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ONE POT ENZYMATIC SYNTHESIS OF UDP-GLCA AND UDP-GALA AND CHEMOENZYMATIC SYNTHESIS OF A LIBRARY OF HUMAN MILK OLIGOSACCHARIDES AND O-ANTIGEN FROM *P. AERUGINOSA* SEROTYPE O11

by

Yuxi Guo

Under the Direction of Peng George Wang, PhD

ABSTRACT

Carbohydrates are ubiquitous in nature and provide the function of protection and recognition in many biological systems. Therefore, the efficient synthesis of carbohydrate-based compounds is of considerable interest for both research and commercial purposes. However, unlike DNA and protein, successful synthesis of glycan is still challenging, due to the large number of sugar nucleotides and the several different linkage and conformation. Therefore, to tackle the challenge of glycosynthesis, chemists are increasingly turning their attention towards enzymes, which are exquisitely adapted to the intricacy of these biomolecules. In this dissertation, we will focus on practical biosynthesis of glycome such as UDP-GlcA and UDP-GalA, HMOcomics, O-Polysaccharides.

Chapter 1 provides a general introduction of function of glycosylation of protein and glycan synthetic approaches.

Chapter 2 describes that innovative approach of biosynthesis of UDP-GlcA and UDP-GalA by utilizing salvage pathway. UDP-HexA is active form of monosaccharide for further glycosylation which occurs in the biosynthesis of numerous cell components. Limited availability of these sugar nucleotides has been hindering the development of facile synthesis of bioactive glycans. In this study, we have developed an efficient and facile one-pot multi-enzyme method to harvest hundreds milligram of UDP-GlcA and UDP-GalA.

In Chapter 3, Human milk sugar library synthesis is the footstone for future manufacturing specific human milk sugar macro array which can be used for screening blood and virus. A tremendous human milk sugar library is the foundation for application of macro-array in multiple research area. we accomplished a work of chemoenzymatic synthesize 31 HMOs based on 3 chemically synthesized core structures. A more comprehensive 100 HMOs library by 7 robust glycosyltransferases have been accomplished for printing array to screen virus and bacteria.

In Chapter 4, chemoenzymatic synthesis of O-antigen from *P. aeruginosa* serotype O11 was achieved by two glycotransferases WbjE and WbjA. The bacterial cell surface is decorated with polysaccharides whose structures show remarkable diversity. These polysaccharides play critical role of interaction between Host and bacterium. Many are important virulence factors, adhesion mediators, or immunomodulators. Successful reconstitution of pure homogenous repeat unit of PS can facilitate development of multivalence bacterial vaccine.

INDEX WORDS: Structurally-Defined, HMOs, CSEE, Glycans, Chemoenzymatic Synthesis, O-Antigen

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YUXI GUO

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in the College of Arts and Sciences

Georgia State University

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May 2019

DEDICATION

To my parents, Jianjun Guo, Renxiu Tian and my wife Qun Wang for their unconditional support and love.

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It is great honor that I can study, grow and perform my graduate research under the direction of Prof. Peng George Wang. Thus, I would like to deeply thank my advisor Dr. Peng George Wang for his insightful and creative guidance and continuous financial support these years. His enthusiasm and encouragement dramatically influence my attitude and achievement during all these five years of frustration and joy.

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LIST OF ABBREVIATIONS

Amp	ampicillin
Ka	Kanamycin
ATCC	American Type Culture Collection
bp	base pair
cm	centimeter
DTT	dithiothreitol
Fuc	fucose
Gal	galactose
Glc	glucose
GlcN	glucosamine
GlcNAc	*N*-acetylglucosamine
GlcA	Glucuronic acid
GalA	Galacturonic acid
Sia	Sialic acid
GT	glycosyltransferase
h	hour
HRMS	high-resolution mass spectrometry
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilo Dalton
L	liter
LB	Luria-Bertani (medium)
LPS	lipopolysaccharide
LNFP III	lacto-*N*-fucopentaose III
LNnT	lacto-*N*-neotetraose
M	molar
m/z	mass to charge ratio
MALDI	matrix-assisted laser desorption-ionization
g	gram
mg	milligram

min	minute
mL	milliliter
mM	millimolar
s	second
μg	microgram
μL	microliter
MS	mass spectrometry
NMR	nuclear magnetic resonance
^{o}C	degree centigrade
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween
PCR	polymerase chain reaction
PPA	yeast inorganic pyrophosphatase
PPi	pyrophosphate
rpm	round per minute
SDS	sodium dodecyl sulfate
TLC	thin-layer chromatography
UDP	uridine diphosphate
UTP	uridine 5'-triphosphate
ATP	adenosine 5'-triphosphate
GTP	guanosine 5'-triphosphate
UDP-Glc	uridine 5'-diphosphate-glucose
UDP-GlcNAc	uridine 5'-diphosphate-*N*-acetylglucosamine
UDP-Gal	uridine 5'-diphosphate-galactose
UDP-GlcA	uridine 5'-diphosphate-glucoronic acid
UDP-GalA	uridine 5'-diphosphate-galacturonic acid
GTP-Fuc	guanosine 5'-diphosphate-fucose

