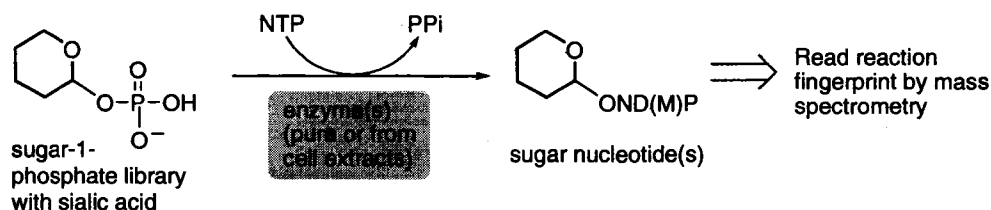
Figure 1. Strategy for identification of multiple sugar nucleotidyltransferase activities in a

cell extract or purified protein preparation.
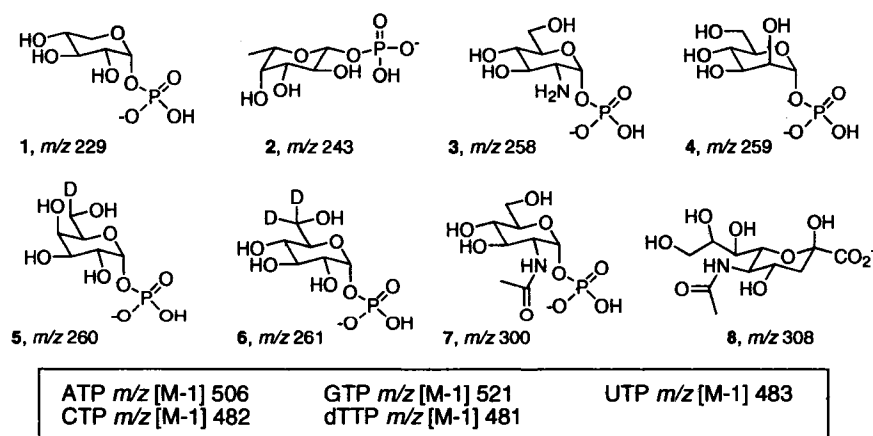
Figure 2. Mass-differentiated library of substrates for sugar nucleotidyltransferases.
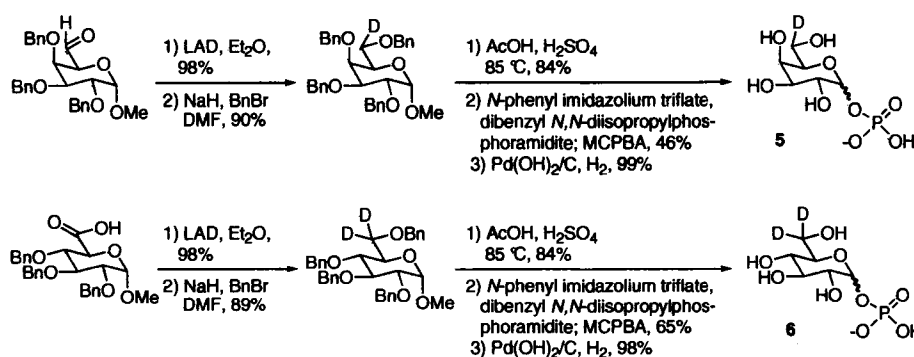
Figure 3. Synthesis of deuterium labeled glucose- and galactose-1-phosphates.

Sugar nucleotidyltransferases are known to operate by a bi-bi mechanism in which the NTP is bound first to the active site, followed by the sugar-1-phosphate.[11] Some sugar NTs display promiscuity with regards to the NTP and therefore can produce more than one product. However, many enzymes can at least bind alternate NTPs even if they are not turned over and therefore be inhibited by the presence of NTPs. Indeed, incubation of the library containing the sugar-1-phosphates and sialic acid with the five nucleotide triphosphates used to make activated sugar nucleotides (Figure 2) and with a well-characterized sugar nucleotidyltransferase [11a] did not result in detection of the expected range of products in the mass spectrum. Even the presence of the correct NTP substrate did not overcome the presence of the inhibitory compounds.
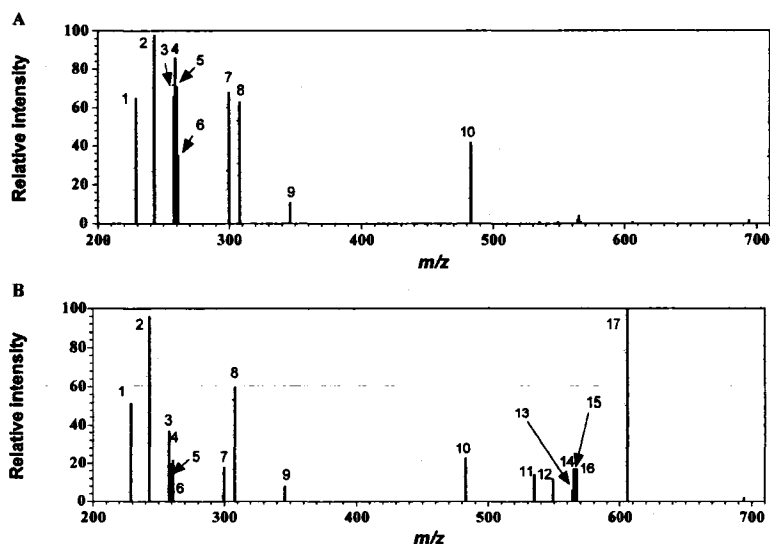
Figure 4. ESI-MS monitoring of the reactants and products in the presence of an *E. coli* K12 cell extract with UTP. A, Reaction at 0 min; B, reaction after 30 min. Compounds 1-8 (see Figure 1); 9, AMP; 10, UTP; 11, UDP-xylose; 12, UDP-fucose; 13, UDP-glucosamine; 14, UDP-mannose; 15, UDP-galactose; 16, UDP-glucose and 17, UDP-*N*-acetylglucosamine.

Next, the sugar substrate library was incubated with only one nucleotide triphosphate at a time and either the yeast sugar nucleotidyltransferase known to produce both ADP- and UDP-activated glucose as well as with a promiscuous archaeal sugar nucleotidyltransferase. An electrospray ionization mass spectrometry (ESI-MS) based technique, developed recently for the sugar NT assay, was employed effectively to monitor the formation of product or disappearance of starting substrates in a one-pot enzymatic reaction. Incubation of the yeast enzyme with substrate library (Figure 1) and UTP or ATP

yielded UDP- or ADP-glucose respectively and no product was detected when the reaction was carried out containing both UTP and ATP in the reaction vessel. In a previous study we have found that yeast enzyme is very specific to its natural substrate and accepts only glucose 1-phosphate in presence of UTP or ATP and ATP was shown to be a competitive inhibitor[6a] which correlates our present finding. Then the same approach was employed to reevaluate the function of a promiscuous hyperthermostable homologous enzyme[11a] from *Pyrococcus furiosus*. The enzymatic reaction, carried out at 80 °C, has turned over all the previously known phosphorylated sugars in presence of UTP including the mono- and dideuterated sugar-1-phosphates and a new substrate, xylose 1-phosphate, which was previously an unknown substrate for this enzyme. It also turned over several sugar 1-phosphates, as expected, in presence of dTTP. No activated sugar was detected in ESI-MS when the reaction was run in presence of ATP, CTP and GTP. These findings completely corroborate our previous report and strongly verify the utility of library approach in order to detect the function of sugar NTs in vitro. The sensitivity and strength of this library approach was utilized further to monitor the sugar NTs activities in crude cell extract from a well known *E. coli* strain. Like wise, soluble cell extract from *E. coli* K12 turned over a variety of sugar 1-phosphates in presence of ATP, GTP, dTTP and UTP. (Figure 4 and Table S1). No product was detected when the reaction was carried out with CTP, the reason of which is unclear. It might be due to that CTP utilizing enzyme was degraded by the proteases or was not induced properly during growth of the *E. coli* strain.

Conclusion

The current library contains the sugars commonly found in many mammalian and bacterial organisms, but it can easily be extended to include sugar-1-phosphates found in plants. In this study we have shown that instead of having any adverse effects, chemically modified deutarated glucose- and galactose-1-phoshates have simplified the ESI-MS detection of activated sugars in one-pot enzymatic reaction. Apart from verifying substrate specificities of known sugar nucleotidyltransferases from eukaryotic and archaeal sources, this substrate library approach is particularly important for screening the sugar NTs activities in cell extract and also to monitor the inhibitors of the particular enzyme. This mass-differentiated library approach would be suitable for screening multiple enzymes in a given cell extract ideally from any sources without having any genetic recombination and extensive purification of the enzymes.

Supporting Information Available: Experimental details for the synthesis of the library, including NMR spectra, as well as conditions for library analysis and additional mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

Experimental Section

General Methods.

General materials and methods. Trizma base and trizma hydrochloride were purchased

from Fisher Scientific Company (Hanover Park, IL). Thermostable inorganic

pyrophosphatase (IPP) from *Thermococcus litoralis* (EC 3.6.1.1, M0296S) was purchased

from New England Biolabs (Ipswich, MA). All other chemicals were obtained from Sigma

Chemical Co. (St. Louis, MO) unless otherwise noted and used without further

purification. Yeast UDP-glucose pyrophosphorylase (EC 2.7.7.9, 9026-22-6) was desalted

by buffer exchange using a Microcon Centrifugal Filter Device, MWCO 10 kDa

(Millipore, Billerica, MA). *E. coli* UDP-glucose pyrophosphorylase was prepared as

previously reported.[1] Nanopure water (18.1 MHz) prepared from a Barnstead E-pure water

purification system was employed throughout. Reaction solvents were distilled from

calcium hydride for dichloromethane and from sodium metal and benzophenone for

diethyl ether. Amberlyst 15 ion-exchange resin was washed repeatedly with methanol

before use. All other commercial reagents and solvents were used as received without

further purification. The reactions were monitored and the R_f values determined using

analytical thin layer chromatography (TLC) with 0.25 mm EM Science silica gel plates

(60F-254). The developed TLC plates were visualized by immersion in *p*-anisaldehyde

solution followed by heating on a hot plate. Flash chromatography was performed with

Selecto Scientific silica gel, 32-63 □m particle size. All moisture-sensitive reactions were

performed in flame- or oven-dried glassware under a nitrogen atmosphere. Bath

temperatures were used to record the reaction temperature in all cases run without

microwave irradiation. All reactions were stirred magnetically at ambient temperature unless otherwise indicated. ^{1}H NMR, ^{13}C NMR, and ^{31}P spectra were obtained with a Bruker DRX400 at 400 MHz, 100 MHz, and 162 MHz respectively. ^{1}H NMR spectra were reported in parts per million (δ) relative to CDCl$_3$ (7.27 ppm) as an internal reference. ^{13}C NMR spectra were reported in parts per million (δ) relative to CDCl$_3$ (77.23 ppm) or CD$_3$OD (49.15 ppm). ^{31}P NMR spectra were reported relative to H$_3$PO$_4$ (0.00 ppm) as an internal reference and were obtained with complete proton decoupling.

Bacterial strain and enzymes. Escherichia coli K12 cells were grown on Luria-Bertani (LB, Sigma, St. Louis, MO) medium at 37 °C for over night on an incubator shaker at 225 rpm. Cells were harvested by centrifugation at 3600 x g for 10 min at 25 °C and were disrupted by sonication (Fisher model 100 Sonic Dismembranator, Fisher Scientific, Pittsburgh, PA), after which unbroken cells and debris were removed by centrifugation (30 min at 10,000 x g). The supernatant was used as cell extract to check sugar nucleotidyltransferase activity. The recombinant UDP-α-D-glucose pyrophosphorylase (UGPase) (UTP:α-D-glucose-1-phosphate uridylyltransferase, EC 2.7.7.9) from *P. furiosus*, expressed in E. coli BL21 (DE3) Codon Plus$^®$ cells, was purified by affinity column chromatography according to the method described previously.[2] Purified yeast UGPase was obtained from Sigma (St. Louis, MO) and was used after buffer exchange as described previously.[12]

Mass Spectrometry. A Shimadzu LCMS 2010 quadrupole mass spectrometer (Shimadzu

Scientific Instruments, Columbia, MD) equipped with an electrospray ionization (ESI)

source was used in negative ion mode. The capillary temperature and the spray voltage

were kept at 220 °C and 4.5 kV, respectively. The instrument was calibrated by direct

infusion of polyethylene glycol (PEG) 200, 600, 1000, (1.5 µL/L, 2 µL/L, and 15 µL/L,

respectively) and raffinose (50 mg/L) in water/methanol (1:1, v:v) containing ammonium

acetate (0.19 mM), 0.1% formic acid and 0.1% acetonitrile. For sample analysis the

solvent acetonitrile/water/triethylamine, (35/65/0.2) was constantly infused into the ion

source at 250 µL/min by the attached Shimadzu HPLC pump and the samples were

injected (30 µL) via the autosampler adapted to fit two 96-well plates. A preliminary MS

chromatogram was obtained by scanning from 50-700 *m/z*. To increase the signal to noise

ratio, the instrument was set for selected ion monitoring (SIM) mode and all relevant *m/z*

ions were monitored for further analysis of the enzymatic reactions. Inclusion of an

Agilent Extend C18 column (2.1x50 mm, Agilent, Palo Alto, CA) in the system further

increased the signal to noise ratio and decreased the appearance of sodium ion adducts by

10-15% without separation of reaction components. Postrun software (LCMS Postrun

version 2.02, Shimadzu Scientific Instruments, Columbia, MD) was used to analyze the

data from the ESI-MS chromatogram. Peaks were integrated to determine the relative

intensity of each ion species monitored as compared to an internal standard.

Sugar nucleotidyltransferase activity screening assay. The enzymatic activity of UGPases

from *P. furiosus* and yeast were determined as was described previously by the

consumption of glucose-1-phosphate in the direction of UDP-glucose biosynthesis.[13] The

reaction mixture in a total volume of 100 µL for sugar nucleotidyltransferase substrate

screening assay consisted of the following components:

a) Library of sugars containing eight substrates (5 mM for each, except for GlcNAc1P

and NeuAc, for which 1 and 2 mM concentrations were used respectively).

b) NTP (ATP/CTP/GTP/dTTP/CTP) (5 mM). Each NTP was used independently in a

separate reaction.

c) Inorganic pyrophosphatase (0.2U/mL).

d) MgCl₂ (3 mM for *P. furiosus* enzyme and 10 mM for yeast enzyme and *E. coli*

extract.

e) Buffer (25 mM, Phosphate buffer pH 7.5 for *P.furiosus* enzyme and Tris-HCl pH

7.6 for the other enzyme or extract).

f) Appropriate concentration of purified enzyme or cell extract.

For the control reaction, nanopure water was used instead of an enzyme or cell extract.

The reaction components were preincubated at the appropriate temperature (90 °C for the

P. furiosus enzyme; 37 °C for yeast and *E. coli*) for 5 min before addition of the sugar

substrate library. Fifteen (15) µL of the reaction mixture was quenched by the addition of

70% methanol/water (30 µL). The quenched solutions were centrifuged for 10 min at

10,000 x g to precipitate the protein. Aliquots of the reaction mixtures were diluted with

135 µL of acetonitrile/water/triethylamine (35/65/0.2). These samples (5 µL) were

subjected to analysis via ESI-MS to detect the formation of NDP-sugars in comparison

to a control reaction containing no enzyme.

Table S1. NDP-sugars formed after 30 min incubation of the sugar substrate library with individual nucleotide triphosphates (NTP) and the specified enzyme or cell extracts. Nd= No product was detected.

Source of enzyme	ATP product (m/z)	CTP product (m/z)	GTP product (m/z)	dTTP product (m/z)	UTP product (m/z)
E. coli extract	ADP-GlcN (587)	Nd	GDP-Fuc (588)	dTDP-Glc (565)	UDP-Xyl (535)
	ADP-Man (588)				UDP-Fuc (549)
	ADP-Gal (589)				UDP-Man (565)
	ADP-Glc (590)				UDP-Gal (566)
					UDP-Glc (567)
					UDP-GlcNAc (606)
P. furiosus	Nd	Nd	Nd	dTDP-Man (563)	UDP-Xyl
				dTDP-Gal (564)	UDP-Fuc
				dTDP-Glc (565)	UDP-GlcN (558)
				dTDP-GlcNAc (604)	UDP-Man
				Nd	UDP-Gal
					UDP-Glc
					UDP-GlcNAc
					UDP-Glc
Yeast	ADP-Glc	Nd	Nd		

Table S2. The *m/z* ratio of the sugar-1-phosphate library and their corresponding products

after reaction with various nucleotide triphosphates (NTPs).

Sugar-1-phosphate	ATP	CTP	GTP	dTTP	UTP
Xylose1P (229) **1**	558	534	574	533	535
Fucose1P (243) **2**	572	548	588	547	549
GlcN1P (258) **3**	587	563	603	562	564
Man1P (259) **4**	588	564	604	563	565
Deu-Gal1P (260) **5**	589	565	605	564	566
Deu2-Glc1P (261) **6**	590	566	606	565	567
GlcNAc1P (300) **7**	629	605	645	604	606
Sialic acid (308) **8**	716	613	732	692	694

Synthetic Procedures.
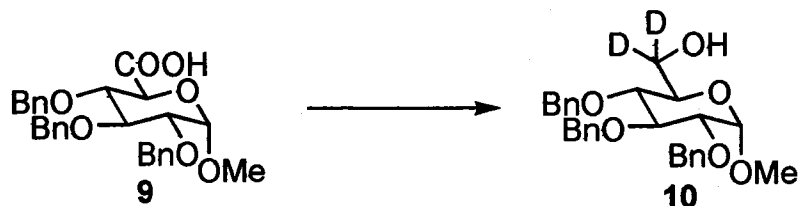
Methyl-2,3,4-tri-O-benzyl-α-D-(6,6-^{2}H$_2$)glucopyranoside[14]

To a solution of compound **9**[3] (0.50 g, 1.04 mmol) in Et$_2$O (15 mL) was added a solution of lithium aluminum deuteride (43.0 mg) at ambient temperature for 2 h. The mixture was diluted with Et$_2$O (2 x 50 mL), acidified with 2 N HCl (2 x 45 mL), and washed with water (2 x 50 mL). The solvent was removed under reduce pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 1/1, v/v) to afford **10** as a syrup (0.48 g, 1.03 mmol, 98%)

R$_f$ 0.72 (ethyl acetate/hexane, 1/1 v/v).

MS (ESI) m/z = 489 [M+Na]$^+$; ^{1}H NMR and ^{13}C NMR spectra match those previously reported.[3]
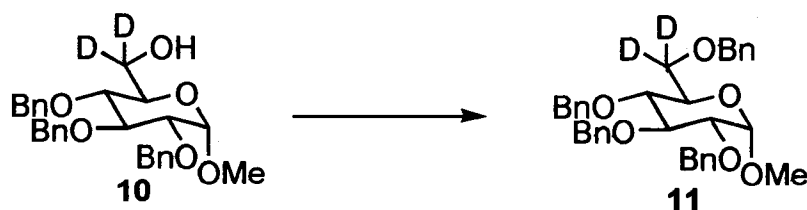
Methyl-2,3,4,6-tetra-O-benzyl-α-D-(6,6-^{2}H$_2$)glucopyranoside

Sodium hydride (27.0 mg, 1.13 mmol) was slowly added into a solution of compound (**10**, 0.44 g, 0.94 mmol) in DMF (30 mL) at ambient temperature. Benzyl bromide (0.14 mL, 1.13 mmol) was added to the solution. The mixture was stirred at ambient temperature for

4 h, diluted with ethyl acetate (60 mL), and then washed with water (3 x 40 mL) and saturated aqueous sodium bicarbonate (15 mL). The solution was dried over magnesium sulfate. The solvent was removed under reduced pressure. The product was isolated by silica gel column chromatography (ethyl acetate/hexane, 7/3, v/v) to afford **11** as the white solid (0.47 g, 0.86 mmol, 89%).

R_f 0.72 (ethyl acetate/hexane, 3/7 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.48-7.81 (m, 20H), 4.99 (d, 1H, J = 10.8 Hz, PhCH), 4.83 (d, 1H, J = 4.0 Hz, PhCH), 4.80 (m, 2H, PhCH$_2$), 4.69 (m, 2 H, PhCH$_2$), 4.63 (d, 1H, J = 3.6 Hz, C$_1$-H), 4.48 (m, 2H, PhCH$_2$), 4.60 (m, 2 H), 4.50 (d, 1H, J = 4.0 Hz), 3.99 (t, 1H, J = 9.2 Hz, C$_3$-H), 3.75 (d, 1H, J = 10.0 Hz, C$_5$-H), 3.65 (t, 1H, J = 10.0 Hz, C$_4$-H), 3.55 (dd, 1H, J = 9.6, 3.6 Hz, C$_2$-H), 3.38 (s, 3H, -OCH$_3$).

^{13}C NMR (100 MHz, CDCl$_3$) δ 138.9, 138.4, 138.3, 138.0, 128.5, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 98.3 (C$_1$), 82.2 (C$_3$), 79.7 (C$_2$), 77.7 (C$_4$), 75.9 (PhCH$_2$), 75.1 (PhCH$_2$), 73.5 (PhCH$_2$), 73.5 (PhCH$_2$), 70.0 (C$_5$), 55.3 (-OCH$_3$).