1 INTRODUCTION

1.1 Function of glycan and glycoconjugate

Glycan and glycoconjugates are the fundamental mediator in nature which play pivotal roles in signaling, cell-cell interaction, cell differentiation, cell proliferation, immunological responses. [1] The chemical structure diversity and complexity of glycans underlie their functional variety. The function exerted are also very diverse containing: (1) organizational and stability role, (2) protective and barrier functions, (3) provision of specific receptors for microorgamisms

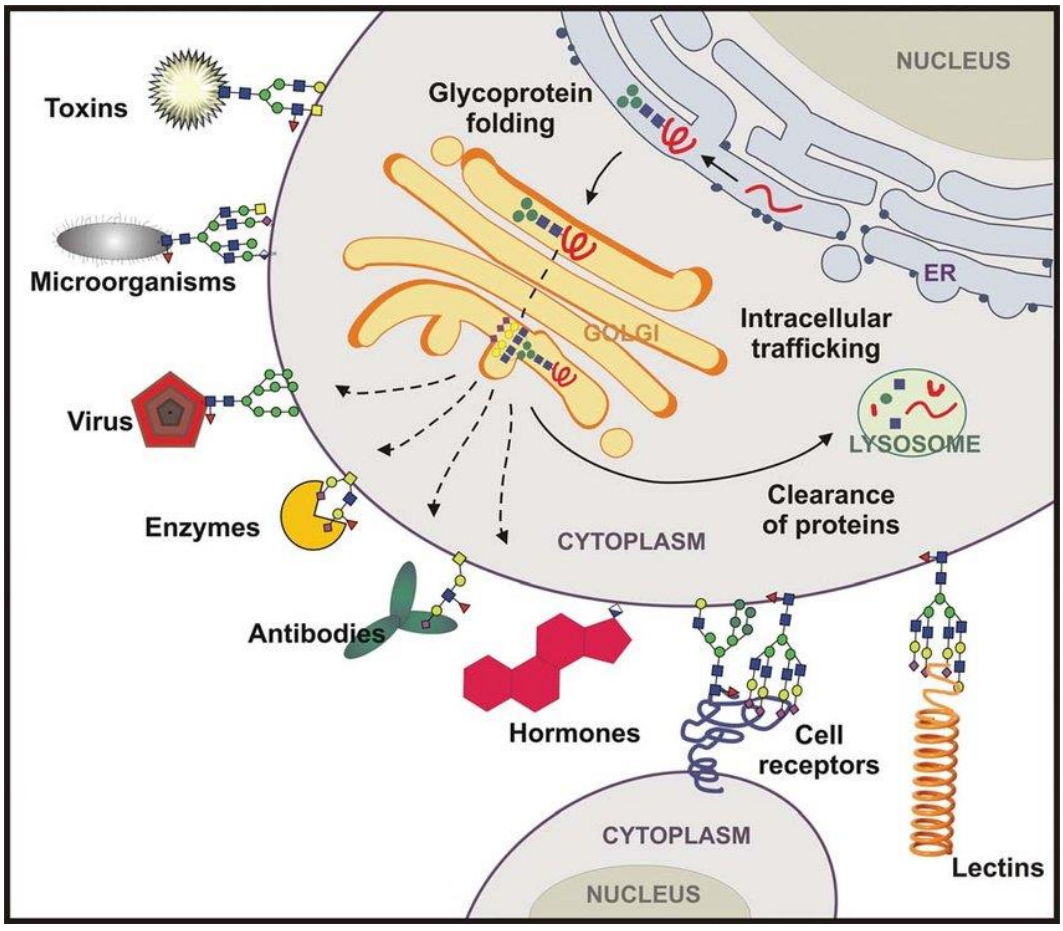

Figure 1.1 glycans mediate multiple mechanisms of cellular interaction.

Toxin, virus, antibodies, enzymes to attack, shield and lure, (4) modulating protein functions, (5) intra and inter cellular trafficking, (6) mediator of cell-cell and cell-matrix interactions. [2](**Figure 1.1)**

Glycosylation is a most important post-translational modification of protein that increase protein diversity and function. In general glycosylation is defined as the addition of one or more sugar residues to a protein or lipid to convey additional information, structure or function. There are five types of glycosylation in mammalian cell: (1) N-glycosylation in which glycan is attached to nitrogen of asparagine (Asn), (2) O-Glycosylation in which glycan is attached to hydroxyl oxygen of serine (Ser) or threonine (Thr), (3) Phosphoserine glycosylation in which sugar is linked to the phosphate of a phosphoserine, (4) C-linked glycosylation in which glycan is attached to a carbon of a tryptophan side chain, (5) glypiation (glycosylphosphatidylinositol-anchored proteins). [3] (**Figure 1.2)**

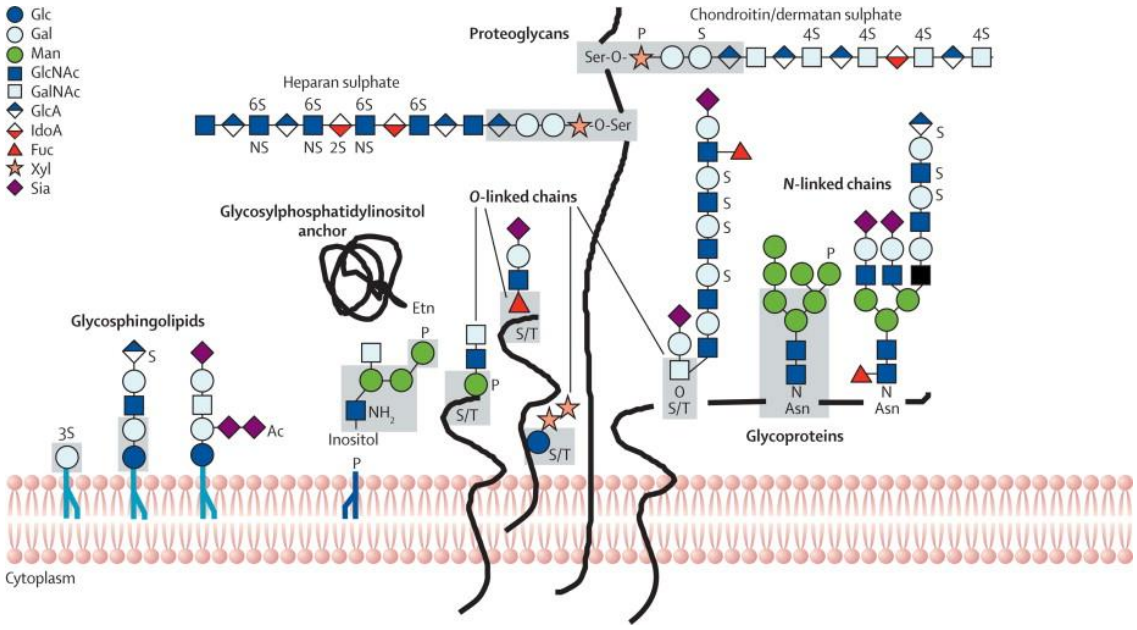

Figure 1.2 Different types of glycosylated proteins

Unlike DNA, composed of adenine(A), cytosine (C), guanine (G), thymine (T), and peptide or protein, composed of 20 amino acid, glycan can be either linear or branched which is controlled by certain number of glycotransferases which belong to intrinsic biosynthesis pathway. [4] these specific glycotransferases can transfer corresponding monosaccharide to another one by forming glycosidic bond. Every monosaccharide has several hydroxyl groups which can link to hydroxyl group of another monosaccharide's anomeric carbon by either α or β configuration. There are more than ten types of monosaccharide existing in nature. Based on above several characters, numerous sequence and structure of glycans were found in bacteria, mammalian cell and organism. The diversity of glycan enables it to carry more complicate signal information and convey it through specific interaction.

1.2 Preparation of glycome

Glycome is a whole collection of different form of carbohydrate existing in cell, tissue, organism. [5] Successful preparation of such big carbohydrate collection will be tremendous benefit to glycoscience. Until now the lack of structure defined glycan as standard has impeded the interpretation of oligosaccharides or glycan function and their mechanism behind scene. [6-7] To address this fundamental problem, isolation and synthesis are two major direction. Each one has their own advantages and disadvantages. Currently human milk oligosaccharides isolated from natural human milk, glycans from glycolipid and glycoprotein, and polysaccharides from bacteria are some examples by isolation approach. [8-11] although glycan isolated from natural product is reliable large scale of source for industry application, most of them are mixtures which need further separation. [12] Especially when some isolated glycans are same molecular weight but different structure, separation is very challenging. In contrast synthesized glycan by either chemical or enzymatic approach are structurally defined glycan which are widely utilized in

biological and biomedical studies.[13-14] Chemical synthesis are mutil-steps process, time consuming, low yield and strict reaction conditions. [15] those aspects determine that chemical synthesis mostly fits small molecular synthesis. Oligosaccharides over 1000 KDa prefer enzymatic synthesis. [16] The key factor of enzymatic synthesis is robust glycostransferases (GT) from mammalian or bacteria and corresponding sugar nucleotides. [17] (**Figure 1.3)**