MS (ESI) m/z = 579 [M+Na]$^+$
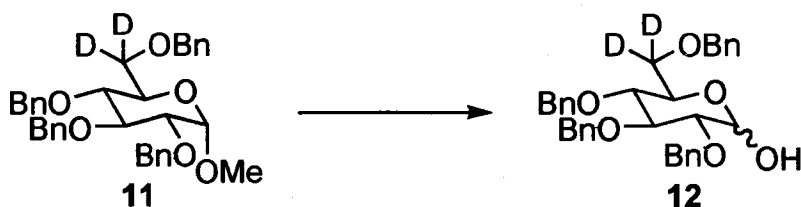
**2,3,4,6-tetra-_O_-benzyl-α/β-D-(6,6-^{2}H$_2$)glucopyranose**

Compound **11** (0.4 g, 0.7 mmol) was treated with glacial acetic acid (25 mL) and 3.0 M

sulfuric acid (0.7 mL) at 85 °C for 1.5 h. The mixture was cooled to ambient temperature

and then diluted with ice water (45 mL), extracted with toluene (50 mL), and then washed

with saturated aqueous sodium bicarbonate (3 x 25 mL), water (2 x 30 mL), and brine (30

mL). The solvent was removed under reduced pressure. The product was isolated by silica

gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford **12** as a white solid

(0.33 g, 0.68 mmol, 84%).

R_f 0.46 (ethyl acetate/hexane, 2/3 v/v).

¹H NMR (400 MHz, CDCl₃) δ 7.41-7.20 (m, 40 H), 5.27 (d, 1 H, J = 2.8 Hz), 4.99 (m, 3

H), 4.86 (m, 4 H), 4.74 (m, 3 H), 4.60 (m, 3 H), 4.52 (m, 3 H), 4.10 (dd, 2 H, J = 20.8, 10.0

Hz), 3.80 (s, 1H), 3.62 (m, 4 H), 3.44 (m, 1 H).

¹³C NMR (100 MHz, CDCl₃) δ 138.8, 138.6, 138.5, 138.3, 138.1, 138.0, 137.9, 137.8,

128.6, 128.5, 128.5, 128.5, 128.2, 128.1, 128.1, 128.1, 128.0, 128.0, 128.0, 127.9, 127.8,

127.8, 127.8, 127.8, 127.7, 97.6, 91.3, 84.7, 83.2, 81.8, 80.0, 77.9, 77.9, 75.8, 75.8, 75.1,

75.1, 74.8, 74.5, 73.4, 73.2, 70.1.

MS (ESI) m/z = 542 [M]⁺
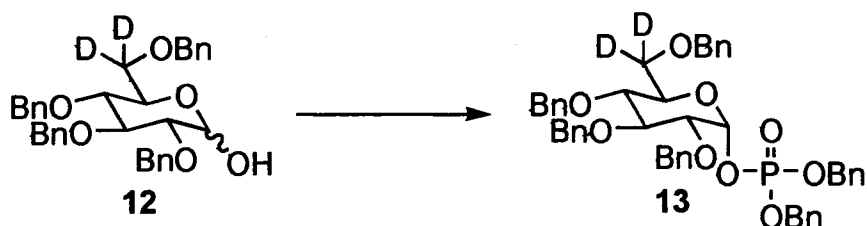
Dibenzyl-(2,3,4,6-tetra-*O*-benzyl-α-D-(6,6-^{2}H$_2$)glucopyranosyl) phosphate

The compound of 2,3,4,6-tetra-*O*-benzyl-6-dideuterium-α-D-glucopyranose (0.30 g, 0.55 mmol) in dichloromethane (4 mL) was added to a mixture of dibenzyl *N*,*N*-diisopropylphosphoramidite (0.25 mL) and *N*-phenyl imidazolium triflate (0.21 g) in anhydrous acetonitrile-dichloromethane (1:1) (8 mL). The TLC showed no starting material left (R$_f$ 0.75, ethyl acetate/hexane, 1/1, v/v) and then the mixture was cooled down to –50 °C. 3-chloroperoxybenzoic acid (0.15 g) in anhydrous dichloromethane (5 mL) was added. The resulting solution was stirred at 0 °C for 30 min. The mixture was diluted with 30 mL CH$_2$Cl$_2$, 40 mL 20% Na$_2$SO$_3$ solution was added and the stirred at ambient temperature for 3 h. Organic layer was washed with saturated NaHCO$_3$ (3x 30 mL), water (3 x 30 mL), brine (40 mL) and dried over magnesium sulfate. The product was isolated by silica gel column chromatography (chloroform/diethylether, 9/1, v/v) to afford the desired phosphate (**13**) as a white solid (0.29 g, 0.36 mmol, 65%). The β anomer could also be isolated (0.12 g, 0.15 mmol, 27%).

R$_f$ 0.32 (chloroform/diethylether, 9/1 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.41-7.20 (m, 30 H), 6.05 (dd, 1 H, *J* = 6.4, 3.2 Hz), 5.11 (m, 4 H), 4.99 (dd, 2H, *J* = 23.2, 19.6 Hz), 4.86 (dd, 2H, *J* = 9.2, 5.2 Hz), 4.71 (d, 1 H, *J* = 7.6 Hz), 4.58 (dd, 2 H, *J* = 17.2, 10.8 Hz), 4.49 (d, 1 H, *J* = 12.4 Hz), 3.99 (dd, 2 H, *J* = 9.6, 9.6 Hz), 3.77 (t, 1 H, *J* = 9.6 Hz), 3.70 (m, 1 H).

^{13}C NMR (100 MHz, CDCl$_3$) δ 137.9, 137.7, 136.0, 136.0, 135.9, 135.9, 128.8, 128.7, 128.6, 128.6, 128.5, 128.5, 128.3, 128.2, 128.1, 128.1, 128.0, 128.0, 128.0, 127.9, 127.9,

127.8, 127.8, 127.7, 127.6, 127.5, 127.0, 95.8 (d, J_{CP} = 6.0 Hz), 81.2, 79.4, 79.4, 75.8,

75.3, 73.6, 73.5, 73.2, 72.7, 72.6, 69.5, 69.4, 69.4, 69.4, 69.3, 69.2, 68.1, 67.4, 67.4, 65.1.

MS (ESI) m/z = 825 [M+Na]$^+$
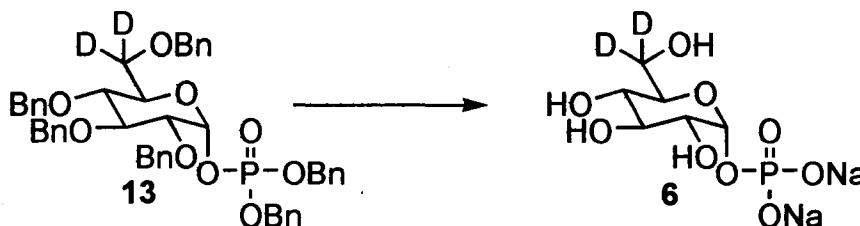
Disodium α-D-(6,6-^{2}H$_2$)glucopyranosyl phosphate

A solution of **13** (0.30 g, 0.38 mmol) in MeOH (15 mL) was stirred with 20 wt. %

palladium hydroxide on carbon (80.0 mg) and saturated aqueous sodium bicarbonate (3

mL) under atmospheric H$_2$ (gas) at ambient temperature overnight. The mixture was

filtered over Celite and the filtrate was concentrated under reduced pressure to afford **6** as

a white solid (0.11 g, 0.37 mmol, 98%).

R_f 0.13 (methanol/ethyl acetate/hexane, 5/1/1 v/v).

^{1}H NMR (400 MHz, D$_2$O) δ 5.28 (dd, 1 H, J = 7.2, 3.2 Hz), 3.74 (d, 1 H, J = 10.0 Hz),

3.61 (t, 1 H, J = 9.6 Hz), 3.35 (m, 1 H), 3.24 (t, 1 H, J = 9.6 Hz).

^{13}C NMR (100 MHz, D$_2$O) δ 93.5 (d, J_{CP} = 5.2 Hz), 73.0, 72.0, 71.9, 71.8, 69.6.

^{31}P NMR (162 MHz, D$_2$O) δ 2.78

MS (ESI) m/z = 239 [M-Na]$^-$
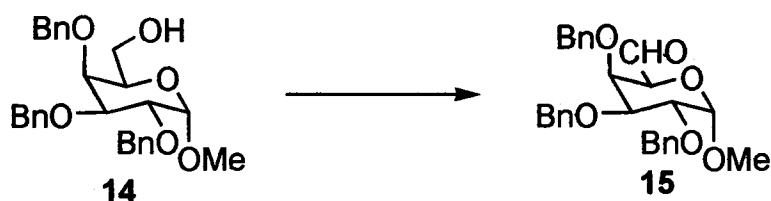
Methyl-2,3,4-tri-*O*-benzyl-6-aldehyde-α-D-galactopyranoside[15]

To a solution of **14** (0.78 g, 1.69 mmol) in CH_2Cl_2 (15 mL) was added a solution of Dess-Martin periodinane (0.86 g) with stirring for 30 min. The reaction mixture was diluted with diethyl ether (40 mL) and added sodium thiosulfate (8 mL) in saturated aqueous sodium bicarbonate. The layers were separated, and the organic phase was washed sequentially with saturated aqueous sodium bicarbonate (2 x 35 mL) and water (2 x 35 mL), and extracted with diethylether (2 x 40 mL). The combined organic phases were dried over magnesium sulfate to provide the crude aldehyde (**15**) which was carried on directly without further purification (0.78 g, 1.7 mmol, quantitative).

R_f 0.64 (ethyl acetate/hexane, 3/7 v/v).

MS (ESI) m/z = 485 $[M+Na]^+$; ^1H NMR and ^{13}C NMR spectra match those previously reported.[4]
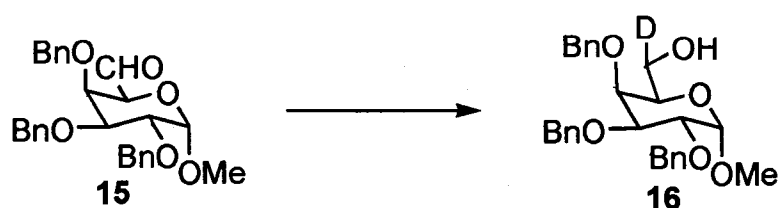
Methyl-2,3,4-tri-*O*-benzyl-α-D-(6-$^2H_1$)galactopyranoside

To a solution of compound **15** (0.50 g, 1.04 mmol) in Et$_2$O (15 mL) was added a solution of lithium aluminum deuteride (43.0 mg) at ambient temperature for 2 h. The mixture was diluted with Et$_2$O (2 x 50 mL), acidified with 2 N HCl (2 x 45 mL), and washed with water (2 x 50 mL). The solvent was removed under reduce pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 1/1, v/v) to afford **16** as a syrup (0.48 g, 1.0 mmol, 98%)

R$_f$ 0.72 (ethyl acetate/hexane, 1/1 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.51-7.34 (m, 15 H), 5.02 (d, 1 H, J = 11.2 Hz), 4.99 (dd, 2H, J = 18.4, 12.0 Hz), 4.75 (m, 2H), 4.66 (dd, J = 25.6, 12.4 Hz), 4.11 (dd, 1 H, J = 10.0, 3.6 Hz), 3.95 (dd, 1H, J = 10.0, 3.2 Hz), 3.88 (d, 1 H, J = 2.4 Hz), 3.67 (m, 1 H), 3.49 (m, 1H), 3.36 (s, 3H), 2.03 (s, 1 H).

^{13}C NMR (100 MHz, CDCl$_3$) δ 138.8, 138.5, 138.4, 128.6, 128.5, 128.5, 128.5, 128.2, 128.2, 128.0, 127.9, 127.8, 127.7, 127.7, 98.9, 79.2, 76.6, 75.3, 75.2, 74.6, 73.6, 73.6, 70.5, 62.0(m), 55.4.

MS (ESI) m/z = 488 [M+Na]$^+$
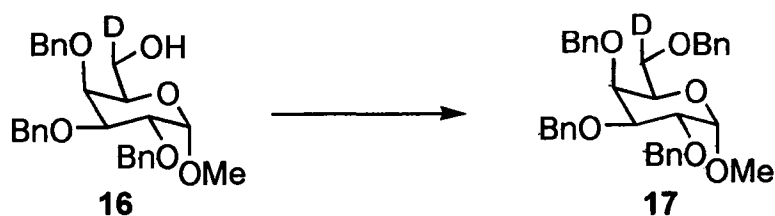
Methyl-2,3,4,6-tetra-*O*-benzyl-α-D-(6-^{2}H$_1$)galactopyranoside

Sodium hydride (0.04 g, 1.8 mmol) was slowly added into a solution of a compound (16, 0.72 g, 1.56 mmol) in DMF (30 mL) at ambient temperature. Benzyl bromide (0.22 mL, 1.8 mmol) was added to the solution. The mixture was stirred at ambient temperature for 4 h under N_2, diluted with ethyl acetate (60 mL), and then washed with water (3 x 40 mL) and aqueous saturated sodium bicarbonate (35 mL). The solution was dried over magnesium sulfate. The solvent was removed under reduced pressure. The product was isolated by silica gel column chromatography (ethyl acetate/hexane, 7/3, v/v) to afford 17 as a solid (0.78 g, 1.4 mmol, 90%).

R_f 0.71 (ethyl acetate/hexane, 1/1 v/v).

¹H NMR (400 MHz, CDCl₃) δ 7.44-7.24 (m, 20 H), 5.10 (d, 1 H, J = 11.6 Hz), 4.88 (dd, 2H, J = 11.6, 6.8 Hz), 4.75 (dd, 2H, J = 18.4, 12.0 Hz), 4.73 (d, J = 3.2 Hz), 4.63 (t, 1H, J = 14.0 Hz), 4.45 (dd, 2 H, J = 34.0, 11.6 Hz), 4.08 (dd, 1 H, J = 10.0, 3.6 Hz), 3.98 (m, 2 H), 3.92 (d, 1H, J = 6.4 Hz), 3.54 (m, 1 H), 3.40 (s, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 128.9, 138.8, 138.6, 138.1, 128.5, 128.5, 128.4, 128.4, 128.3, 128.2, 128.2, 127.9, 127.9, 127.8, 127.8, 127.8, 127.7, 127.7, 127.6, 98.9, 79.2, 76.6, 75.3, 74.8, 73.7, 73.6, 73.4, 73.2, 69.3, 69.2(m), 55.5.

MS (ESI) m/z = 578 [M+Na]⁺
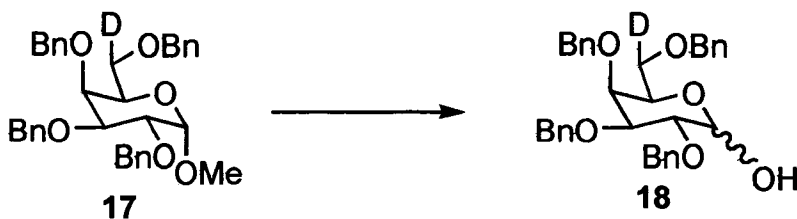
2,3,4,6-tetra-*O*-benzyl-α-D-(6-²H₁)galactopyranose

Compound **17** (0.4 g, 0.72 mmol) was treated with glacial acetic acid (25 mL) and 3.0 M

sulfuric acid (0.7 mL) at 85 °C for 1.5 h. The mixture was cooled to ambient temperature

and then diluted with ice water (45 mL), extracted by toluene (50 mL) and then washed

with saturated aqueous sodium bicarbonate (3 x 25 mL), water (2 x 30 mL), and brine (30

mL). The solvent was removed under reduced pressure. The product was isolated by silica

gel column chromatography (ethyl acetate/hexane, 2/3, v/v) to afford **18** as a white solid

(0.33 g, 0.68 mmol, 84%).

R_f 0.68 (ethyl acetate/hexane, 3/7 v/v).

¹H NMR (400 MHz, CDCl₃) δ 7.51-7.34 (m, 20 H), 5.31 (d, 1 H, J = 3.6 Hz), 4.99 (dd,

2H, J = 13.2, 2.4 Hz), 4.86 (m, 2H), 4.74 (m, 2 H), 4.62 (m, 2H), 4.49 (d, 1 H, J = 11.6, 4.8

Hz), 4.40 (dd, 1 H, J = 16.4, 7.6 Hz), 4.20 (d, 1 H, J = 6.0 Hz), 4.07 (dd, 1 H, J = 9.6, 3.6

Hz), 3.99 (m, 2H), 3.80 (m, 1 H), 3.56 (m, 1 H), 3.47 (d, 1 H, J = 6.4 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 138.8, 138.6, 138.5, 138.3, 138.1, 138.0, 137,9, 137.8,

128.5, 128.5, 128.5, 128.4, 128.4, 128.4, 128.3, 128.1, 128.1, 127.9, 127.8, 127.8, 127.7,

127.7, 127.6, 127.6, 127.6, 127.0, 97.9, 91.9, 82.3, 80.8, 78.9, 76.9, 76.7, 75.2, 74.9, 74.8,

74.6, 73.7, 73.6, 73.5, 73.4, 73.1, 73.0, 69.4, 69.3(m).

MS (ESI) m/z = 541 [M]⁺
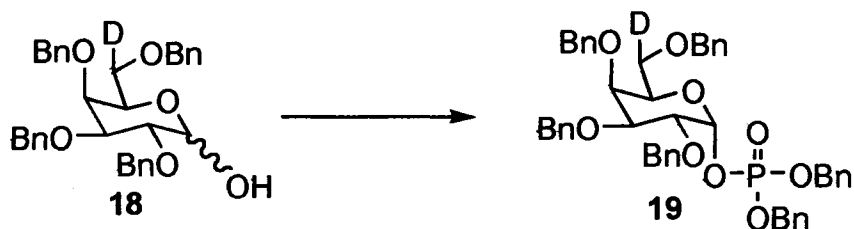
Dibenzyl-(2,3,4,6-tetra-O-benzyl-α-D-(6-^{2}H$_1$)galactopyranosyl) phosphate

The compound of 2,3,4,6-tetra-O-benzyl-6-deuterium-α-D-galactopyranose (0.32 g, 0.59 mmol) in acetonitrile (4.0 mL) was added to a mixture of dibenzyl N,N-diisopropylphosphoramidite (0.35 mL) and N-phenyl imidazolium triflate (0.33 g) in dry acetonitrile (8.0 mL). After the TLC showed no remaining starting material (R$_f$ 0.75, ethyl acetate/hexane, 1/1, v/v), the solvent was evaporated under reduced pressure and then a solution of 3-chloroperoxybenzoic acid (0.13 g, 0.76 mmol) in dichloromethane (15 mL) was added to the mixture at –50 °C. The resulting solution was stirred at 0 °C for 30 min. The mixture was diluted with CH$_2$Cl$_2$ (30 mL), treated with a 20% Na$_2$SO$_3$ solution (40 mL), and then stirred at ambient temperature for 3 h. The organic layer was washed with saturated NaHCO$_3$ (3 x 30 mL), water (3 x 30 mL), and brine (40 mL) and then dried over magnesium sulfate. The product was isolated by silica gel column chromatography (chloroform/diethylether, 9/1, v/v) to afford the desired phosphate **19** as a white solid (0.21 g, 0.27 mmol, 46%). The β anomer could also be isolated (0.20 g, 0.25 mmol, 42%).

R$_f$ 0.34 (chloroform/diethylether, 9/1 v/v).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.46-7.19 (m, 30 H), 5.98 (dd, 1 H, J = 6.4, 3.2 Hz), 5.07 (m, 6 H), 4.75 (m, 4 H), 4.55 (d, 1 H, J = 11.2 Hz), 4.34 (dd, 2H, J = 23.2, 11.6 Hz), 4.14 (m, 2 H), 3.98 (m, 1H), 3.87 (dd, 1 H, J = 10.0, 2.8 Hz), 3.51 (d, 1 H, J = 7.2).

^{13}C NMR (100 MHz, CDCl$_3$) δ 138.5, 138.4, 138.1, 137.8, 135.9, 135.9, 128.6, 128.5, 128.5, 128.4, 128.3, 128.3, 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.7, 127.6, 127.5, 96.8 (d, J$_{CP}$ = 6.2 Hz), 78.1, 75.8, 75.7, 74.9, 74.6, 73.4, 73.3, 73.0, 71.5, 69.3, 69.3, 69.2, 69.2, 69.0, 69.0.

MS (ESI) m/z = 824 [M+H]$^+$
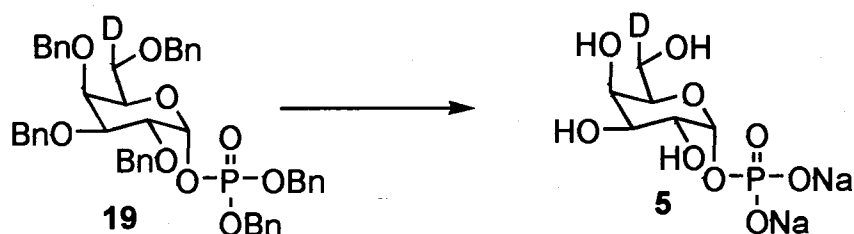
Disodium α-D-(6-^{2}H$_1$)galactopyranosyl phosphate

A solution of **19** (0.20 g, 0.25 mmol) in MeOH (15 mL) was stirred with 20 wt. % palladium hydroxide on carbon (0.08 g) with saturated sodium bicarbonate (3 mL) under atmospheric H$_2$ (gas) at ambient temperature overnight. The mixture was filtered over Celite and the filtrate was concentrated under reduced pressure to afford **5** as a white solid (65 mg, 0.25 mmol, 99%).