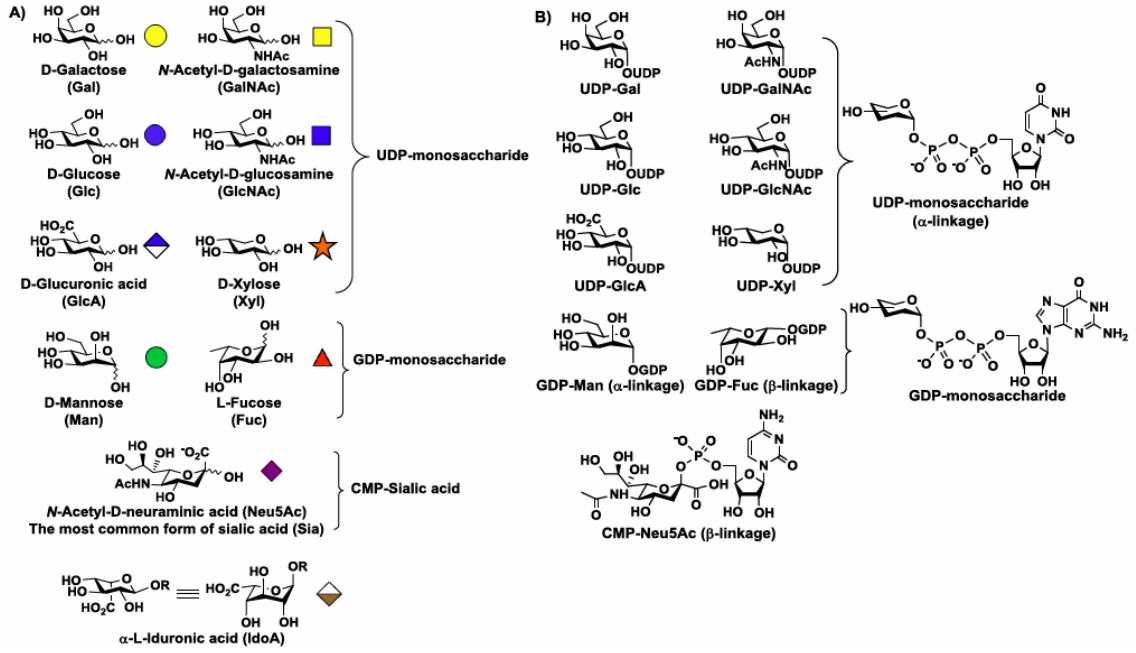

Figure 1.3 Common monosaccharides and corresponding sugar nucleotides

Glycotransferase catalyzed enzymatic and chemoenzymatic synthesis are highly specific and regio and stereo control. Recently Dr. Xi Chen and her coworkers employ a new strategy called One-pot multienzyme (OPME) system to boost yield of carbohydrate synthesis. In OPME, glycotransferases for forming specific glycosylic bond and enzymes involved in biosynthetic pathway of corresponding sugar nucleotides are combined into one reaction system to drive reaction forward and avoid tedious separation. [17]

1.3 Outline of the work in this dissertation

The work in this dissertation mainly focuses on biosynthesizing sugar nucleotides, oligosaccharides from human milk and O-antigen from bacteria. In chapter 2, we biochemically characterized two UDP-sugar pyrophosphorylases from *Arabidopsis thaliana* (AtUSP) and *Bifidobacterium infantis* ATCC15697 (BiUSP). Both enzymes showed significant pyrophosphorylation activities towards GlcA-1-phosphate, and AtUSP also exhibited comparable activity towards GalA-1-phosphate. By combing with monosaccharide-1-phosphate kinases, we have developed an efficient and facile one-pot three-enzyme approach to quickly obtain hundreds mg of UDP-GlcA and UDP-GalA. In chapter 3, an efficient chemoenzymatic strategy, namely Core Synthesis/Enzymatic Extension (CSEE), for rapid production of diverse HMOs was reported. 3 core structures were chemically synthesized. Each of these core structures was then extended to up to 11 HMOs by 4 robust glycosyltransferases. A library of 31 HMOs were chemoenzymatically synthesized and characterized by MS and NMR. CSEE indeed provides a practical approach to harvest structurally defined HMOs for various applications. In chapter 4, we have successfully reconstituted pure homogenous O-repeat unit of *P. aeruginosa* O11, which will facilitate development of multi-valence bacterial vaccine towards opportunistic *P. Aeruginosa* infection in hospital.

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2 ONE POT ENZYMATIC SYNTHESIS OF UDP-GLCA AND UDP-GALA

2.1 Introduction

Hexuronic acids (HexAs), a class of sugar acids with both carbonyl and carboxylic acid functional groups, are the oxidation products of the corresponding monosaccharides. Glucuronic acid (GlcA) is one of the most commonly found HexAs in the plant and animal kingdoms.[18] It is also prevalent in carbohydrate chains of proteoglycans.[19] Galacturonic acid (GalA) is a major sugar residue of plant pectic polysaccharides and a minor component of certain plant arabinogalactan proteins.[20] GalA is also found in various cell surface polysaccharides of different gram-negative bacteria,[21-22] as well as capsular polysaccharide of the gram-positive human pathogens, such as type I *Streptococcus pneumonae*.[23]

Because of the biological significance and structure complexity, glycans containing HexAs have attracted considerable attention in past decades.[24-26] One of the major bottlenecks in basic research and application of these components is the limited and cost-effective availability of uridine 5'-diphosphate glucuronic acid (UDP-GlcA) and uridine 5'-diphosphate galacturonic acid (UDP-GalA), the active forms of corresponding monosaccharides. UDP-GlcA and UDP-GalA are essential glycosyl donors for glycosyltransferases in the biosynthesis of various cell components including glycosaminoglycans, some N- and O-linked glycans, glycosphingolipids, lectins, etc.[27-28] Recently, several chemical or coupled enzymatic approaches have been developed for the synthesis of UDP-GlcA and UDP-GalA.[29-30] However, low yield, tedious steps and strict condition requirements have restricted the application of these approaches in large scale sugar nucleotide preparation.[31] Therefore, a simple, cost-effective and efficient approach for the preparation of UDP-GlcA and UDP-GalA is highly desirable for subsequent glycosyltransferase-catalyzed reactions.

Enzymatic synthesis, which mimics the biosynthesis pathway, is considered more attractive with advantages of high conversion rate and mild reaction conditions. As shown in **Figure 1**, the *de novo* biosynthesis of UDP-GlcA initiates from glucose-6-phosphate (Glc-6-P), an intermediate of the glycolytic pathway. Three enzymatic catalyzed steps were involved, including the equilibrium conversion of Glc-6-P and Glc-1-P, the pyrophosphorylation step to yield UDP-Glc, and the oxidoreduction step catalyzed by UDP-Glc 6'-oxidoreductase. The complication of the pathway, the similarities between the final product and intermediates, as well as the consuming of expensive NAD^+ or $NADP^+$ have highly limited the application of such a pathway in large scale UDP-GlcA preparation. The *de novo* biosynthetic pathway of UDP-GalA is even more complicated (**Fig. 1**).

In contrast, salvage biosynthetic pathways of sugar nucleotides, usually involving only two reactions (sugar-1-phosphate kinase catalyzed formation of sugar-1-P from monosaccharide and ATP, and NDP-sugar pyrophosphorylase catalyzed formation of NDP-sugar from sugar -1-P and NTP) were found much more practical in large scale synthesis of sugar nucleotides. Using recombinant enzymes from salvage pathways, we have successfully developed efficient approaches for 100 mg scale synthesis of UDP-Gal, UDP-GlcNAc, UDP-GalNAc, GDP-Fuc, GDP-Man and derivatives.[32-39] Recently, a salvage biosynthetic pathway of UDP-GlcA had been discovered in *Arabidopsis thaliana* (**Fig. 1**).[40] In such a pathway, GlcA is first catalyzed by a glucuronokinase (AtGlcAK) to form GlcA-1-P in the presence of ATP and Mg^{2+}. Subsequently, a UDP-sugar pyrophosphorylase (AtUSP) catalyzes the conversion of GlcA-1-P into UDP-GlcA in the presence of UTP and Mg^{2+}.[41-42] A galacturonokinase (AtGalKA) was also found in *A. thaliana* [40] implies that a salvage biosynthetic way of UDP-GalA may also exist.