R_f 0.14 (methanol/diethylether/hexane, 5/1/1 v/v/v).

^{1}H NMR (400 MHz, D$_2$O) δ 5.35 (dd, 1H, J = 7.2, 3.6 Hz), 4.04 (d, 1H, J = 4.8 Hz), 3.85 (d, 1H, J = 3.2 Hz), 3.78 (m, 1 H), 3.63 (m, 1H), 3.57 (d, 1H, J = 4.0 Hz).

^{13}C NMR (100 MHz, D$_2$O) δ 93.7 (d, J_{CP} = 5.5 Hz), 71.0, 69.6, 69.4, 69.0, 68.9, 61.8(m).

^{31}P NMR (162 MHz, D$_2$O) δ 2.92.

MS (ESI) m/z = 238 [M-Na]$^-$
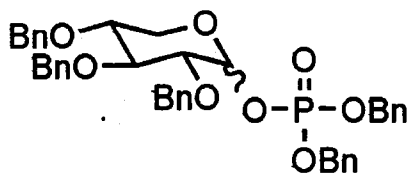
Dibenzyl-(2,3,4-tri-*O*-benzyl-α/β-D-xylopyranosyl) phosphate

The compound 2,3,4-tri-*O*-benzyl-α/β-D-xylopyranose[1] (81.7 mg, 0.2 mmmol) in acetonitrile (4 mL) was added to a mixture of dibenzyl diisopropyl phosphoramidite (0.087mL) and *N*-phenyl immidazolium triflate (60.5 mg) in anhydrous acetonitrile (8 mL). When the tlc showed no remaining starting material (R_f 0.75, ethyl acetate/hexane, 1/1, v/v), the solvent was removed under reduced pressure. Then 3-chloroperoxybenzoic acid (43.7 mg, 0.25 mmol) was added to the solution of the mixture in 15 mL dichloromethane at –50 °C and the resulting solution was stirred at 0 °C for 30 min. The mixture was diluted with CH_2Cl_2 (30 mL). 20 % aqueous Na_2SO_3 (40 mL) solution was added and the mixture was stirred at room temperature for 3 h. The organic layer was washed with saturated aqueous $NaHCO_3$ (3 x 30 mL), water (3 x 30 mL), and brine (40 mL) and then dried over magnesium sulfate. The product was isolated by silica gel column chromatography (chloroform/diethyl ether, 9/1, v/v) to afford 76% of dibenzyl-(2,3,4-tri-*O*-benzyl-α/β-D-xylopyranosyl) phosphate as a white solid (51.2 mg, 0.08 mmol, 52% for α compound, 47.2 mg, 0.07 mmol, 48% for β compound);

R_f 0.34 (chloroform/ethylether, 2/3 v/v).: ^{1}H NMR and ^{13}C NMR spectra match those previously reported.[16]
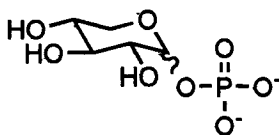
Disodium α/β-D-xylopyranosyl phosphate

A solution of dibenzyl-(2,3,4-tri-*O*-benzyl-α/β-D-xylopyranosyl) phosphate (80.3 g, 0.12 mmol) in MeOH (15 mL) was stirred with 20 wt. % palladium hydroxide on carbon (0.08 g) with saturated sodium bicarbonate (3 mL) under atmospheric H$_2$ (gas) at ambient temperature overnight. The mixture was filtered over Celite and the filtrate was concentrated under reduced pressure to afford disodium α/β-D-xylopyranosyl phosphate as a white solid (27.1 mg, 0.12 mmol, quantitative).

R$_f$ 0.14 (methanol/ethyl ether/hexane, 5/1/1 v/v/v).: ^{1}H NMR and ^{13}C NMR spectra match those previously reported.[17]

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CHAPTER 6

Synthesis of Isobutyl-C-galactoside(IBCG) as an Isopropylthiogalactoside(IPTG) Substitute for Increased Induction of Protein Expression

A paper published in the *Organic Letter*[4]

Kwang-Seuk Ko, Jerred Kruse, and Nicola L. Pohl*

Abstract

Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to bacterial cultures is often used to induce expression of plasmid-based genes for the production of recombinant proteins under control of the lac promoter, but a simple method to circumvent the inherent instability of this compound has not been addressed experimentally. Herein we report the first synthesis of isobutyl-C-galactoside (IBCG), the C-glycoside analogue of IPTG, and show that IBCG is superior to IPTG in inducing protein expression over long induction times.

Introduction

Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to bacterial cultures is a long-standing way to induce expression of plasmid-based genes for the production of recombinant proteins under the control of the *lac* promoter, but is not without its share of

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problems.[1] The S-glycoside analogue binds to the *lac* repressor in *Escherichia coli*, thereby preventing binding of the repressor protein to DNA and blocking gene transcription (Figure 1).[2] From the standpoint of experimental practicality, IPTG and its solutions should be stored below room temperature to prevent decomposition over time. In addition, multiple additions of IPTG are often necessary for longer induction times as the compound degrades under culture conditions. A more stable version of IPTG would be desirable to circumvent these issues and provide greater control of protein expression, especially over long periods of induction. Herein we report the first synthesis of the C-glycoside analogue of IPTG and show that it cannot only serve as a functional replacement of IPTG in inducing expression of proteins under control of the *lac* promoter, but it actually appears to be superior to IPTG for long induction times.
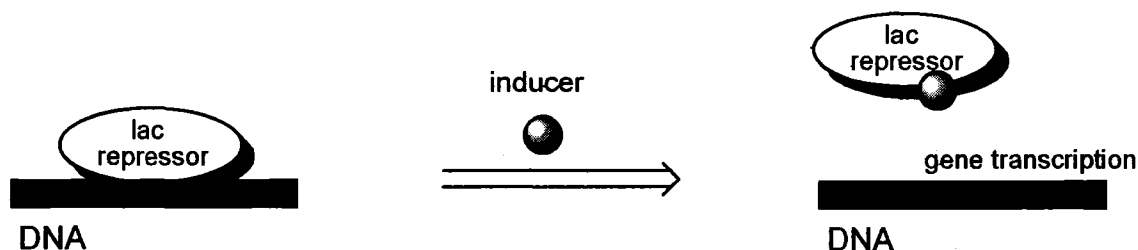
Figure 1 Small molecules such as IPTG, allolactose, and IBCG can bind to the lac repressor to allow gene transcription machinery access to DNA for the production of proteins.

Replacement of the C-O anomeric linkage in naturally occurring O-glycosides with a C-S or C-C bond is known to engender hydrolytic stability, providing derivatives capable of adopting similar conformations and hence retaining function.[3] However, the S-glycosidic linkage suffers from its susceptibility to both chemical and enzymatic oxidation and

cleavage reactions. The S-glycoside IPTG serves as an analogue of allolactose, which is formed from lactose in the bacterial cell. Apparently, only the galactose portion of the larger carbohydrate is necessary to disrupt the repressor protein/DNA binding interaction. Substitution of the 6-OH of galactose with methyl or hydrogen, for instance, destroys its induction capabilities.[1b] In contrast, the sulfur of the galactosidic bond is not implicated in a particular binding interaction that would be destroyed in the absence of lone pairs as seen by inspection of the recent X-ray structure of the E. coli lac repressor bound to IPTG.[4] Therefore, substitution of the anomeric atom appeared feasible provided the increased conformational mobility of the carbon side chain did not interfere with binding or significantly diminish cell permeability.

Synthetic routes to IPTG[5] do not lend themselves directly to the synthesis of the C-glycoside. Fortunately, recent advancements in the synthesis of these types of saccharide structures provide many practical options.

Results and discussion

Two synthetic routes to the isobutyl-C-galactoside (IBCG, 1) analogue were pursued in parallel (Scheme 1). In the first, treatment of galactose pentaacetate 2 with methallyltrimethylsilane in the presence of boron trifluoride etherate[6] gave a mixture of α and βanomers, which were separated by silica gel column chromatography to provide the β-anomer 3 in 48% yield. While this scheme did provide the desired compound after hydrogenation of the alkene and Zemplen deacylation, separation of the anomeric mixture was tedious. Alternatively, an approach relying on a Grignard reaction previously applied

to glucose derivatives[7] was developed. Commercially available bromoacetogalactose[8] **4** was treated with excess isobutylmagnesium bromide to provide exclusively the desired β-anomer of the C-linked glycoside. A proton-proton COSY NMR spectrum[9] confirmed the anomeric configuration of the isolated product by evidence of the 9 Hz coupling between protons 1 and 2 of the galactose ring. Deprotection of the acetyl groups with sodium methoxide resulted in the desired IBCG.[9] The latter route easily allowed gram-scale synthesis of the analogue.

Scheme 1. Synthesis of IBCG[a]
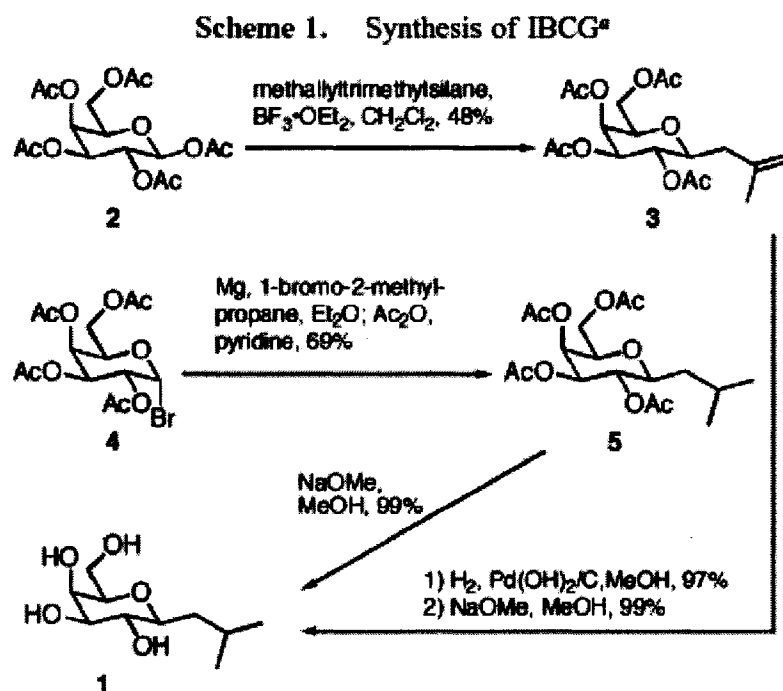
To compare the ability of the C-glycoside to take the place of IPTG in the induction of protein expression, assays of promoter activity that rely on production of a fluorescent protein were pursued.[10-13] Reporters for gene activity in bacteria that rely on green fluorescent protein (GFP) and its variants have become very popular as they have been

validated by direct comparison to traditional reporter assays that use either chloroamphenicol acetyltransferase[10] or β-galactosidase.[11] For bacterial studies, the enhanced green fluorescent protein[12] (EGFP) is particularly useful as it is not toxic to *E. coli* like the wild-type GFP itself and the protein folds and autocatalyzes formation of its fluorophore with a half-life of less than 45 min rather than hours. Therefore, the gene for EGFP (BD Biosciences) was ligated into a pET vector plasmid, using NcoI and EcoRI to have control of EGFP protein expression with a *lac* promoter system and to have low background protein expression levels. This plasmid was transformed into *E. coli* BL21 DE(3) cells for protein expression studies. The cells were grown to an optical density of 0.7 (at 600 nm) and then protein production was induced with either IBCG or IPTG. The fluorescence emission at 507 nm after excitation at 488 nm, normalized for the number of cells, was plotted as a function of time (Figure 2) as previously reported.[13] As expected, in the absence of IBCG and IPTG, the culture showed no activity at the *lac* promoter. In contrast, EGFP fluorescence was considerably larger among induced cells in the presence of IBCG or IPTG. After induction times of greater than 4 h, the IBCG induced cells show even greater fluorescence than the IPTG induced cells. This surprising result suggests that the *C*-glycoside analogue may act as a superior promoter or that it is more stable in the culture conditions over time.
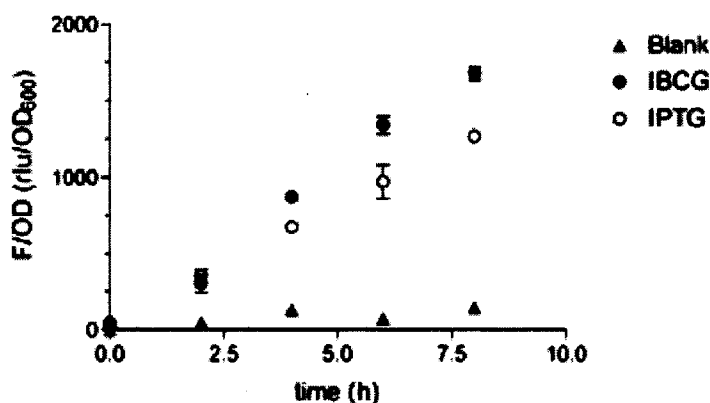
Figure 2 Comparison of the expression of the enhanced green fluorescent protein under *lac*

promoter control in BL21 DE(3) cells over time by the increase of fluorescence emission

(at 507 nm after excitation at 488 nm) corrected for cell densities: blank, uninduced cells;

IPTG, addition of 5.78 mM final concentration of IPTG; and IBCG, addition of 5.78 mM

final concentration of IBCG. Experiments were run in duplicate. Rlu = relative light units.

Conclusion

Clearly, the more stable *C*-glycoside analogue IBCG serves as a surrogate for the

commonly used inducer of protein expression IPTG and shows advantages at long

induction times. In addition, solutions of the analogue do not need to be made as frozen

aliquots, but can be autoclaved in water and stored at room temperature for ready addition

of the inducer to cell cultures. The latter property is especially valuable for small-scale

culture induction and for cases in which a more precise concentration of inducer is

desirable. These results and the earlier X-ray structural work are both consistent with the

supposition that the anomeric linkage of the natural inducer allolactose does not form any

critical binding interactions with the *lac* repressor protein. In addition, the *C*-glycoside

retains cell permeability. This work suggests that, in studies on biological systems dependent on O-glycosides, lone pairs in the glycosidic linkage are not necessarily required and the C-glycosides may offer distinct advantages beyond stability over S-glycosides in mimicking carbohydrate activities and functions.

Experimental Section

General methods: Reaction solvents were distilled from calcium hydride for dichloromethane and from sodium metal and benzophenone for diethyl ether. Amberlyst 15 ion-exchange resin was washed repeatedly with methanol before use. The Grignard reaction was performed in oven-dried glassware. The 1-bromo-α-D-galactose-tetraacetate was dissolved in anhydrous toluene and the solvent was removed under reduced pressure; the sample was then dried under high vacuum for 3 days. All other commercial reagents and solvents were used as received without further purification. The reactions were monitored and the R_f values determined using analytical thin layer chromatography (tlc) with 0.25 mm EM Science silica gel plates (60F-254). The developed tlc plates were visualized by immersion in *p*-anisaldehyde solution followed by heating on a hot plate. Flash chromatography was performed with Selecto Scientific silica gel, 32-63 μm particle size. All moisture-sensitive reactions were performed in flame- or oven-dried glassware under a nitrogen atmosphere. Bath temperatures were used to record the reaction temperature in all cases. All reactions were stirred magnetically at ambient temperature unless otherwise indicated. ^{1}H NMR and ^{13}C NMR spectra were obtained with a Bruker

DRX400 at 400 MHz and 100 MHz respectively. ^{1}H-^{1}H correlation experiments were obtained with a Bruker Advance DRX500.
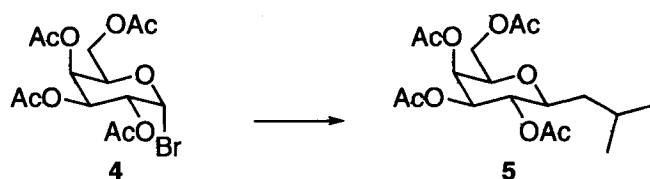
1-isobutyl-β-D-galactose tetraacetate (5): Magnesium turnings (4.2 g) were suspended in diethyl ether (45 mL) and then initiated with a crystal of I_2. 1-Bromo-2-methylpropane (1.37 mL, 12 mmol) was then added to the magnesium suspension. The mixture turned a cloudy white after the flask was heated with a heat gun. A solution of 1-bromo-α-D-galactose tetraacetate **4** (4.307 g, 10.5 mmol) in diethyl ether (10 mL) was added dropwise into the Grignard reaction mixture. The reaction went to completion after the mixture was heated at reflux for 4 h. The mixture was slowly poured into water (300 mL). Glacial AcOH (11 mL) was added. The mixture was separated and the aqueous layer was concentrated. The residue was treated with acetic anhydride (150 mL) and pyridine (150 mL) overnight. The resulting mixture was diluted with water (300 mL), extracted with diethyl ether (3 x 250 mL), washed with water (2 x 150 mL), and dried with magnesium sulfate. The solvent was removed under reduced pressure. The product was purified by flash chromatography (silica gel, 2:3 ethylacetate/hexane) to afford **5** as a white solid (2.80 g, 7.24 mmol, 69%).
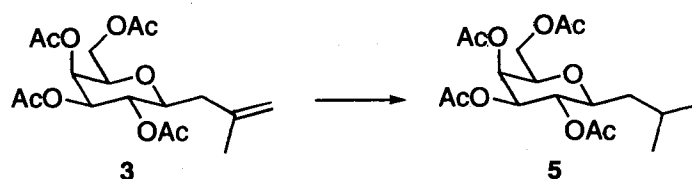
1-isobutyl-β-D-galactose tetraacetate (5): A solution of **3** (0.90 g, 2.3 mmol) in MeOH (15 mL) was stirred with 20 wt. % palladium hydroxide on carbon (0.08 g) under atmospheric H_2 at ambient temperature for 5 h. The mixture was filtered over Celite and the filtrate was concentrated under reduced pressure to afford **5** as a white solid (0.90 g, 2.3 mmol, 97%).

R_f 0.35 (ethyl acetate/hexane, 3/7 v/v).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (d, 1 H, J = 3.2 Hz, H-4), 5.01 (dd, 1 H, J_1 = 8.0 Hz, J_2 = 8.8 Hz, H-2), 4.96 (dd, 1 H, J_1 = 3.2 Hz, J_2 = 8.0 Hz, H-3), 4.10 (dd, 1 H, J_1 = J_2 = 6.8 Hz, H-6), 3.99 (dd, 1 H, J_1 = 4.8 Hz, J_2 = 6.4 Hz, H-6), 3.79 (m, 1 H, H-5), 3.39 (dd, 1 H, J_1 = 9.0 Hz, J_2 = 2.0 Hz, H-1), 2.11 (s, 3 H, acyl), 2.00 (s, 3 H, acyl), 1.98 (s, 3 H, acyl), 1.94 (s, 3 H, acyl), 1.78 (m, 1 H, H-2'), 1.48 (m, 1 H, H-1'), 1.14 (m, 1 H, H-1'), 0.83 (2 d, 6 H, J = 5.1 Hz, H-3', H-4').

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 170.6, 170.4, 170.1, 74.4, 72.5, 70.0, 68.0, 66.5, 61.9, 40.4, 24.5, 23.7, 21.7, 21.1, 20.9, 20.9, 20.8.

MS (EIMS) *m/z* 389 $[M+H]^+$
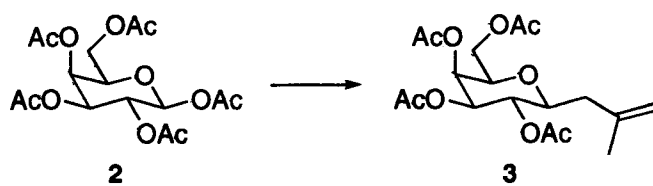
1-(2'-methylallyl)-β-D-galactose tetraacetate (3): To solution of β-D-galactose-pentaacetate (2.02 g, 5.18 mmol) in dichloromethane (60 mL) was added methallyl-trimethylsilane (2.66 g, 20.7 mmol) followed by slow addition of $BF_3 \cdot Et_2O$ (1.64 mL) into the solution at –20 °C. The mixture was stirred under N_2 overnight. The reaction was poured into a saturated solution of $NaHCO_3$. The product was extracted with dichloromethane (3 x 60 mL) and the solvent was removed under reduced pressure. The product was purified by flash chromatography (silica gel, ethyl acetate/hexane gradient) to yield a white solid (0.96 g, 2.5 mmol, 48%).

R_f 0.23 (ethyl acetate/hexane, 3/7 v/v).

¹H NMR (400 MHz, $CDCl_3$) δ 5.38 (dd, 1 H, J = 1.2 Hz, H-4), 5.08 (t, 1 H, J_1 =9.9 Hz, J_2 = 6.9 Hz, H-2), 4.99 (dd, 1 H J_1 = J_2 = 3.6 Hz, H-3), 4.73 (d, 2 H, J = 17.1 Hz, H-3'), 4.11 (m, 1 H, H-6), 4.01 (dd, 1 H, J_1 = J_2 = 6.6 Hz, H-6), 3.82 (t, 1 H, J = 5.7 Hz, H-5), 3.54 (m, 1 H, H-1), 2.19 (m, 2 H, H-1'), 2.13 (s, 3 H, acyl), 2.03 (s, 3 H, acyl), 2.01 (s, 3 H, acyl), 1.96 (s, 3 H, acyl), 1.70 (s, 3 H, H-4').