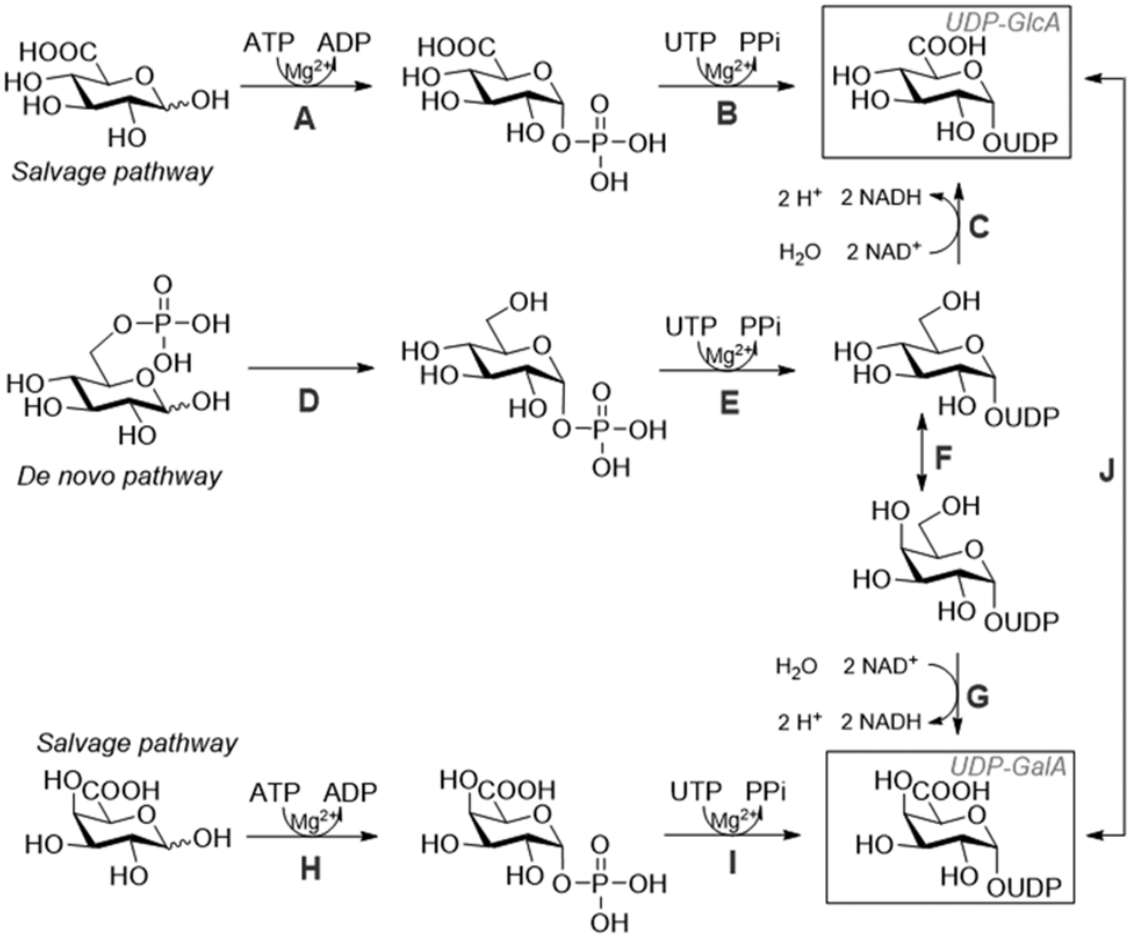


Figure 2.1 De novo and salvage biosynthetic pathway of UDP-GlcA and UDP-GalA
A), Glucuronokinase (GlcAK); B) UDP-sugar pyrophosphorylase (USP); C) UDP-glucose 6-dehydrogenase; D) Phosphoglucomutase; E) UTP: glucose-1-phosphate uridylyltransferase (GalU); F) UDP-Glc 40-epimerase (GalE); G) UDP-galactose 6-dehydrogenase; H) galacturonokinase (GalAK); I) UDP- galacturonate pyrophosphorylase.

In this study, we cloned and characterized a UDP-sugar pyrophosphorylase from *Bifidobacterium infantis* ATCC15697 (BiUSP) which exhibited significant activity towards GlcA-1-P. In addition, we codon optimized the gene encoding AtUSP, and performed heterogeneous overexpression. AtUSP showed significant activities towards both GlcA-1-P and GalA-1-P. Furthermore, substrate specificity study exhibited that both USPs were promiscuous towards a panel of sugar-1-phosphates. Finally, by combing sugar-1-P kinases with USPs as well

as pyrophosphatase, we developed an efficient one-pot three-enzyme approach to prepare hundreds of milligrams of UDP-GlcA and UDP-GalA.

2.2 Results and discussion

Genes encoding AtGlcAK and AtUSP from *A. thaliana* were codon optimized (see DNA sequences in **supporting information**) and synthesized to achieve better heterologous expression in *E. coli*. The synthesized genes were cloned into pQE80L, a tightly controlled *E. coli* protein expression vector with a T5 promoter. Given that inductions were performed at a low temperature (16 °C) and with a low concentration of IPTG (0.2 mM), AtGlcAK and AtUSP were expressed almost entirely in soluble form. As shown in **Figure 2**, after one-step Ni-NTA affinity chromatography, both enzymes with N-terminal His_6-tag were purified to 90% with high yields (90 mg for AtGlcAK, 42 mg AtUSP per L of LB cultures). The apparent molecular weights of the enzymes on SDS-PAGE (**Fig. 2**, Lane 1 & 2) are consistent with theoretical values.

A bacterial UDP-sugar pyrophosphorylase gene, *Blon_1169*, was cloned from *B. infantis* ATCC 15697. The enzyme (BiUSP) encoded by this gene is highly identical (98%) to a previously reported UDP-sugar pyrophosphorylase, BLUSP.[43] BiUSP was expressed and purified with a C-terminal His_6-tag followed the abovementioned methods. The yield of pure BiUSP from 1 L of cultures is 43 mg. An approximate molecular weight of 60 KDa was observed on SDS-PAGE (**Fig. 2**, Lane 4), consistent with theoretical value (58 KDa).