¹³C NMR (100 MHz, $CDCl_3$) δ 170.6, 170.5, 170.4, 170.0, 141.6, 112.9, 77.3, 74.3, 72.4, 69.7, 68.0, 61.8, 40.4, 23.0, 21.2, 21.1, 20.9, 20.8.
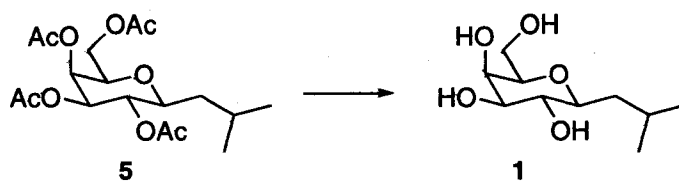
1-isobutyl-β-D-galactose (1): 1-isobutyl-β-D-galactose tetraacetate **5** (1.21 g, 3.1 mmol) in MeOH (30 mL) was stirred with NaOH (0.22 g) at room temperature for 1.5 h. Amberlyst 15 ion-exchange resin was added to neutralize the solution. The mixture was filtered over Celite and methanol was evaporated under reduced pressure to afford **1** as a white solid (0.68 g, 3.1 mmol, 99%).

R_f 0.28 (methanol/ethyl acetate/hexane, 1/5/5 v/v/v).

¹H NMR (400 MHz, D_2O) δ 3.76 (d, 1 H, J = 4.4 Hz, H-4), 3.51 (m, 2 H, H-2, H-3), 3.41 (m, 1 H, H-5), 3.42 (m, 2 H, H-6), 3.25 (m, 1 H, H-1), 1.70 (m, 1 H, H-2'), 1.30 (m, 1 H, H-1'), 1.27 (m, 1 H, H-1'), 0.75 (dd, 6 H, $J_1 = J_2 = 2.4$ Hz, H-3', H-4').

¹³C NMR (100 MHz, D_2O) δ 78.4, 78.2, 74.1, 71.5, 69.2, 61.3, 40.2, 23.9, 23.2, 20.9.

MS (EIMS) *m/z* 221 [M+H]⁺

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CHAPTER 7

Conclusions and Future Directions

In this dissertation, the solution, solid-phase, and fluorous-phase synthesis of carbohydrates has been studied with applications in biology. We developed the new nitrophthalimidobutyric (NPB) acid protecting group to allow colorimetric monitoring of glycosylation/deprotection cycles. Although the NPB group overcame the reaction monitoring problem, the solution phase synthesis of oligosaccharides using a fluorous tag proved superior as it allowed the use of equivalent donor to acceptor group instead of a large excess of the donor. This new fluorous tag-assisted solution phase strategy allows the rapid, and potentially automated, modular synthesis of carbohydrates and also their use in forming microarrays. A fluorous-based microarray method facilitates the formation of a range of carbohydrate chips for the plant and other sciences using synthetic sugars produced with the aid of fluorous-tagged synthesis. Ultimately, automation of the fluorous-phase synthesis protocols initially developed here will allow full exploitation of the power of the new fluorous-based microarray technology. In future work, these new techniques should be easily applicable to study biological systems including vaccine development, cancer drug development, and other compound microarrays.

In addition, we demonstrated that sugar nucleotidyltransferases could make use of not only differences in substrate binding affinity, but also kinetic differences in discriminating among carbohydrate substrates. Chemoenzymatic reactions might benefit from an increase in enzyme concentration rather than substrate concentration to increase

product yields. Sugar nucleotidyltransferases also provided means for the facile chemoenzymatic synthesis of carbocyclic versions of activated sugars. Further work should include studies of the effects of this substitution on the conformations and properties of carbasugars and cocrystallization studies with glycosyltransferases and their respective glycosyl acceptors. In addition, combinations of this enzymatic work with the fluorous-phase work should be promising.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my major professor, Dr. Nicola Pohl, for her encouragement, kind patience, and financial support throughout my graduate studies at Iowa State University. I want to thank all my committee members, Dr. Keith Woo, Dr. Yan Zhao, Dr. Malika Jeffries-EL and Dr. Aaron D. Sadow for providing me both academic and personal help. They truly made this study better.

I would like to thank the members of the Pohl group, Firoz A. Jaipuri, Dr. Rahman M. Mizanur, Dr. Corbin Zea, Yang Yu, Sreeman K. Mamidyala, Gisun Park, Steve Brokman, Gulden Camci-Unal, EunHo Song, Dr. Beatrice Collet, Eric Testroet, and Amy Nielsen, for enjoying work in the same lab. It has been my great pleasure being with all of you.

Finally, I want to express my deepest thanks to my lovely wife, Kab-Bun So, for her constant love, patience, understanding, and encouragement during my graduate program at Iowa State University. I want to say to her you are a wonderful wife and great mom. I also must thank my sons, Alex (Hee-Kwon) and Nathan (Young-Hoon), for their presence in my life have brought great joy with their laughter.

APPENDIX A. CHAPTER 2 ^{1}H AND ^{13}C NMR SPECTRA

400 MHz ¹H NMR(CDCl₃)

kks9-36-rel(400)

400 MHz ^{13}C NMR(CDCl$_3$)

kks9-3/re2

400 MHz ^{17}F NMR(CDCl$_3$)

400 MHz ^{1}H NMR(CDCl$_3$)

ks9-11/rel

400 MHz ^{13}C NMR(CDCl$_3$)

kks10-28/re2

400 MHz ^{1}H NMR(CD$_3$OD)

kks10-28/re1

400 MHz ^{13}C NMR(CD$_3$OD)

kks10-29/re2

400 MHz ^{1}H NMR(CDCl$_3$)

kks10-6/re5

400 MHz ^{13}C NMR(CDCl$_3$)

kks10-6/re6

400 MHz ^{1}H NMR(CD$_3$OD)

400 MHz ^{13}C NMR(CD$_3$OD)

kks9-31/re2
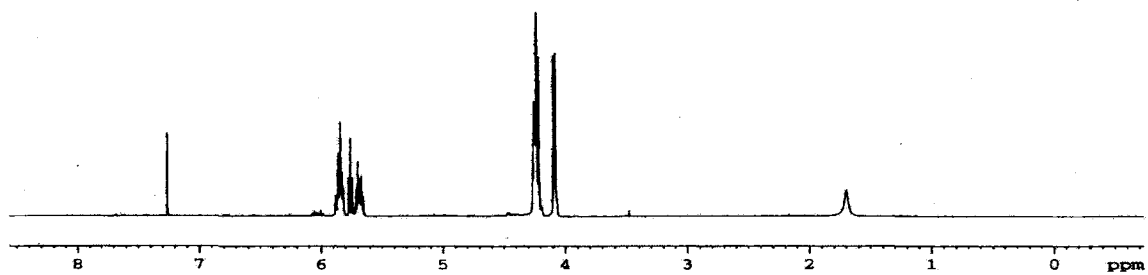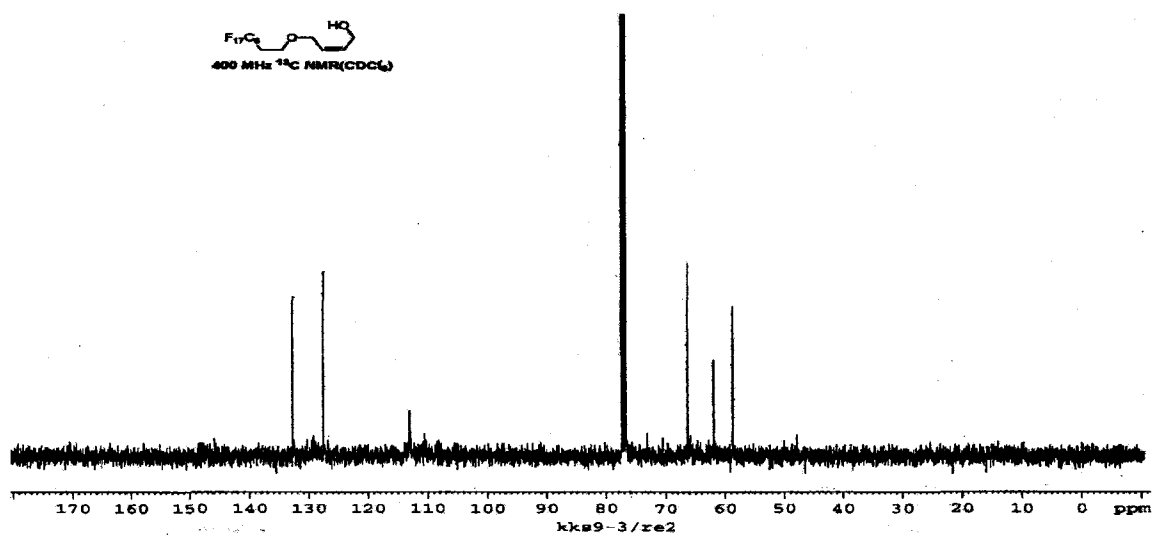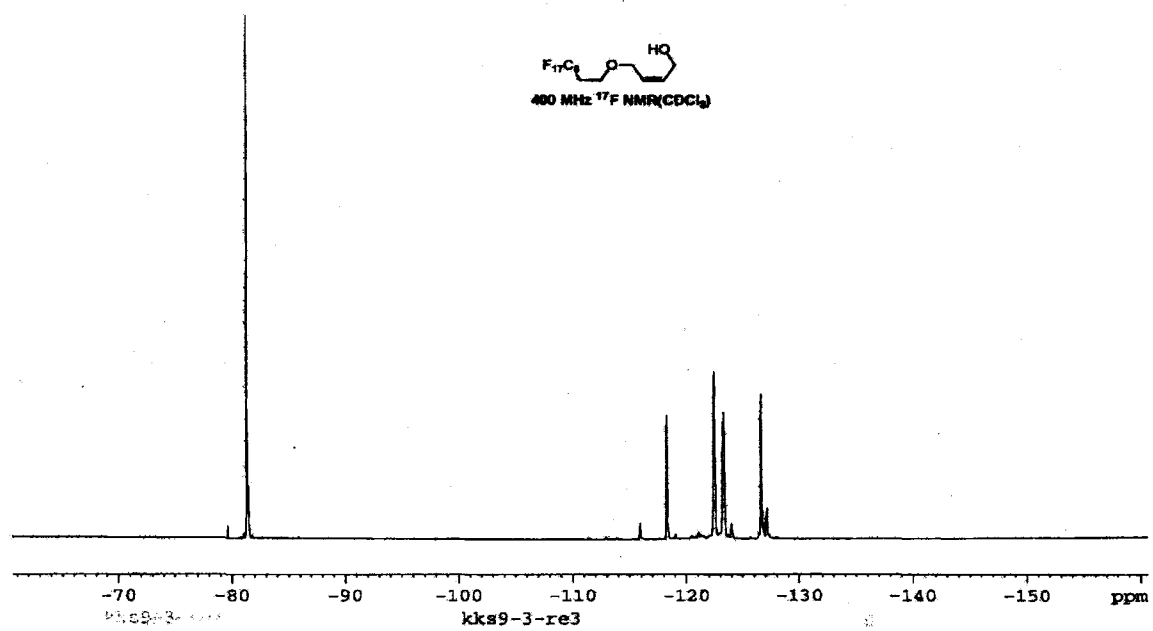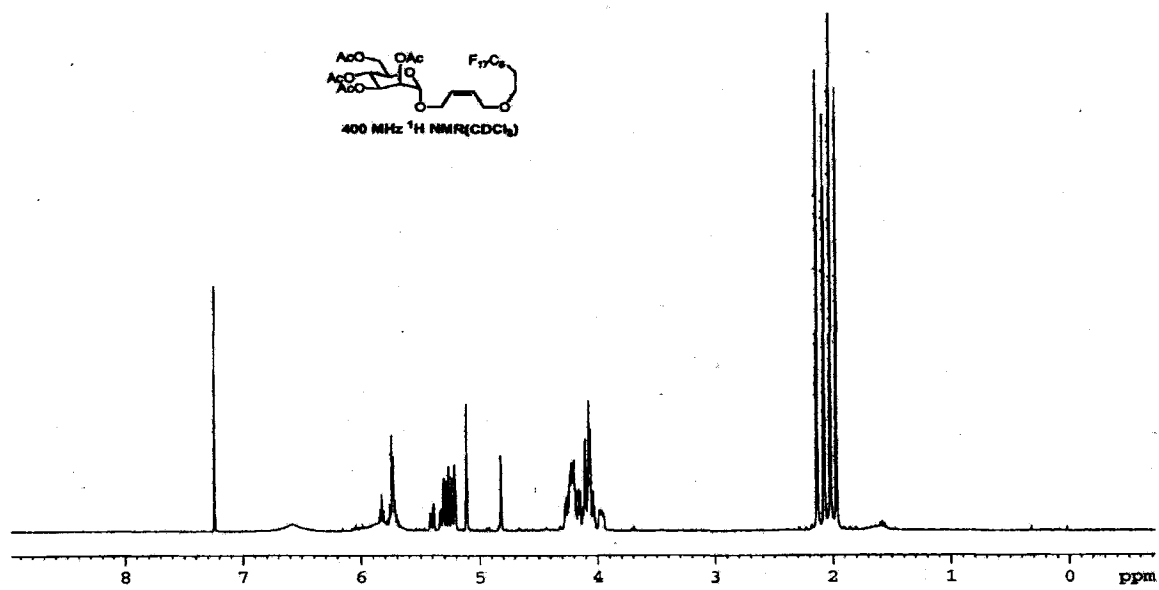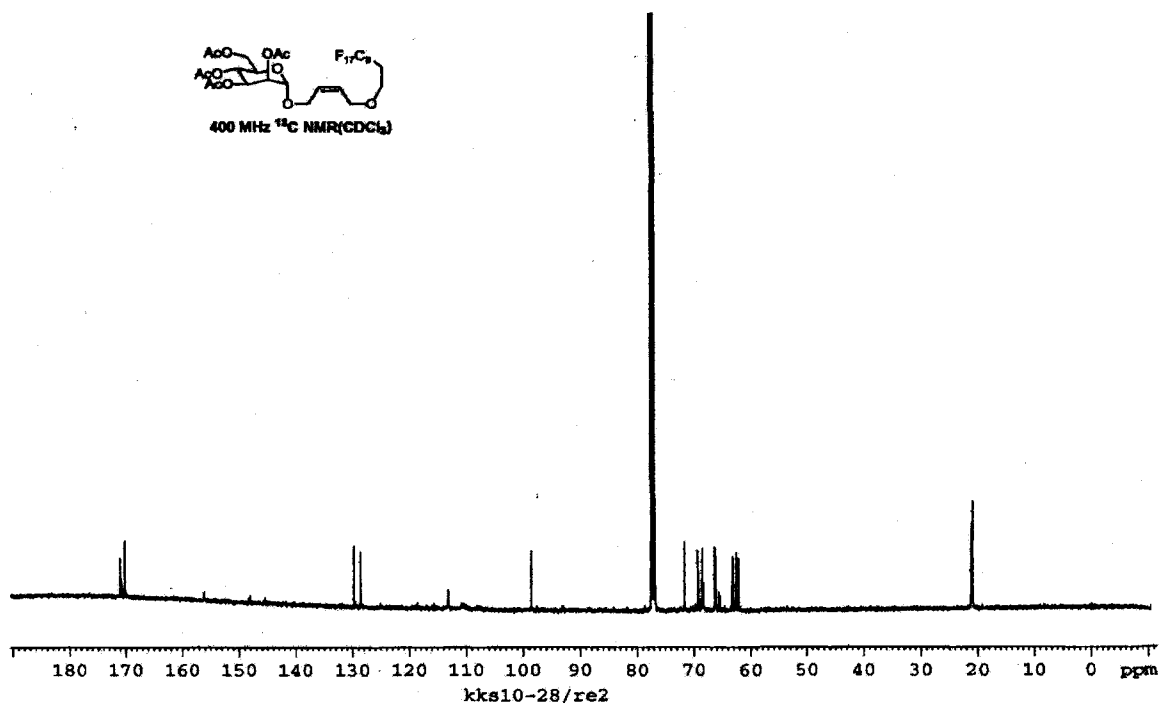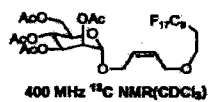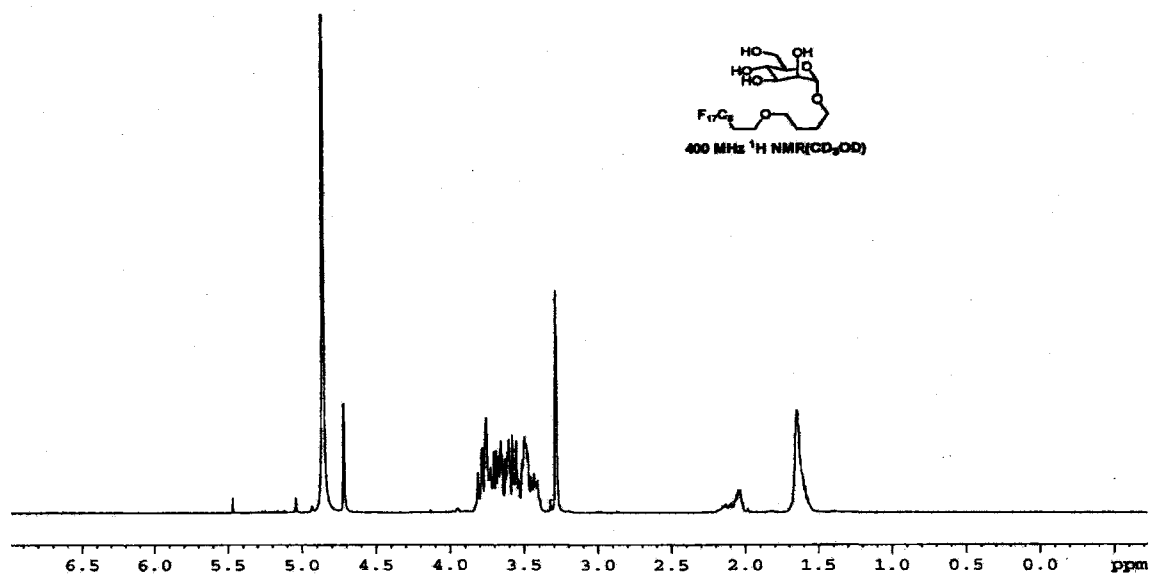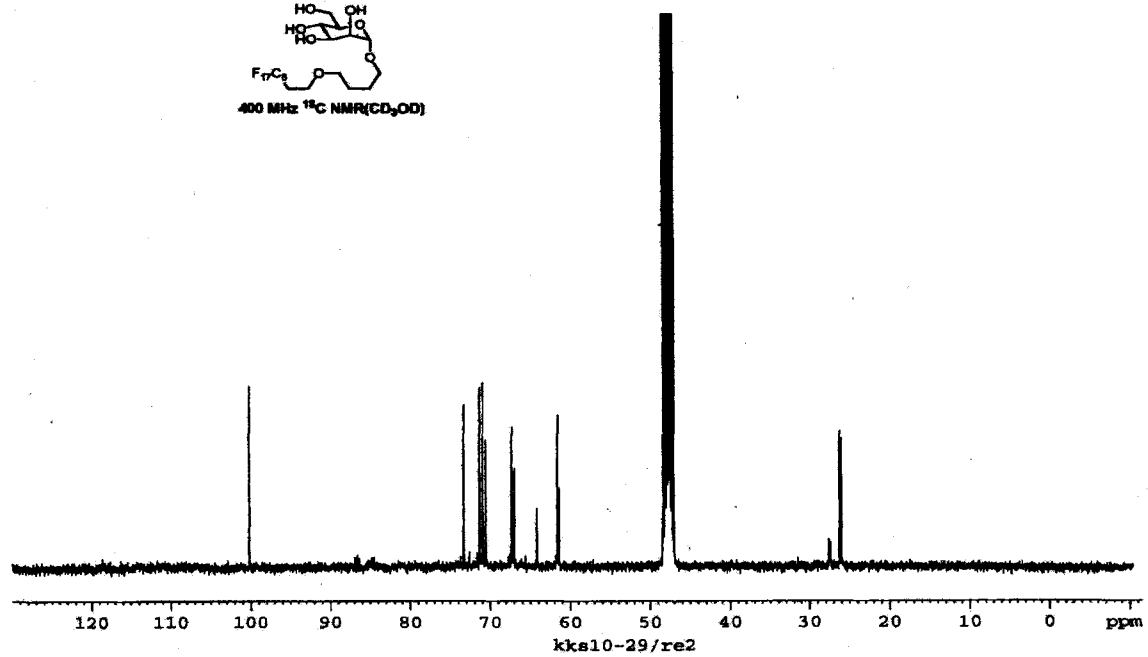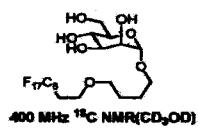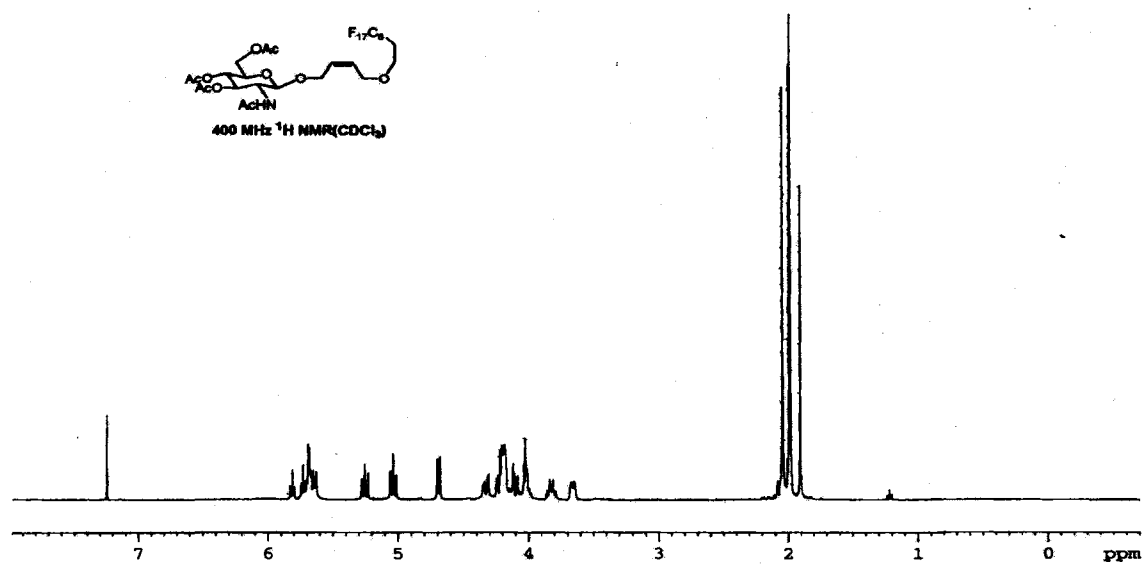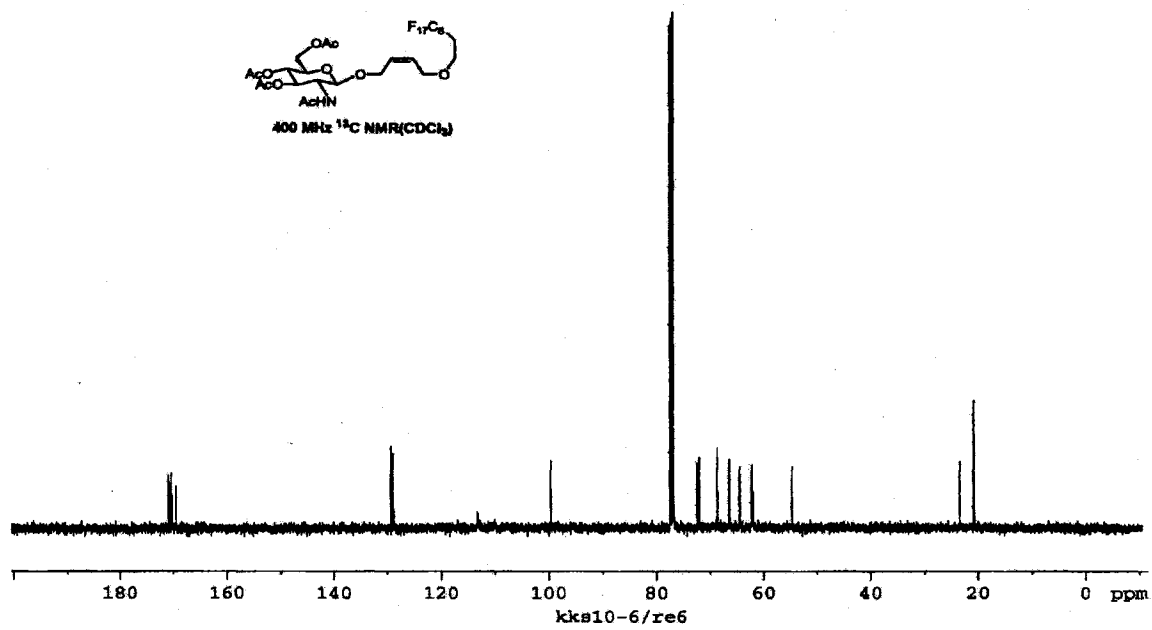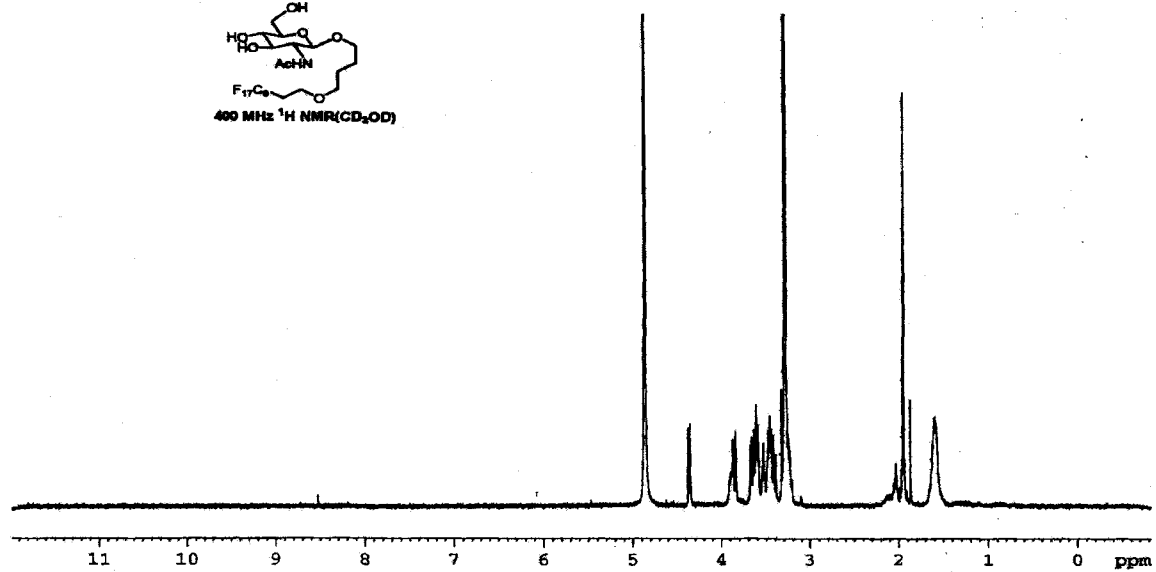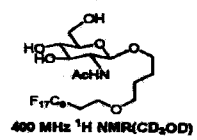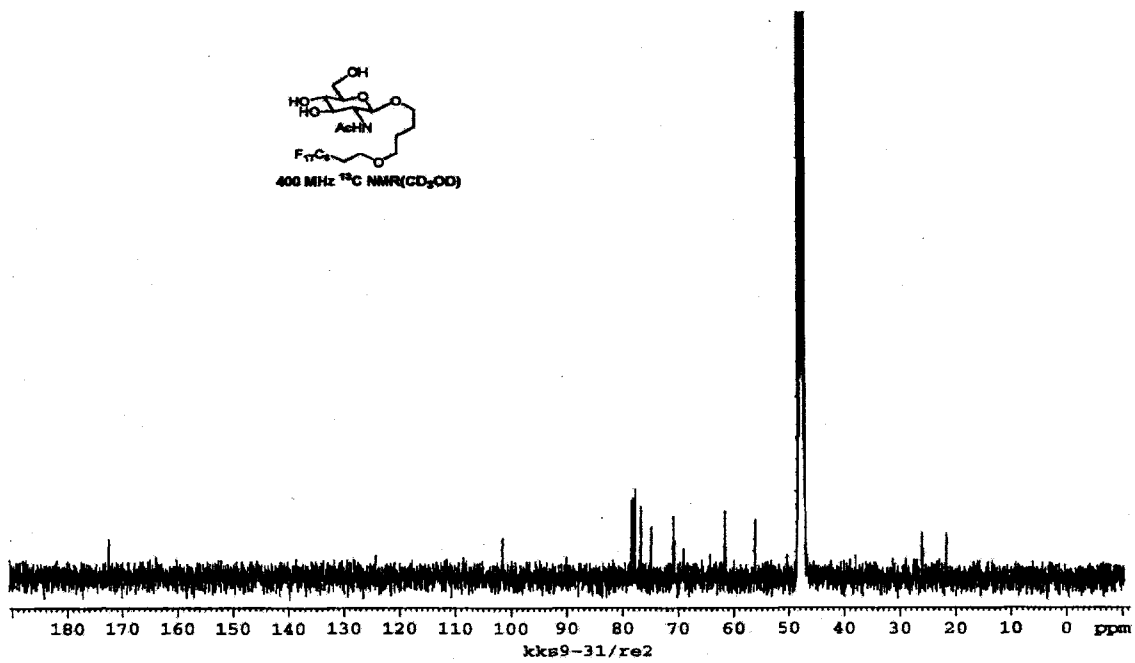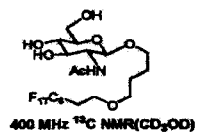
APPENDIX B. CHAPTER 3 ^{1}H AND ^{13}C NMR SPECTRA

9a

400 MHz ^{1}H NMR

400 MHz ^{13}C NMR

10
400 MHz ¹H NMR

10
400 MHz ^{13}C NMR

10a
400 MHz ¹H NMR

400 MHz ^{13}C NMR

400 MHz ^{1}H NMR

5.9 ppm

400 MHz ^{13}C NMR

400 MHz ^{1}H NMR

400 MHz ^{13}C NMR

12

300 MHz ¹H NMR

kks8-17-rel

300 MHz ^{13}C NMR

12

kks8-17-re2

12a

400 MHz ¹H NMR

kks8-29/re1

400 MHz ^{13}C NMR

kks8-21-re2

400 MHz, ^{1}H NMR

ppm 0 1 2 3 4 5 6

400 MHz ^{13}C NMR

vxr400/kks263c

14
300 MHz ^{1}H NMR

kks8-18-re1

14

300 MHz ^{13}C NMR

200 180 160 140 120 100 80 60 40 20 0 ppm

kks8-18-re2

15
300 MHz ^{1}H NMR

kks165H

15
300 MHz ^{13}C NMR

160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

kk#166C

15a
300 MHz ¹H NMR

kks166H

15a
300 MHz ^{13}C NMR

160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

kks166C

16
300 MHz ^{1}H NMR

kks167H

16
300 MHz ^{13}C NMR
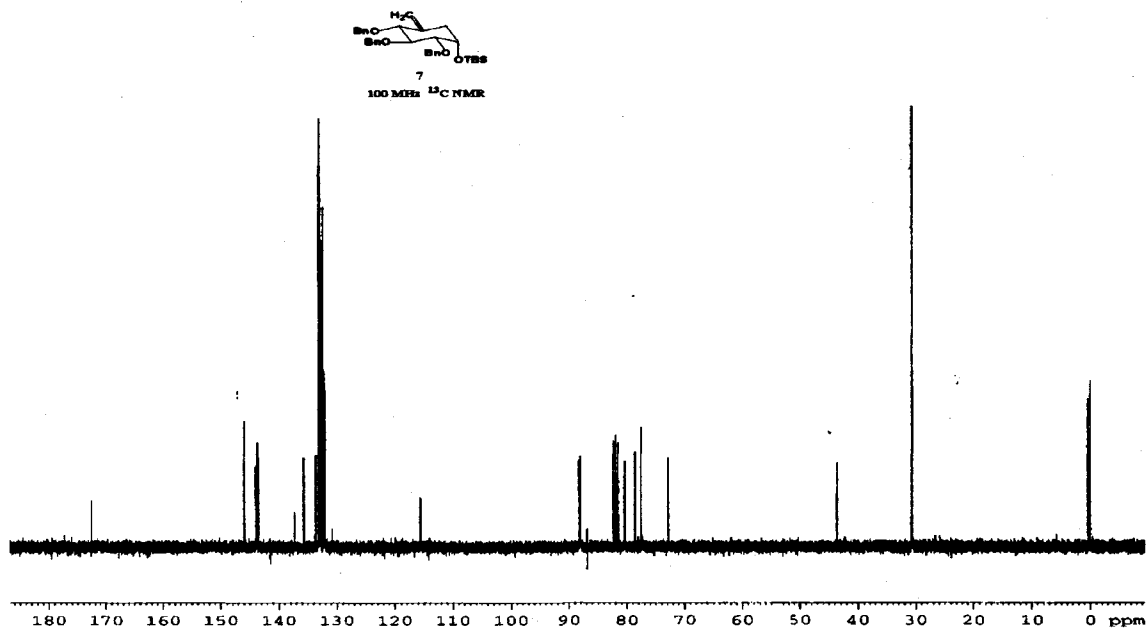
160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

kks167C

16a
300 MHz ^{1}H NMR

kks168H

16a
300 MHz ^{13}C NMR

vxr300/kks168c

400 MHz ^{1}H NMR

kks244H

16b
400 MHz ^{13}C NMR

vxr300/kks168c

17
300 MHz ¹H NMR

kks215H

17
300 MHz ^{13}C NMR

kks215C

17a

360 MHz ^{1}H NMR

kks171H

17a
300 MHz ^{13}C NMR

kks171C

kks172H

kks172C

18a
300 MHz ^{1}H NMR

kks223H

vxr300/kk8223c

ppm 0   20   40   60   80   100   120   140   160

300 MHz 13C NMR
18a

BnO OH
BnO OBn
OBn

18b
400 MHz ^{1}H NMR

kks230H

18b
400 MHz ^{13}C NMR

kks230C

APPENDIX C. CHAPTER 4 ^{1}H AND ^{13}C NMR SPECTRA

400 MHz ^{1}H NMR

kks4-26/rel

100 MHz ^{13}C NMR

kks4-26/re2

5
400 MHz ^{1}H NMR
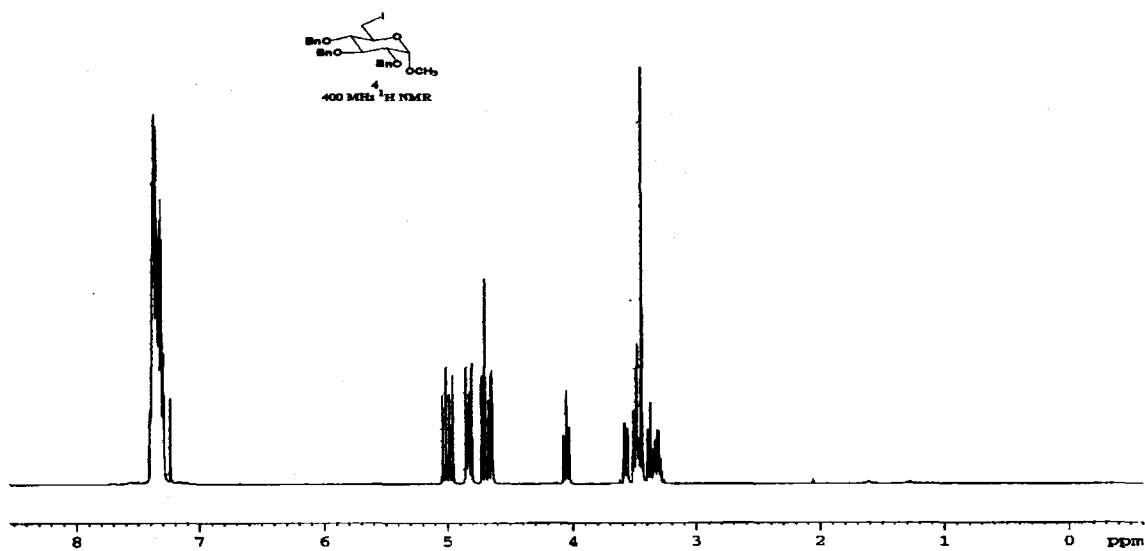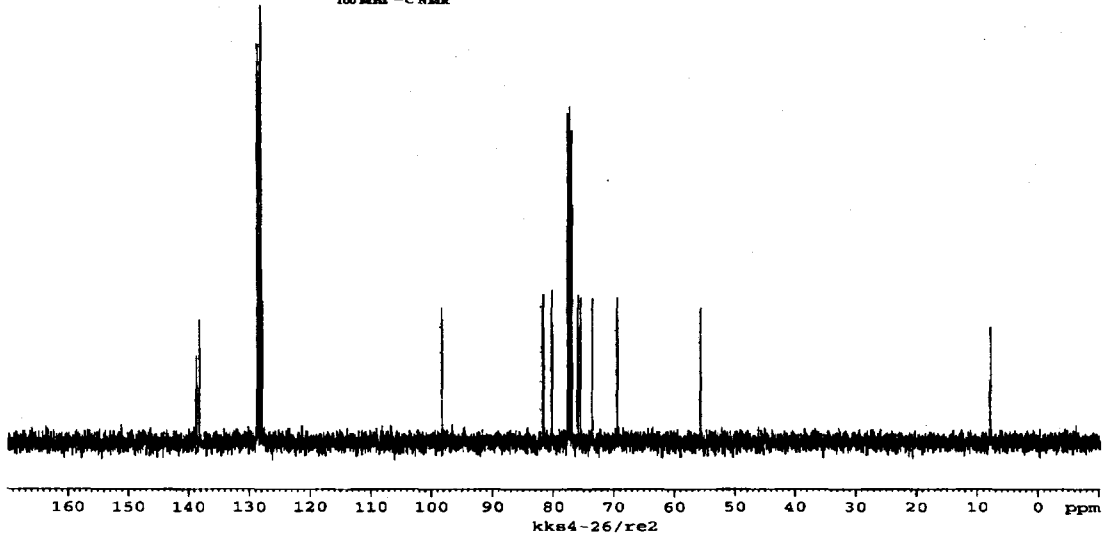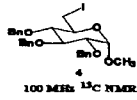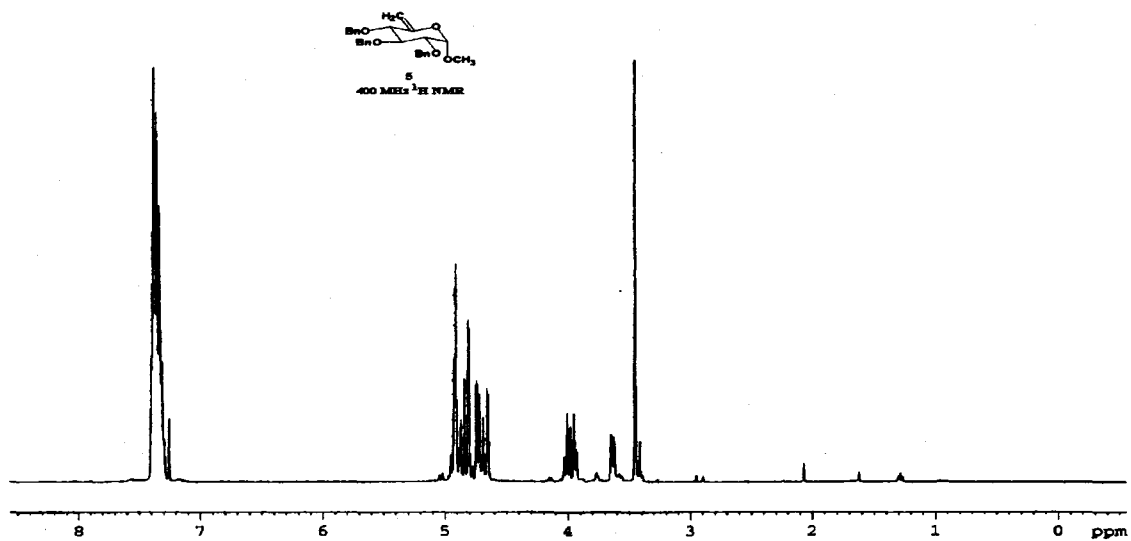
vxr400/kks4-27-rel