2.2.1 Gene cloning, expression, and purification of AtGlcAK, AtUSP and BiUSP

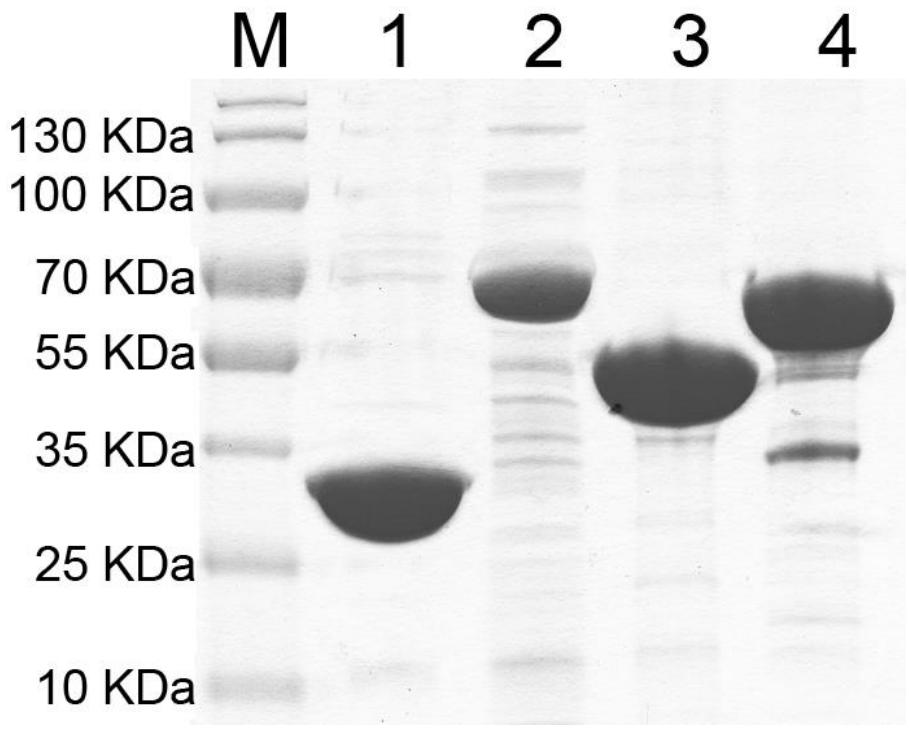


Figure 2.2SDS-PAGE analysis of purified enzymes

Capillary Electrophoresis (CE) was used to analyze the formation of UDP-sugars. Retention times of 21.7, 18.2, 16.6 min were observed on CE profiles for UTP, UDP-GlcA and UDP-GalA standards (**Fig 3A**, **B** & **C**), respectively. The results show that BiUSP could catalyze the formation of UDP-GlcA from GlcA-1-P and UTP efficiently (**Fig**. **3D**), whereas the formation of UDP-GalA was not clearly observed (CE profile not shown), indicating that the C-4 hydroxyl group may be necessary for BiUSP recognition. This is opposite with results yielded form activity assays towards Glc-1-P and Gal-1-P, where both substrates can be well accepted (**Table 2**). One possible explanation could be the bulky carboxylic acid group on C-6 position altered the configuration of C-4 hydroxyl group, makes GalA not tolerable by BiUSP.

2.2.2 Pyrophosphorylase activities of AtUSP and BiUSP

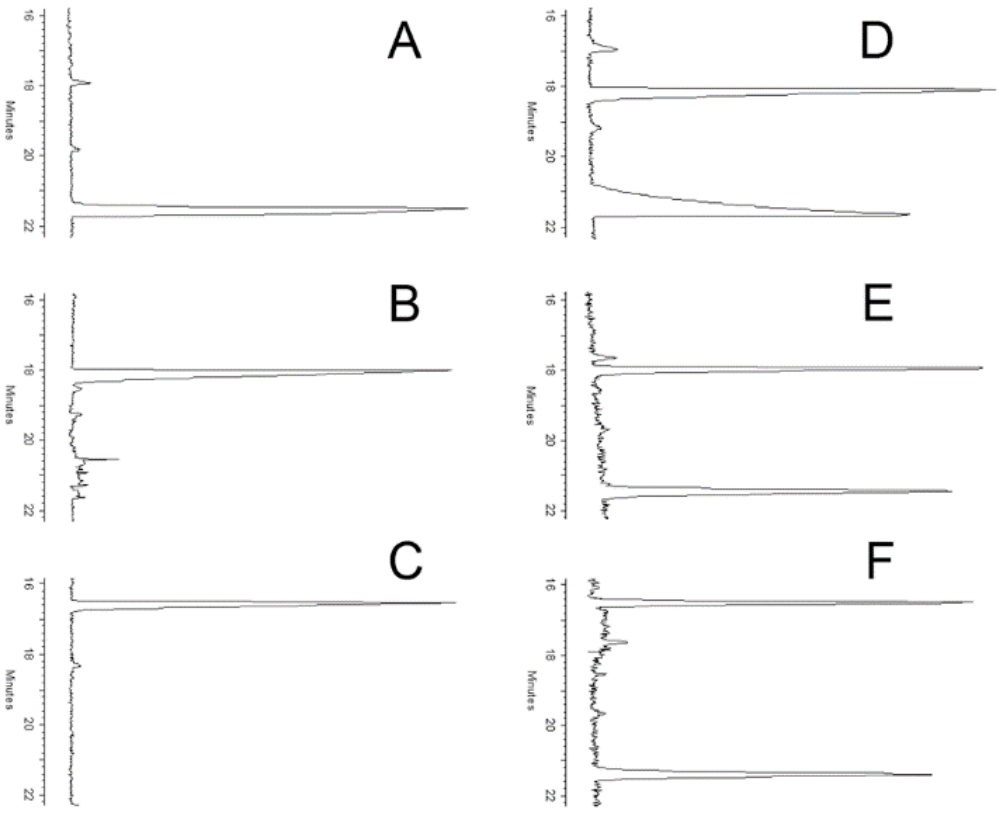


Figure 2.3 CE Profiles of compound standards, and BiUSP/AtUSP catalyzed reactions. (A) 0.5 mM UTP; (B) 0.5 mM UDP-GlcA; (C) 0.5 mM of UDP-GalA; (D) BiUSP catalyzed generation of UDP-GlcA; (E) AtUSP catalyzed generation of UDP-GlcA; (F) AtUSP catalyzed generation of UDP-GalA. For each reaction, 0.8 mM sugar-1-P, 1 mM UTP, and 1 mg enzyme was used in a total 50 mL system, reactions were proceeded at 37 _C for 20 min. UTP has a retention time of 21.7±0.1 min, UDP-GlcA has a retention time of 18.2±0.1 min, and UDP-GalA has a retention time of 16.6±0.1 min.