170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

100 MHz ^{13}C NMR

6a
400 MHz ^{1}H NMR

6a
100 MHz ^{13}C NMR

7
400 MHz ^{1}H NMR

7

100 MHz ^{13}C NMR

400 MHz ^{1}H NMR

100 MHz ^{13}C NMR

400 MHz ^{1}H NMR

100 MHz ^{13}C NMR

139 ppm

160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

kks4-18/re2

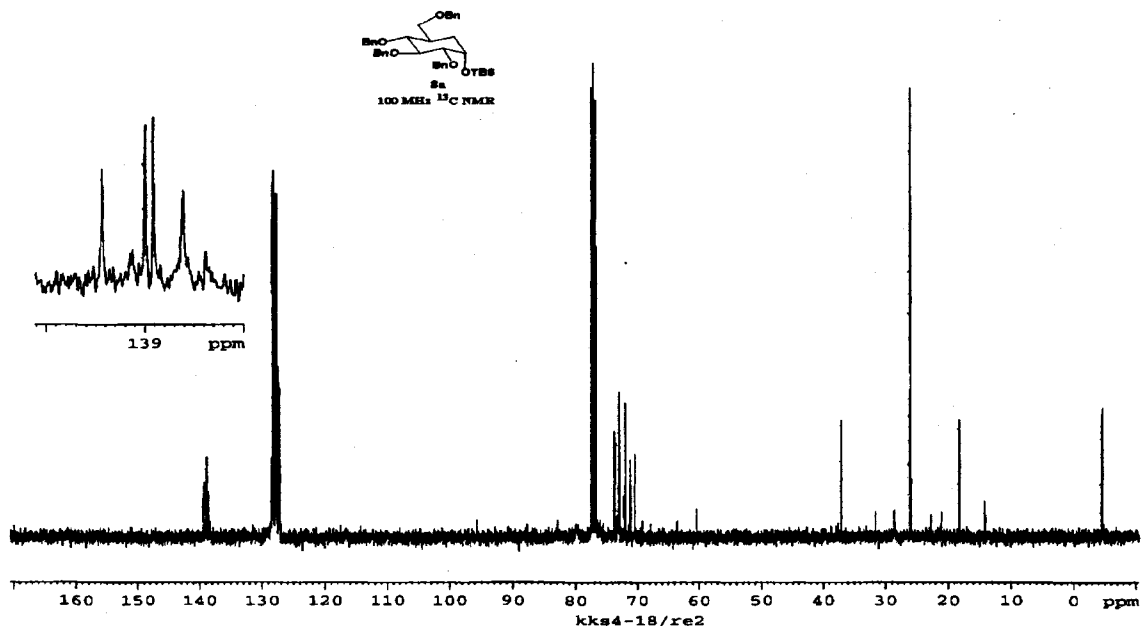400 MHz ^{1}H NMR

kks4-22/re1

100 MHz ^{13}C NMR

kks4-22/re2

9a
400 MHz ^{1}H NMR

100 MHz ^{13}C NMR

400 MHz ^{1}H NMR

2

ppm 0 1 2 3 4 5 6 7

2

100 MHz ^{13}C NMR
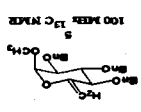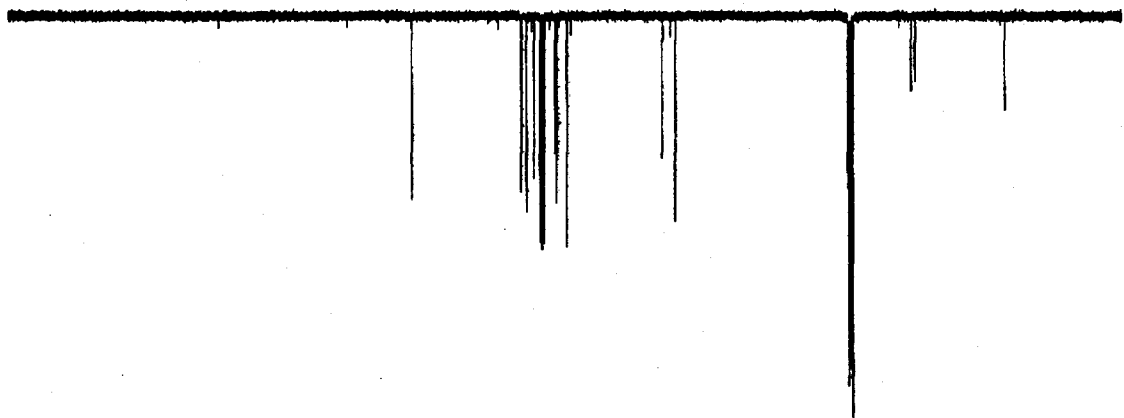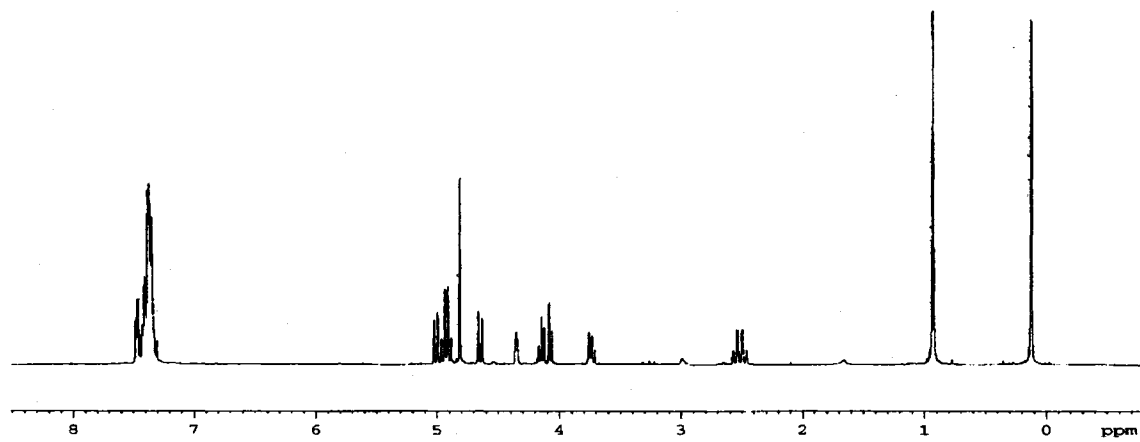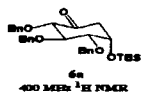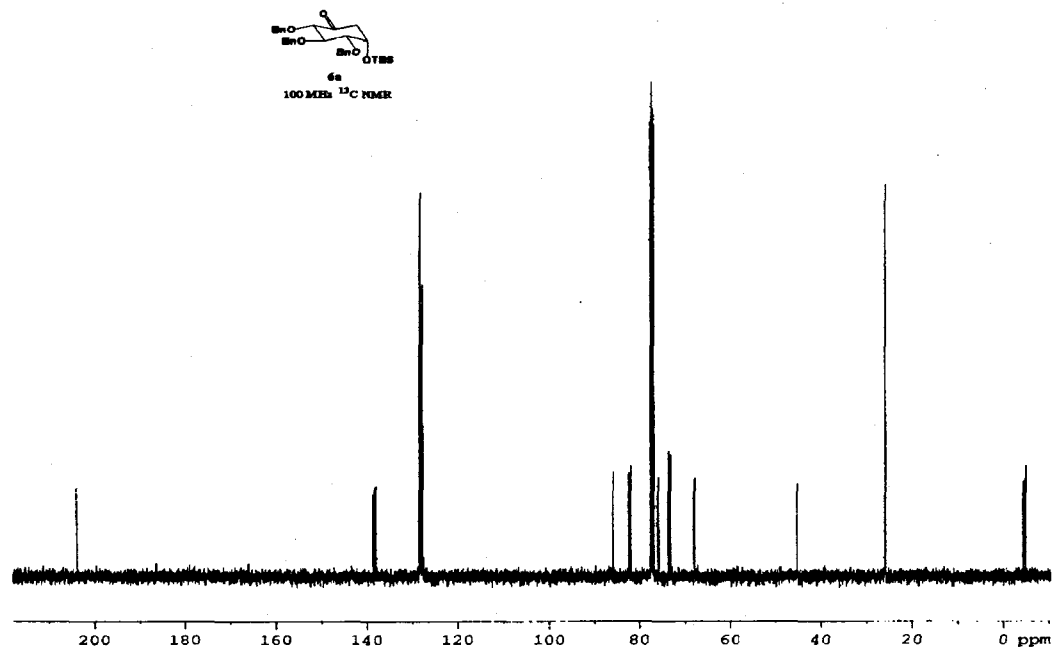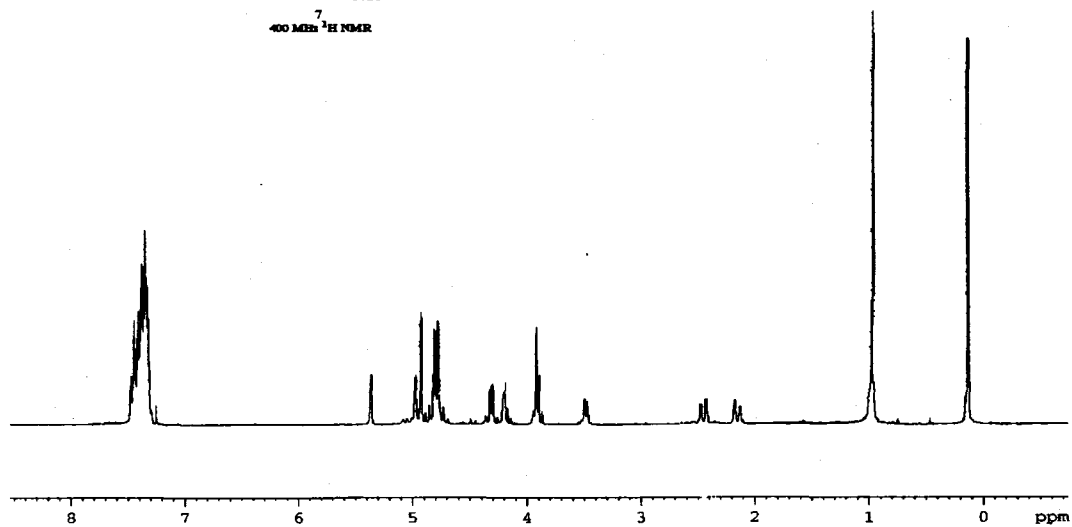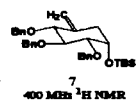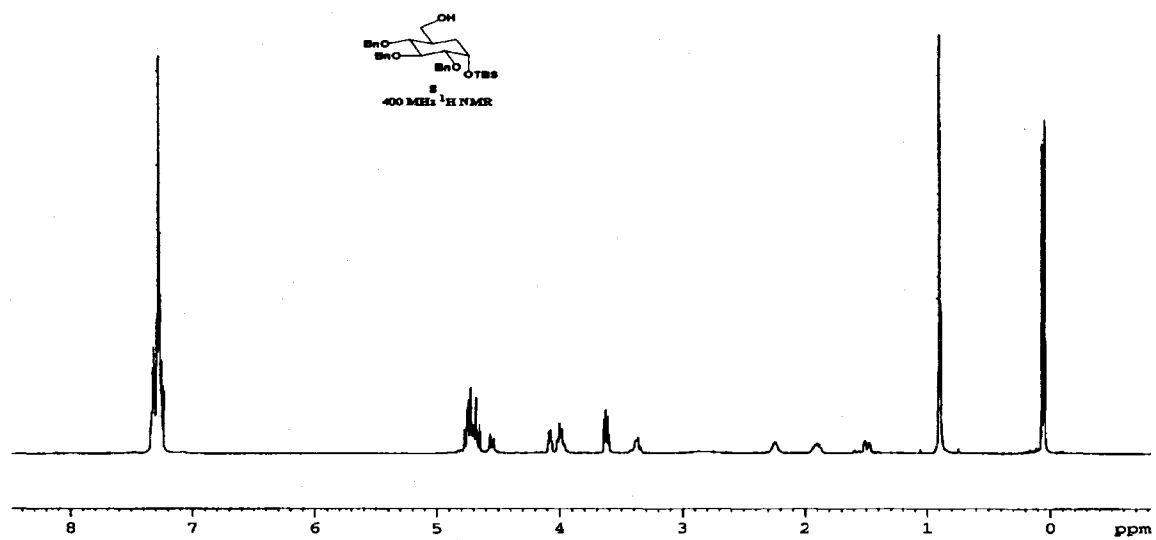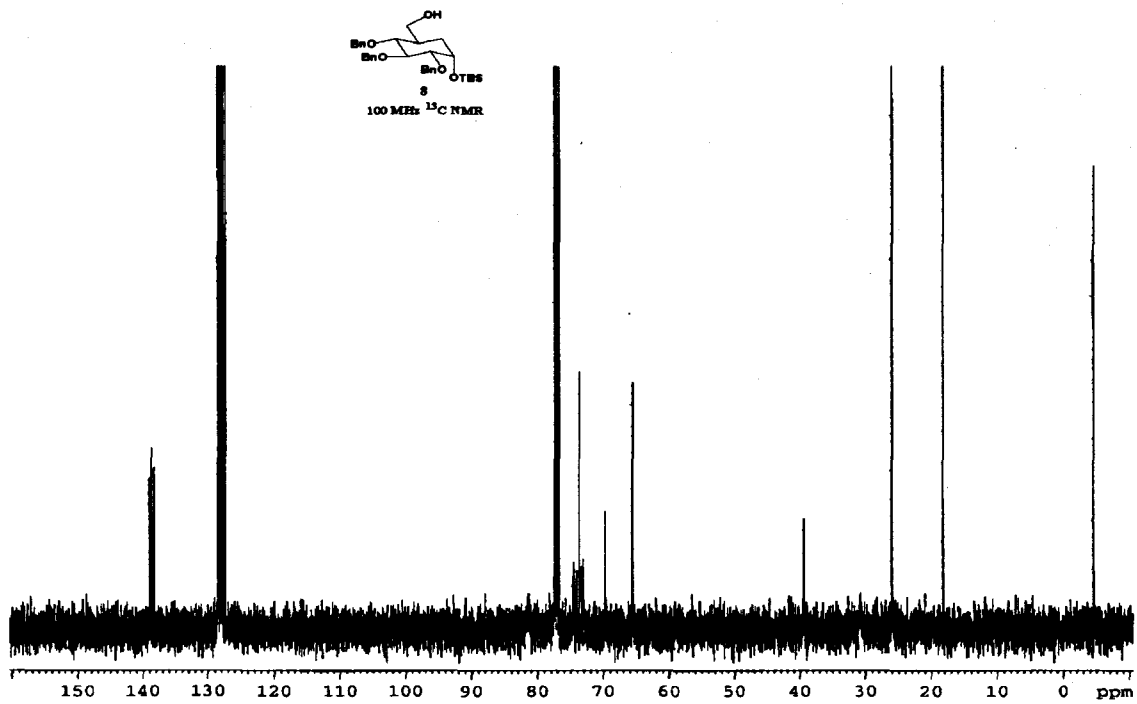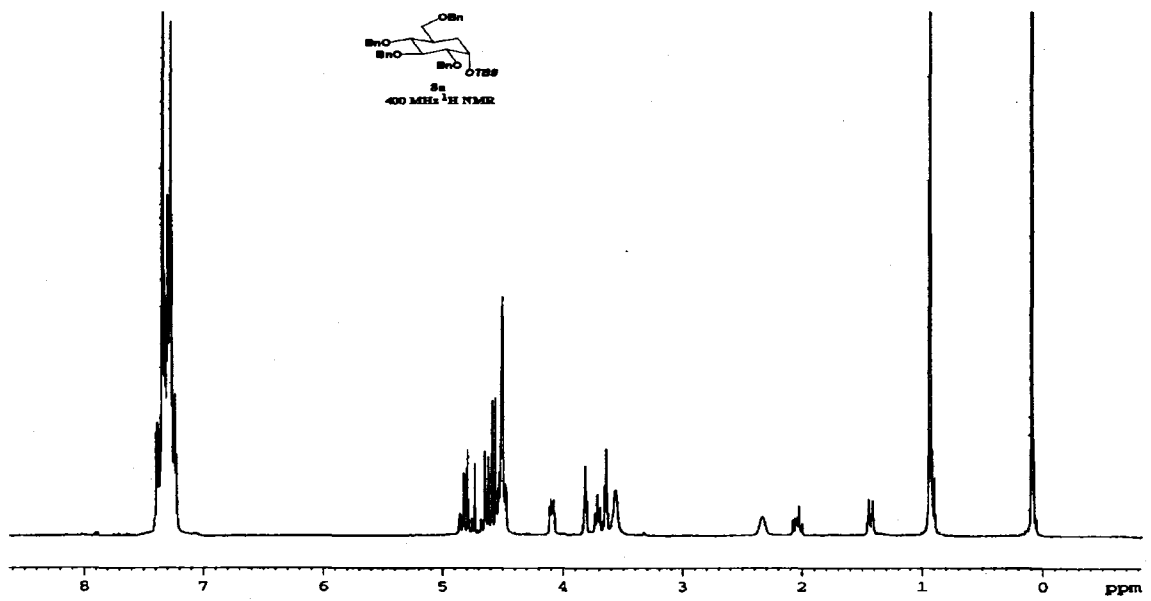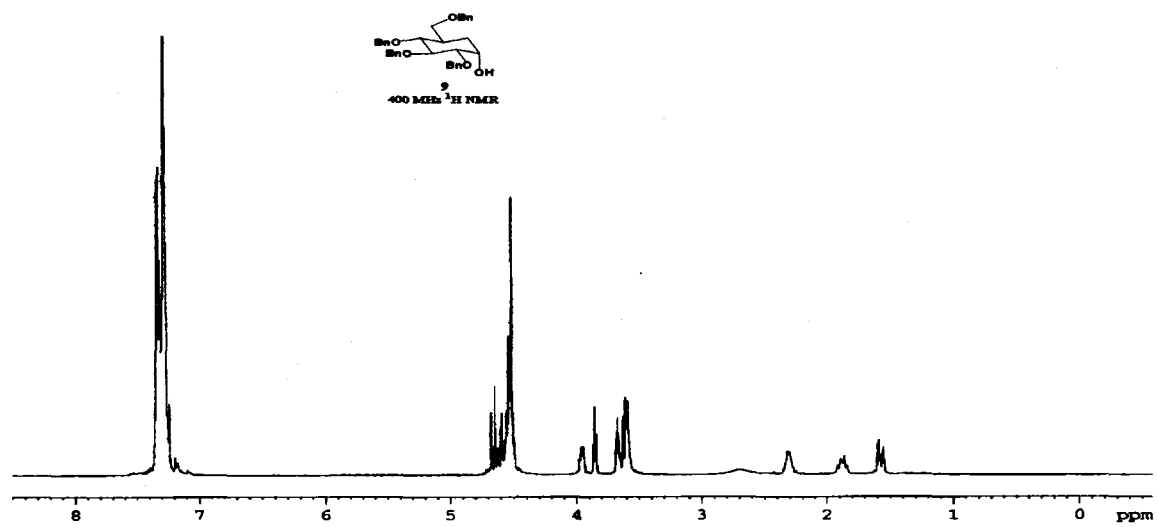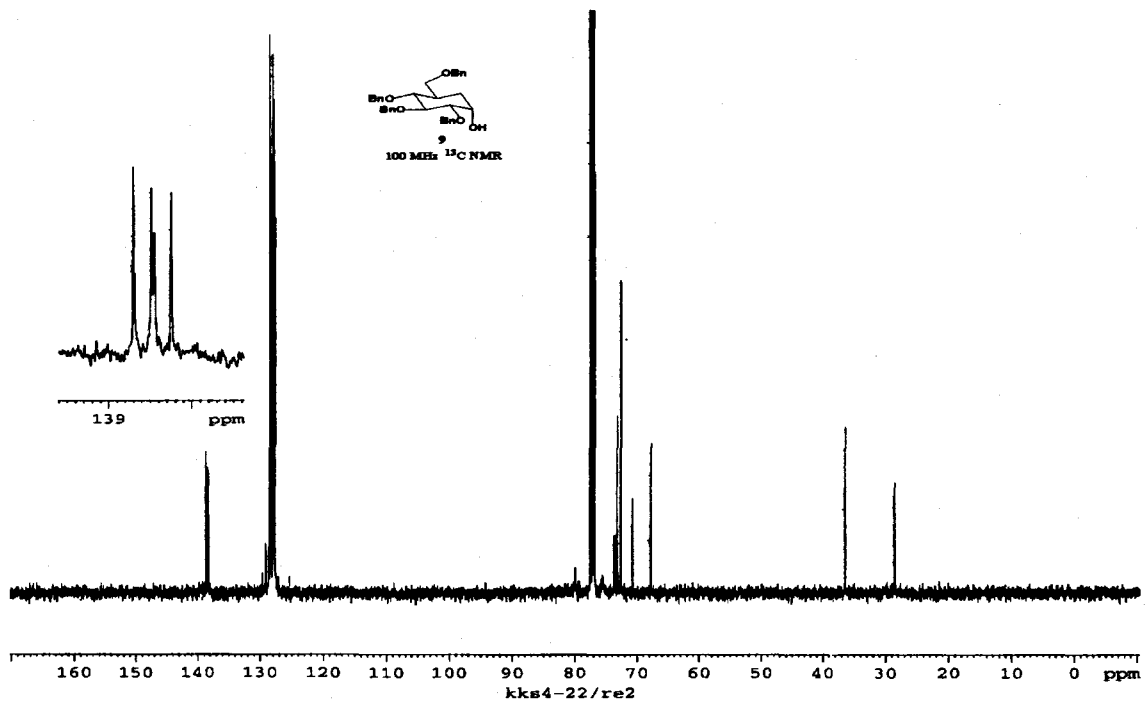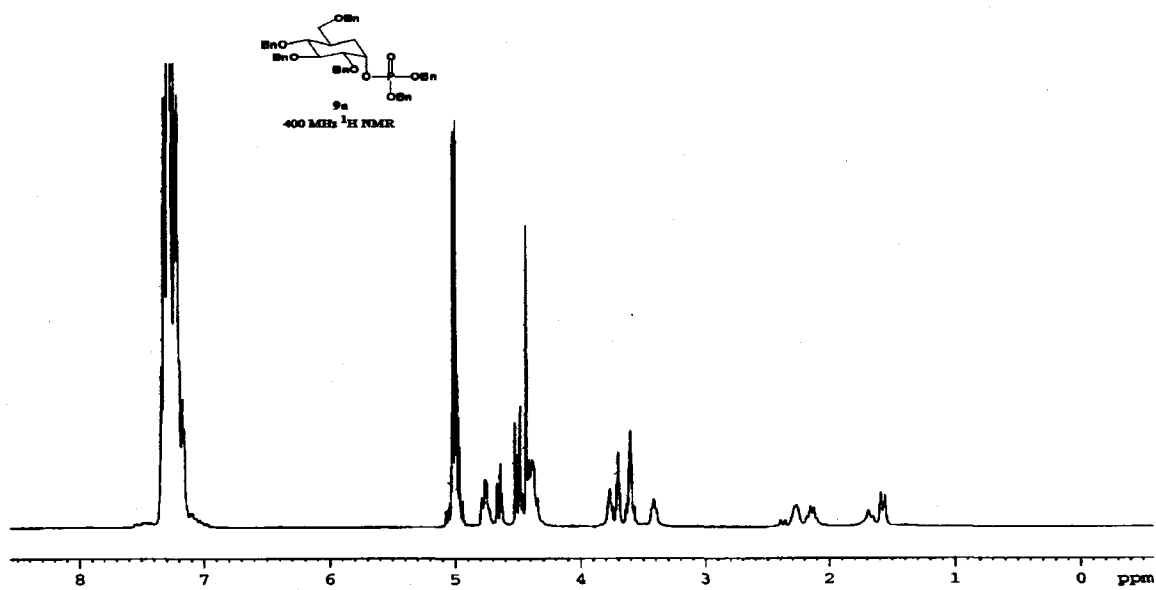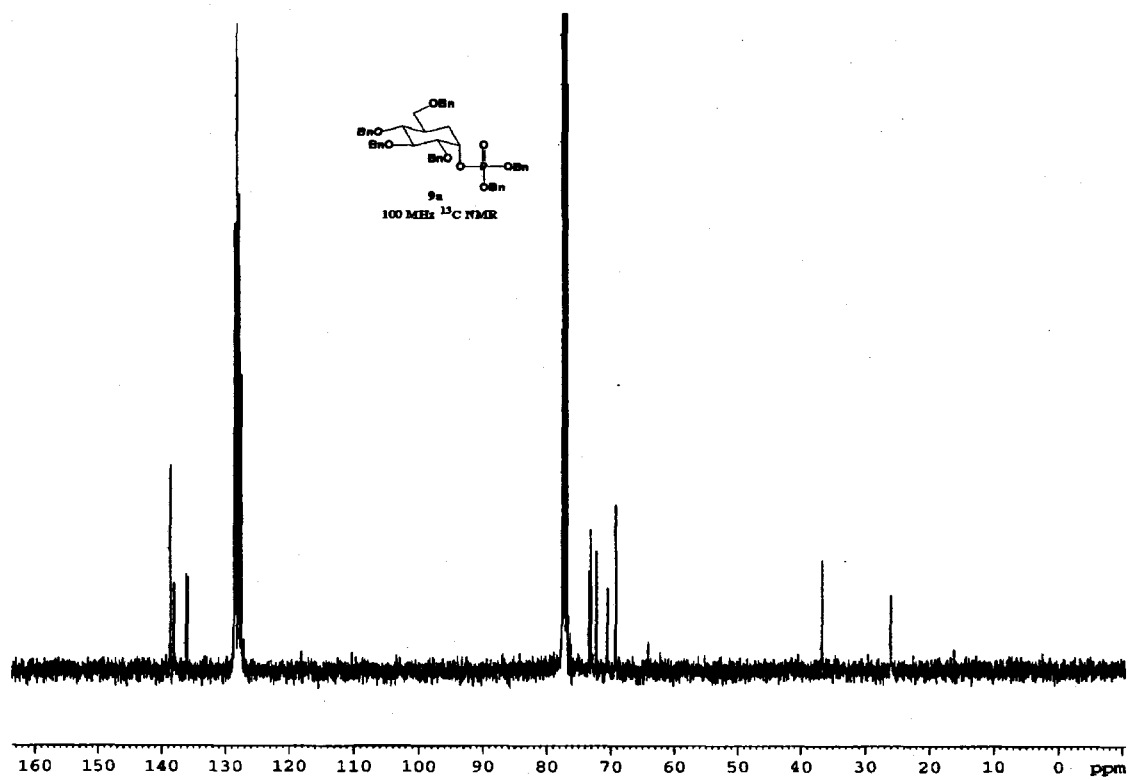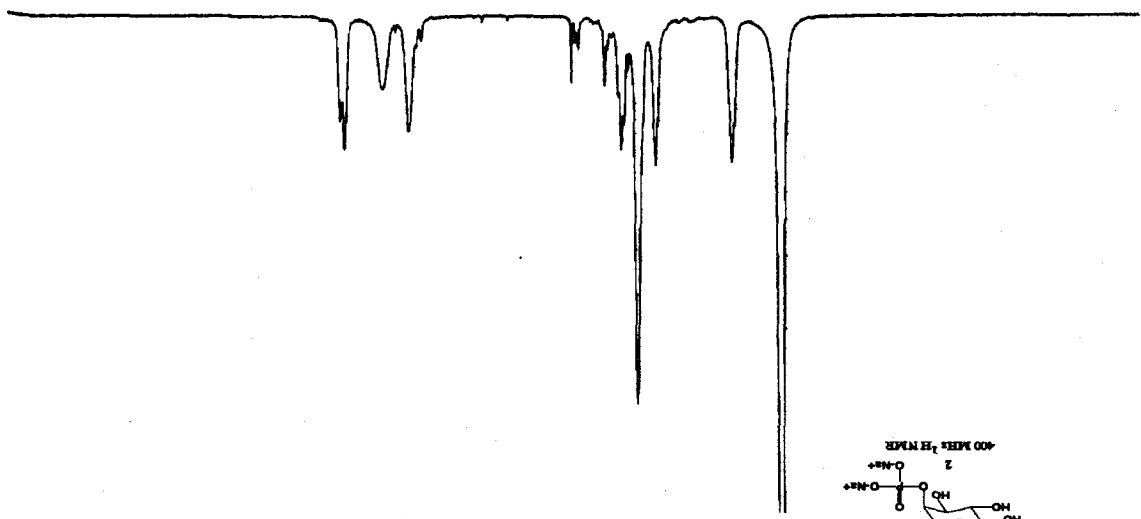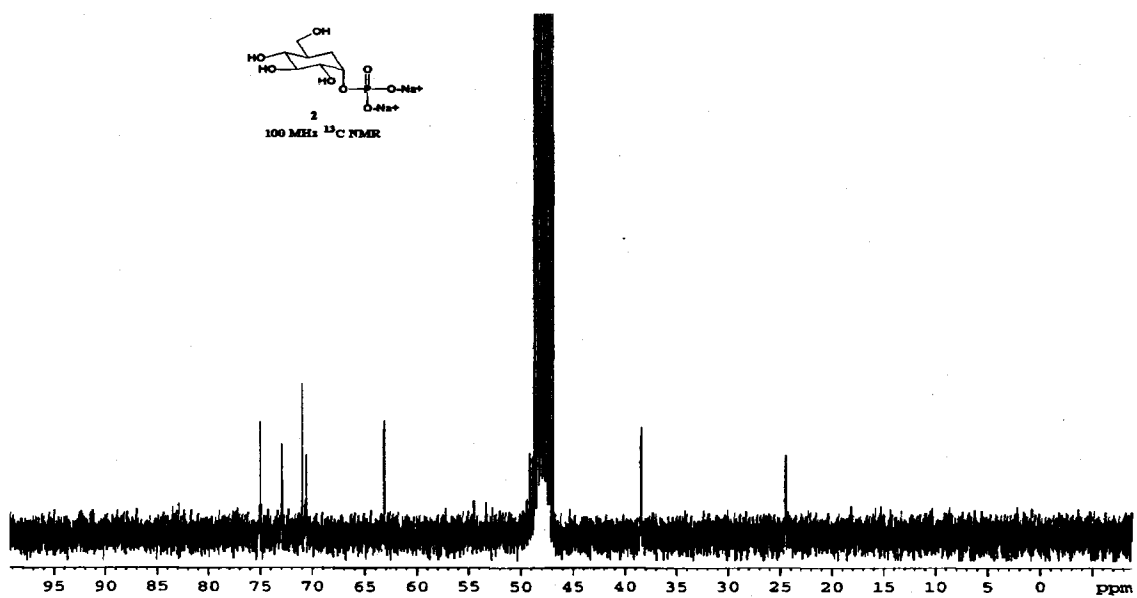
APPENDIX D. CHAPTER 5 ^{1}H AND ^{13}C NMR SPECTRA

400 MHz ^{1}H NMR(CDCl$_3$)

xks10-122/re3

100 MHz ^{13}C NMR(CDCl$_3$)

11

138.942
138.408
138.304
138.074
128.588
128.536
128.496
128.282
128.113
128.041
127.989
127.902
127.822
127.801
127.763
127.731

98.348
82.275
79.963
77.767
77.585
77.267
76.949
75.900
75.164
73.548
73.522
72.226
70.069
55.303

drx400/kks311/re2

225

400 MHz ^{1}H NMR(CDCl$_3$)

12

drx400/kks313/rel

100 MHz ^{13}C NMR(CDCl$_3$)

137.950
137.868
128.617
128.542
128.523
128.503
128.363
128.268
128.179
128.114
128.052
128.022
127.937
127.872
127.851
127.838
127.796
127.762
97.602
91.304
84.717
83.222
81.895
80.078
77.978
77.915
77.592
77.273
76.956
75.859
75.812
75.139
75.102
74.842
74.528
73.488
73.224
70.120

drx400/kks313/re2

400 MHz ¹H NMR(CDCl₃)

13

BnO
BnO
BnO
O
OBn
BnO
O
O=P-OBn
OBn

228

100 MHz ^{13}C NMR(CDCl$_3$)

13

137.921
137.738
135.997
135.953
135.922
135.886
128.834
128.695
128.644
128.593
128.559
128.529
128.477
128.280
128.157
128.135
128.070
128.036
128.020
127.948
127.889
127.859
127.815
127.766
127.713
127.582
127.495
127.013
95.844
95.784
81.245
79.439
79.367
77.614
77.296
76.977
75.777
75.251
73.596
73.537
73.159
72.666
72.554
69.475
69.445
69.421
69.390
69.283
69.230
68.052
67.436
67.377
65.110

150   140   130   120   110   100   90   80   70   60   50   ppm

drx400/kks400/re2

400 MHz ^{1}H NMR(D$_2$O)

5.297
5.289
5.279
5.270

4.665
3.755
3.730
3.633
3.609
3.586
3.357
3.353
3.349
3.345
3.333
3.328
3.325
3.320
3.261
3.237
3.213

1.000

1.070
1.175

1.061
1.053

drx400/kks406/zel

5.4 5.3   ppm

3.8 3.6 3.4   ppm

8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5   ppm

100 MHz ^{13}C NMR(D$_2$O)

93.589
93.537

73.031
72.053
71.984
71.880
69.689

drx400/kks406/rz2

231

400 MHz ^{1}H NMR(CDCl$_3$)
16

drx400/kk=314/rel

140

ppm

160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

drx400/kks314/re2

138.843
138.548
138.373
128.601
128.545
128.500
128.470
128.199
128.159
128.022
127.853
127.815
127.715
127.655

98.895

79.191
77.651
77.332
77.014
76.572
75.250
75.214
74.594
73.659
73.611
70.469

55.419

100 MHz ^{13}C NMR(CDCl$_3$)

16

BnO
BnO
BnO
OMe
O
OH
D

400 MHz ¹H NMR(CDCl₃)

17

kks315

7.288
7.280
7.275
7.269
7.239
5.082
5.074
5.000
4.971
4.899
4.894
4.883
4.870
4.853
4.816
4.799
4.783
4.753
4.737
4.730
4.721
4.707
4.663
4.628
4.599
4.579
4.566
4.526
4.497
4.441
4.411
4.153
4.135
4.117
4.098
4.089
4.073
4.064
3.993
3.986
3.966
3.959
3.941
3.925
3.833
3.827
3.569
3.548
3.531
3.524
3.405
3.366
2.062

23.330

1.000
2.232
1.052
2.070
1.485
1.122
1.044
1.008
1.960
1.196
1.466
2.982

17
400 MHz ^{13}C NMR(CDCl$_3$)

138.948
138.760
138.636
138.108
128.535
128.496
128.443
128.384
128.335
128.217
128.164
127.948
127.873
127.815
127.800
127.764
127.734
127.692
127.606
98.911
79.227
77.541
77.223
76.905
76.556
75.254
74.844
73.678
73.553
73.395
73.209
69.276
55.464

drx400/kks315/re2

400 MHz ^{1}H NMR(CDCl$_3$)

18

Drx400/kks415/rel

100 MHz ^{13}C NMR(CDCl$_3$)

18

drx400/kks415/re2

137.870
128.536
128.507
128.463
128.424
128.382
128.359
128.264
128.106
128.087
127.926
127.871
127.805
127.740
127.714
127.666
127.655
127.626
127.029
97.911
91.908
82.308
80.845
78.855
77.586
77.469
77.267
76.949
76.680
75.171
74.975
74.755
74.648
73.764
73.567
73.508
73.429
73.097
73.066
69.403

400 MHz ^{1}H NMR(CDCl$_3$)

19

238

drx400/kks417/rel

150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

drx400/kks417/re2

- 135.954
- 135.830
- 128.630
- 128.580
- 128.523
- 128.457
- 128.368
- 128.348
- 128.300
- 128.237
- 128.129
- 128.016
- 127.881
- 127.786
- 127.739
- 127.661
- 127.514
- 96.682
- 96.620
- 78.103
- 77.434
- 77.117
- 76.798
- 75.858
- 75.788
- 74.971
- 74.638
- 73.460
- 73.370
- 72.999
- 71.526
- 69.376
- 69.323
- 69.269
- 69.217
- 69.085
- 69.034

19

100 MHz ^{13}C NMR(CDCl$_3$)

400 MHz ^{1}H NMR(D${}_2$O)

5.369
5.360
5.351
5.342
4.689
4.662
4.047
4.035
3.860
3.852
3.805
3.790
3.781
3.764
3.756
3.646
3.642
3.637
3.633
3.621
3.616
3.612
3.607
3.575
3.565

0.959

0.969
0.985
1.155
1.125
0.873

drx400/kks418/re1

100 MHz ^{13}C NMR(D$_2$O)

93.829
93.774

71.046
69.595
69.441
69.015
68.949

drx400/kks418/rel

^{1}H-^{1}H cosy 400 MHz NMR (CDCl$_3$)
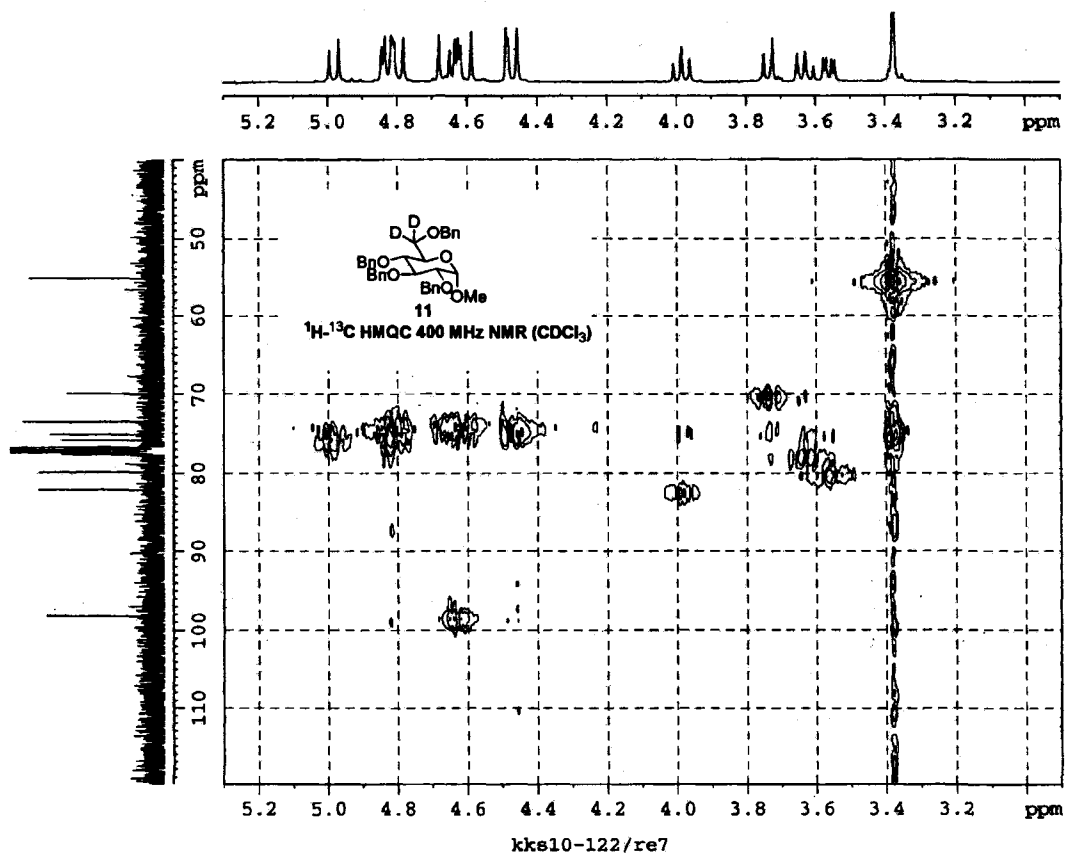
11

kks10-122/re6

11
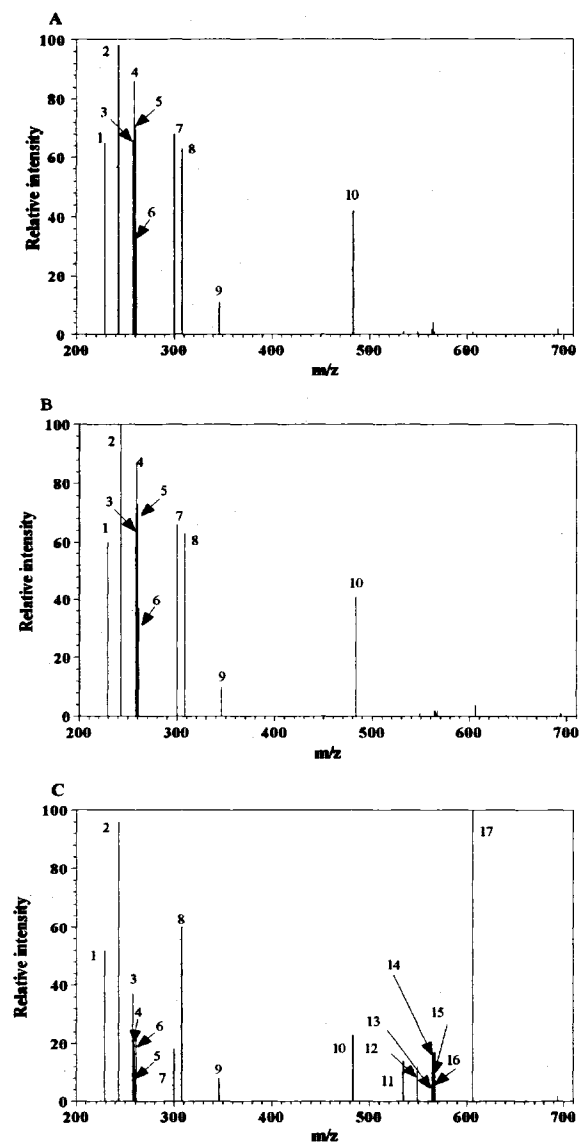
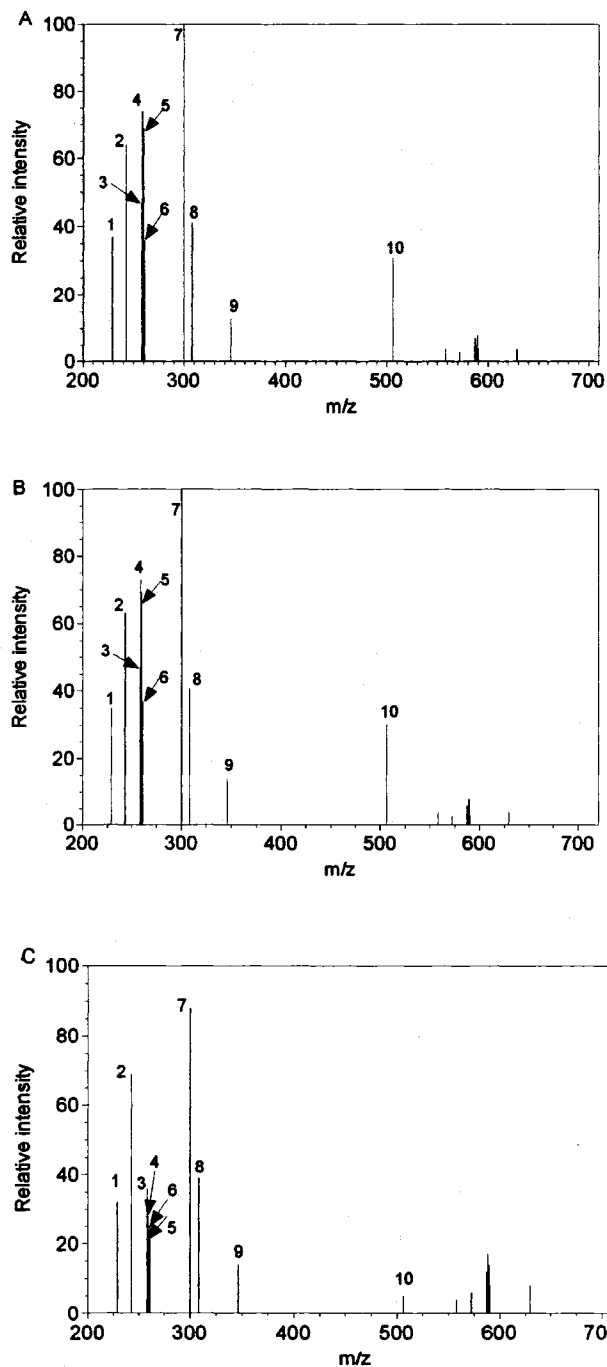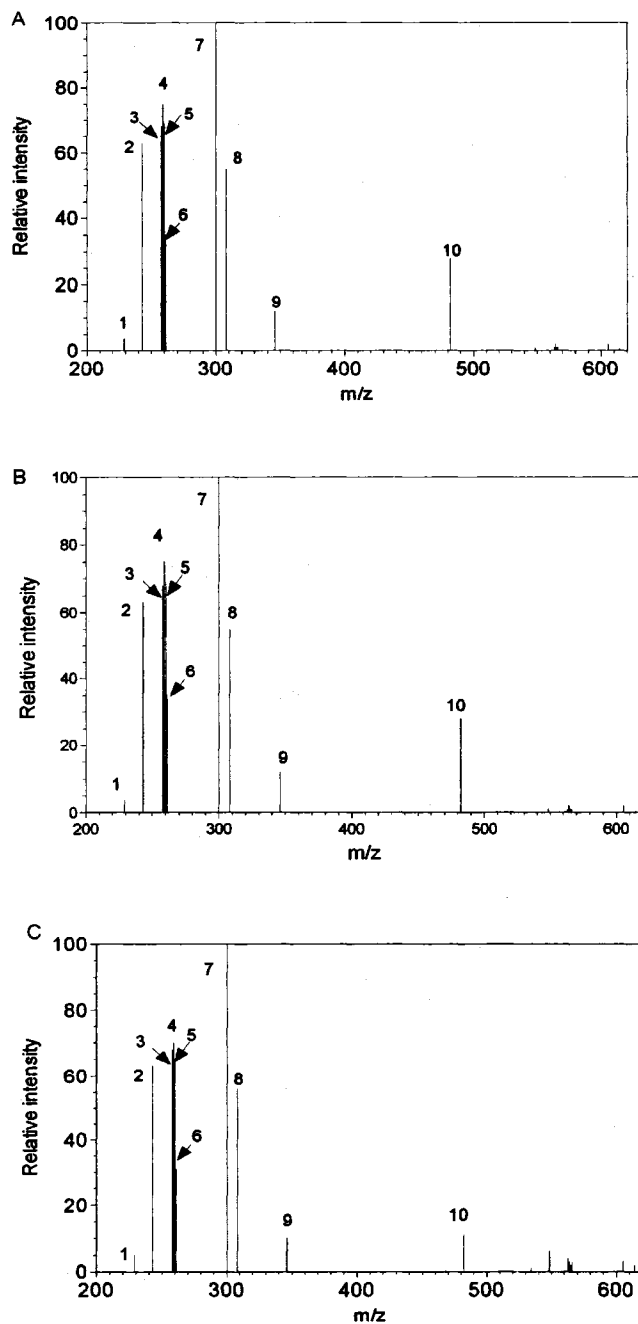^{1}H-^{13}C HMQC 400 MHz NMR (CDCl$_3$)

kks10-122/re7

Figure S1. ESI-MS monitoring of the reactants and products in presence or absence of *E. coli* K12 cell extract with UTP. A. Reaction at 0 min; B, control (without cell extract) after 30 min; C, reaction after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, *N*-acetylglucosamine 1-phosphate; 8, sialic acid; 9, AMP; 10, UTP; 11, UDP-xylose; 12, UDP-fucose; 13, UDP-glucosamine; 14, UDP-mannose; 15, UDP-galactose; 16, UDP-glucose and 17, UDP-*N*-acetylglucosamine. Products of the reactions are indicated in Table S1.
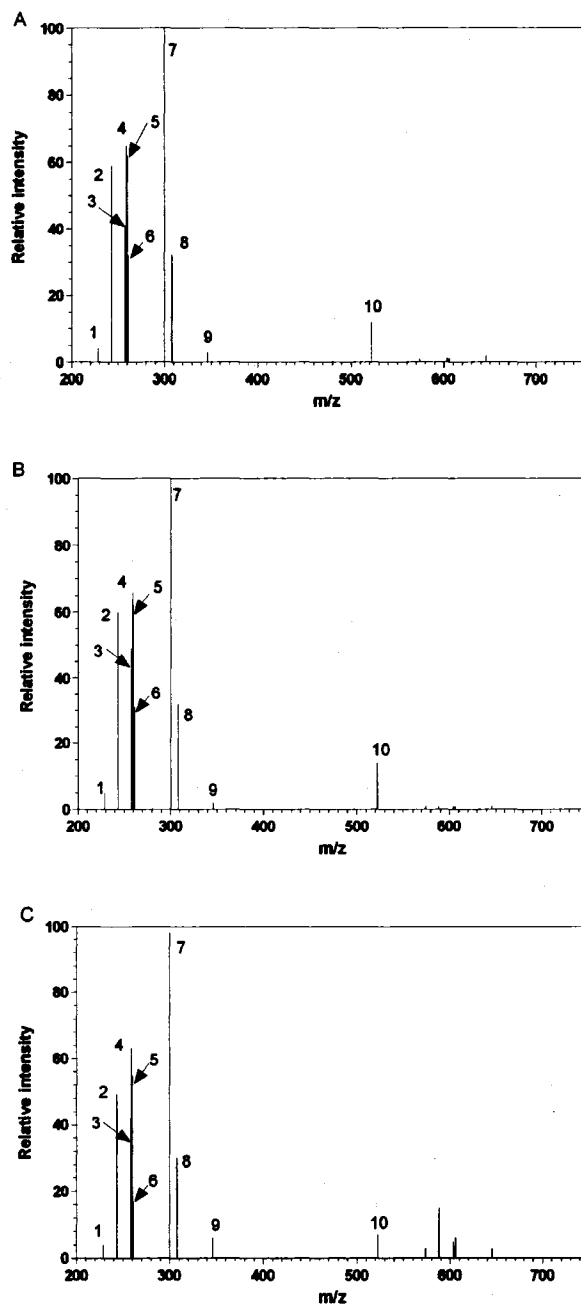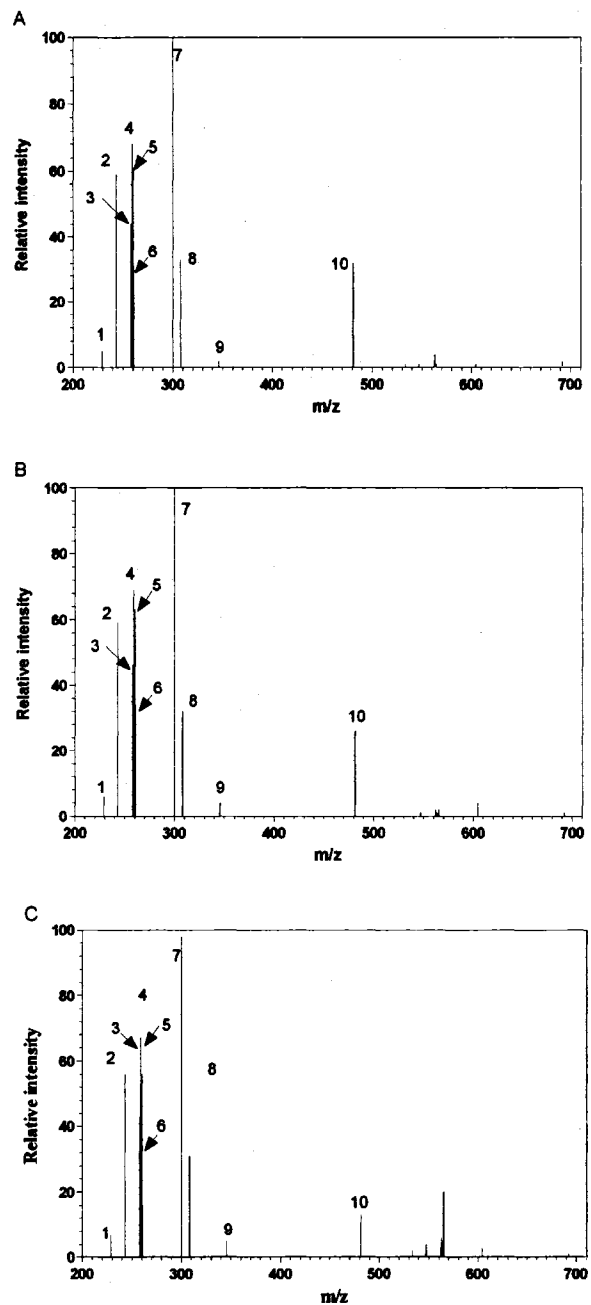
Figure S2. ESI-MS monitoring of the reactants and products in presence or absence of *E. coli* K12 cell extract with ATP. A, Reaction at 0 min; B, control (without cell extract) after 30 min; C, reaction after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, *N*-acetylglucosamine-1-phosphate; 8, sialic acid; 9, AMP; 10, ATP. Products of the reactions are indicated in Table S1.

Figure S3. ESI-MS monitoring of the reactants and products in presence or absence of *E. coli* K12 cell extract with CTP. A, Reaction at 0 min; B, control (without cell extract) after 30 min; C, reaction after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, *N*-acetylglucosamine-1-phosphate; 8, sialic acid; 9, AMP; 10, CTP. Products of the reactions are indicated in Table S1.
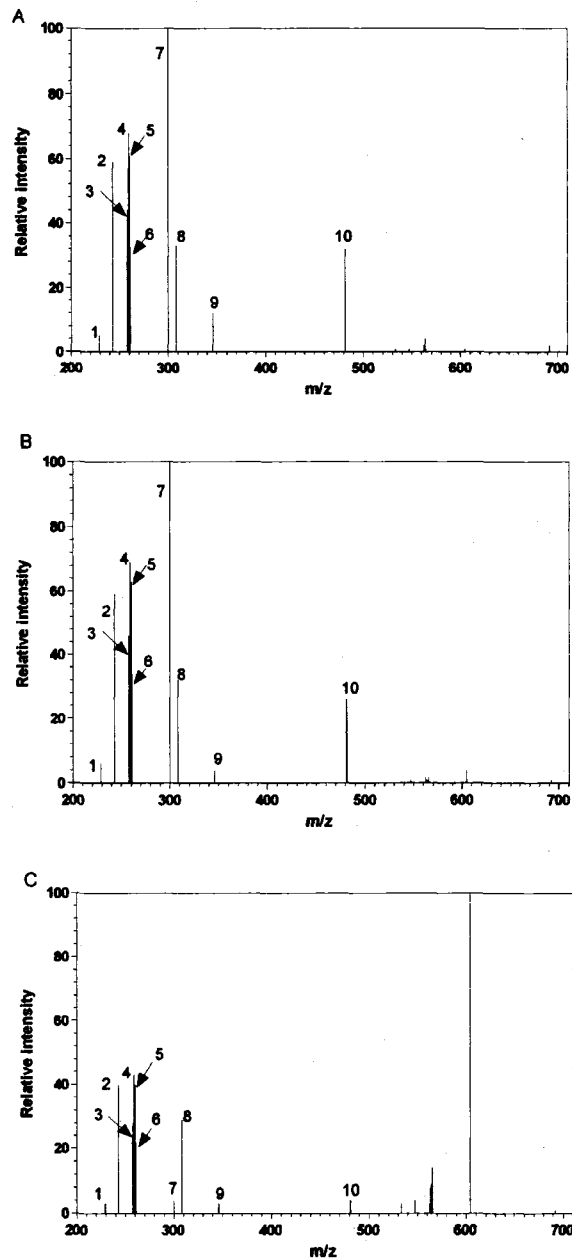
Figure S4. ESI-MS monitoring of the reactants and products in presence or absence of *E. coli* K12 cell extract with GTP. A, Reaction at 0 min; B, control (without cell extract) after 30 min; C, reaction after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, *N*-acetylglucosamine-1-phosphate; 8, sialic acid; 9, AMP; 10, GTP. Products of the reactions are indicated in Table S1.
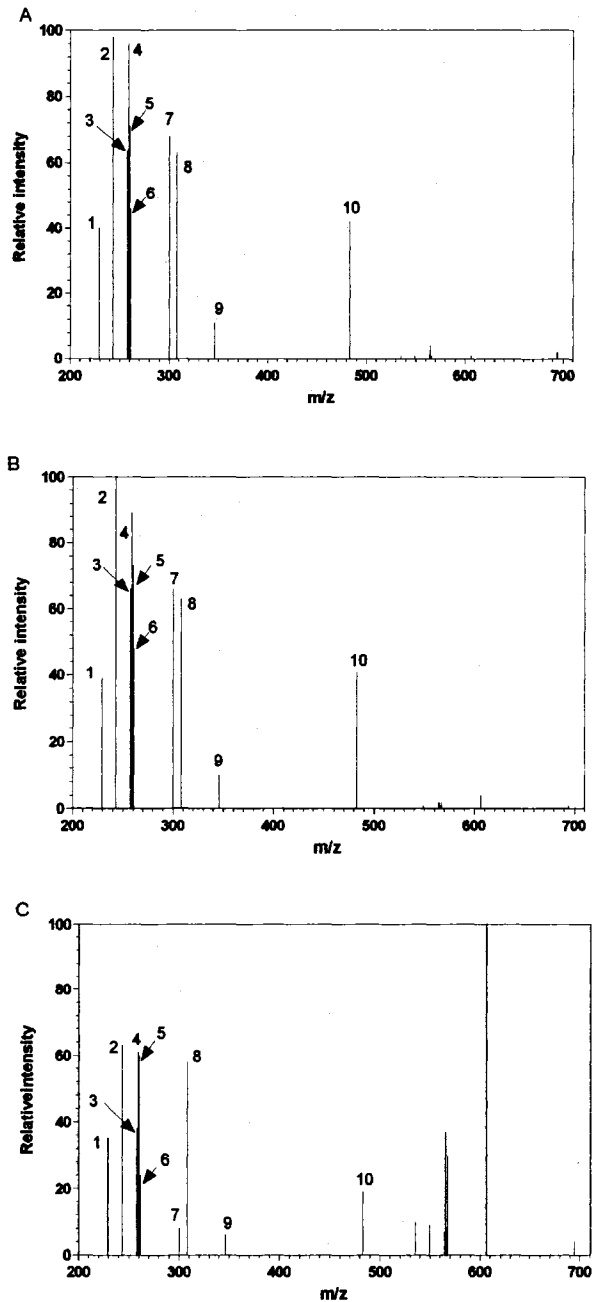
Figure S5. ESI-MS monitoring of the reactants and products in presence or absence of *E. coli* K12 cell extract with dTTP. A, Reaction at 0 min; B, control (without cell extract) after 30 min; C, reaction after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, *N*-acetylglucosamine-1-phosphate; 8, sialic acid; 9, AMP; 10, dTTP. Products of the reactions are indicated in Table S1.

Figure S6. ESI-MS monitoring of the reactants and products in presence or absence of *P. furiosus* enzyme and dTTP. A, Reaction at 0 min; B, control (without enzyme) after 30 min; C, reaction (with enzyme) after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, *N*-acetylglucosamine-1-phosphate; 8, sialic acid; 9, AMP; 10, dTTP. Products of the reactions are indicated in Table S1.
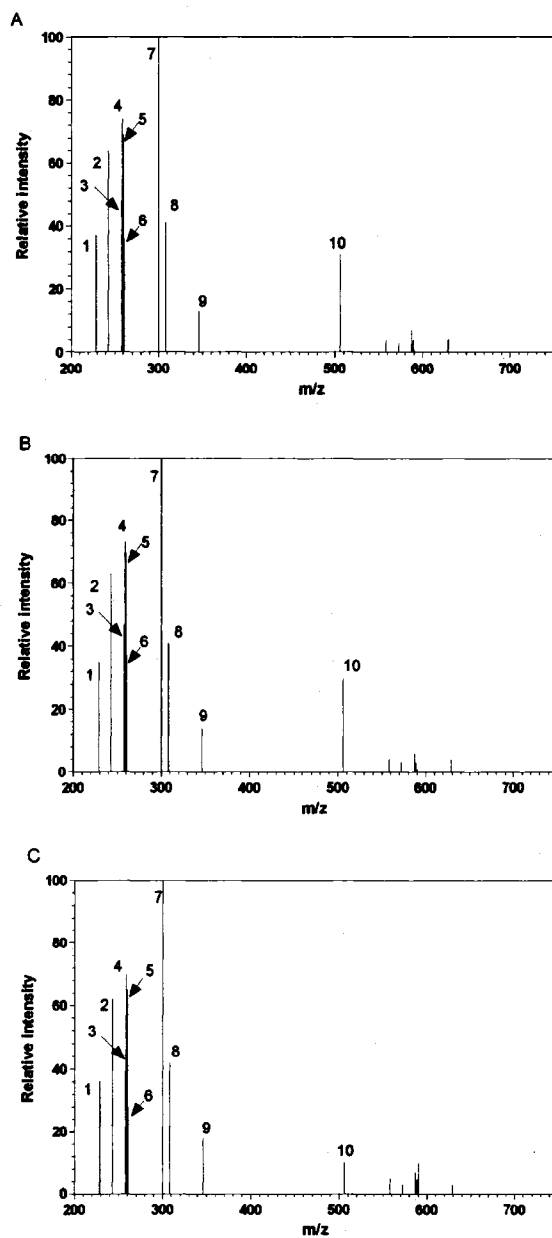
Figure S7. ESI-MS monitoring of the reactants and products in presence or absence of *P. furiosus* enzyme with UTP. A, Reaction at 0 min; B, control (without enzyme) after 30 min; C, reaction (with enzyme) after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, *N*-acetylglucosamine-1-phosphate; 8, sialic acid; 9, AMP; 10, UTP. Products of the reactions are indicated in Table S1.
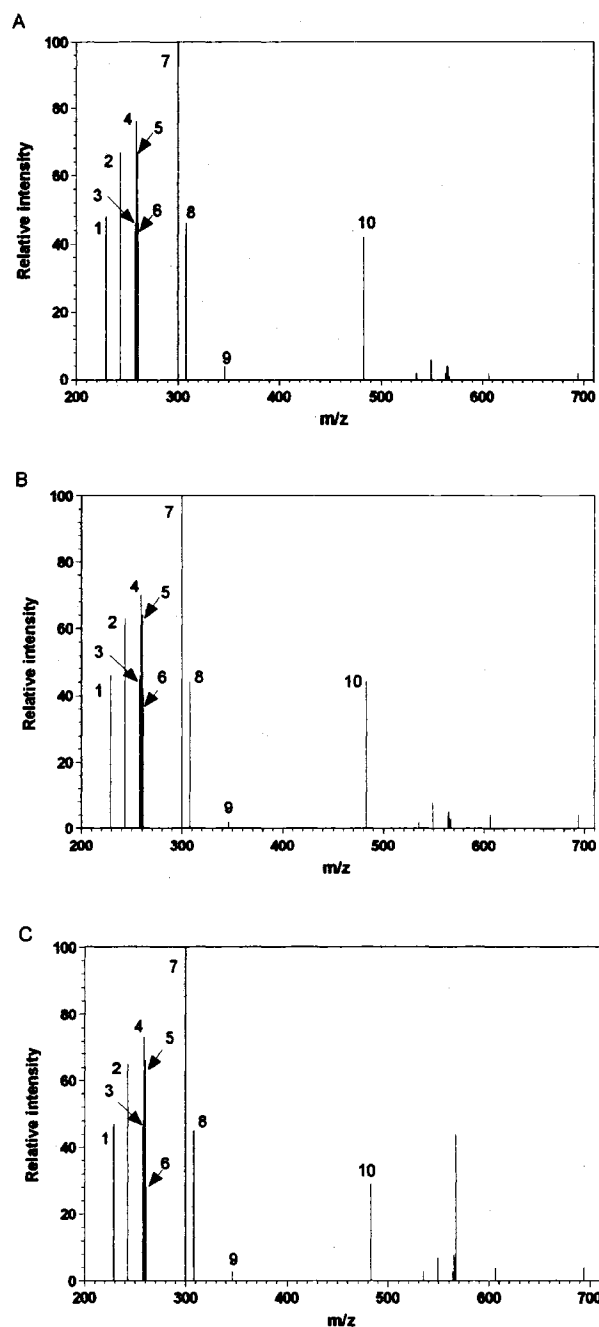
Figure S8. ESI-MS monitoring of the reactants and products in presence or absence of yeast enzyme with ATP. A, Reaction at 0 min; B, control (without enzyme) after 30 min; C, reaction (with enzyme) after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, N-acetylglucosamine-1-phosphate; 8, sialic acid; 9, AMP; 10, ATP. Products of the reactions are indicated in Table S1.

Figure S9. ESI-MS monitoring of the reactants and products in presence or absence of yeast enzyme with UTP. A, Reaction at 0 min; B, control (without enzyme) after 30 min; C, reaction (with enzyme) after 30 min. 1, xylose-1-phosphate; 2, fucose-1-phosphate; 3, glucosamine-1-phosphate; 4, mannose-1-phosphate; 5, galactose-1-phosphate; 6, glucose-1-phosphate; 7, *N*-acetylglucosamine-1-phosphate; 8, sialic acid; 9, AMP; 10, UTP. Products of the reactions are indicated in Table S1.

APPENDIX E. CHAPTER 6 ^{1}H AND ^{13}C NMR SPECTRA

1
400 MHz ¹H NMR

dxr400/kksj028/rel

1
100 MHz ¹³C NMR

400 MHz ^{1}H NMR

3

256

vxr400/kksj026

3
100 MHz ^{13}C NMR

170.410
170.316
170.206
169.786

141.420

112.685

77.455
77.137
77.038
76.818
74.114
72.240
69.514
67.742
61.654

39.807

22.785
20.803
20.706
20.648
20.611

dxr400/kksj025/re5

220   200   180   160   140   120   100   80   60   40   20   ppm

170.5   170.0   ppm

22 ppm   21.0   ppm

400 MHz ^{1}H NMR

5

dxr400/kks]100/rel

100 MHz ^{13}C NMR

^{1}H-^{1}H COSY 500 MHz NMR