On the other hand, AtUSP could efficiently (68% and 65% towards GlcA-1-P and GalA-1-P) catalyze the formation of both UDP-GlcA and UDP-GalA (**Fig**. **3E** & **F**). The activity of BiUSP and AtUSP towards GlcA-1-P are comparable (**Fig**. **3D** & **E**). It is worth to note that AtUSP can be stored for up to 6 months at -20 °C without significant activity loss, whereas BiUSP showed dramatically activity decrease after 6 months storage under the same condition.

Freshly prepared AtUSP or BiUSP was applied in time course experiment and kinetics studies. As shown in **Figure 4**, time course results imply that for AtUSP, the formation rate of UDP-GalA is slightly lower than that of UDP-GlcA. In kinetics studies, to ensure initial reaction rates, enzyme dilutions were selected so that less than 15% of substrate conversion was achieved within 5 min for each reaction. Kinetic parameters were calculated according to Lineweaver–Burk equation (**Table 1**). The K_m value of AtUSP towards GlcA-1-P and GalA-1-P are 2.36×10^{-3} μM and 5.50×10^{-3} μM, indicates that AtUSP has a higher affinity for GlcA-1-P that GalA-1-P. The k_{cat}/K_m values of AtUSP towards GlcA-1-P is 1.8 times as that of GalA-1-P, implies that AtUSP is more efficient for the pyrophosphorylation of GlcA-1-P. The k_{cat}/K_m values of AtUSP ($4.66 \times 10^3\ s^{-1}\ \mu M^{-1}$) and BiUSP ($4.72 \times 10^3\ s^{-1}\ \mu M^{-1}$) towards GlcA-1-P are similar, indicating they are equally efficient in the synthesis of UDP-GlcA. The kinetics of reverse reactions (hydrolysis of UDP-GlcA and UDP-GalA) (**Table 1**) revealed that AtUSP and BiUSP are equally active towards UDP-GlcA, whereas only AtUSP can catalyze the hydrolysis of UDP-GalA. Such results are consistent with that of forward reactions (formation of UPD-GlcA and UDP-GalA).

Table 2.1 Kinetic parameters of AtUSP and BiUSP towards GlcA-1-P, GalA-1-P, UDP-GlcA and UDP-GalA

	K_m (μM)	V_{max} ($\mu M\ mg^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)
AtUSP				
GlcA-1-P	2.36×10^{-3}	3.63×10^{-4}	1.0	4.66×10^{3}
GalA-1-P	5.50×10^{-3}	4.45×10^{-4}	3.5	2.45×10^{3}
UDP-GlcA	2.65×10^{-3}	4.06×10^{-4}	2.3	4.64×10^{3}
UDP-GalA	4.96×10^{-3}	4.59×10^{-4}	3.9	2.80×10^{3}
BiUSP				
GlcA-1-P	1.76×10^{-3}	3.38×10^{-4}	.30	4.72×10^{3}
GalA-1-P	-	-	-	-

UDP-GlcA	2.02×10^{-3}	3.71×10^{-4}	9	4.51×10^{3}
		.12		
UDP-GalA	-	-		-
		-		

2.2.3 Substrate specificity study of AtUSP and BiUSP towards sugar-1-phosphates

It was previously reported that AtUSP possessed relaxed substrate specificity towards sugar-1-phosphate. For example, it could well accept Gal-1-P, Glc-1-P, 4-deoxy-4-azido-Gal-1-P, D-Fuc-1-P, L-Ara-1-P, and Xyl-1-P.[38, 41-42] To further investigate the substrate specificities of AtUSP and BiUSP, the specific activities towards a panel of sugar-1-phosphates were measured. Not surprisingly, BiUSP exhibited similar substrate specificity as BLUSP [43] towards sugar-1-phosphates (**Table 2**). In addition, both AtUSP and BiUSP showed broad substrate specificities, but towards partially different sugar-1-phosphates. For example, AtUSP are highly active towards GlcA-1-P, GalA-1-P, Glc-1-P, Gal-1-P, D-Fuc-1-P, 2-deoxy-Glc-1-P, 2-deoxy-Gal-1-P, whereas BiUSP are highly active towards GlcA-1-P, Glc-1-P, Gal-1-P, Man-1-P, 2-deoxy-Glc-1-P, and 2-deoxy-2-amino-Glc-1-P. Such information is of great importance in applying the USPs in large scale synthesis of UDP-sugar and derivatives.

Table 2.2Substrate specificity of AtUSP and BiUSP towards sugar-1-phosphates
Specific activity of AtUSP towards GlcA-1-P (12.4 mol min^{-1} mg^{-1}) was set as 100. ND, not detected.

Sugar-1-phosphate	Relative activities	
	AtUSP	BiUSP
GlcA-1-P	100	91
GalA-1-P	75.7	ND
Glc-1-P	130	141
Gal-1-P	123	136
GlcNAc-1-P	ND	ND
GalNAc-1-P	ND	ND
Man-1-P	ND	37.2
Talose-1-P	ND	9.65
D-Fuc-1-P	61.7	15.4
2-deoxy-Glc-1-P	73.8	82.0
2-deoxy-Gal-1-P	53.2	ND

6-deoxy-6-azido-Gal-1-P	11.6	ND
2-deoxy-2-amino-Glc-1-P	7.50	41.8
2-deoxy-2-amino-Gal-1-P	ND	ND

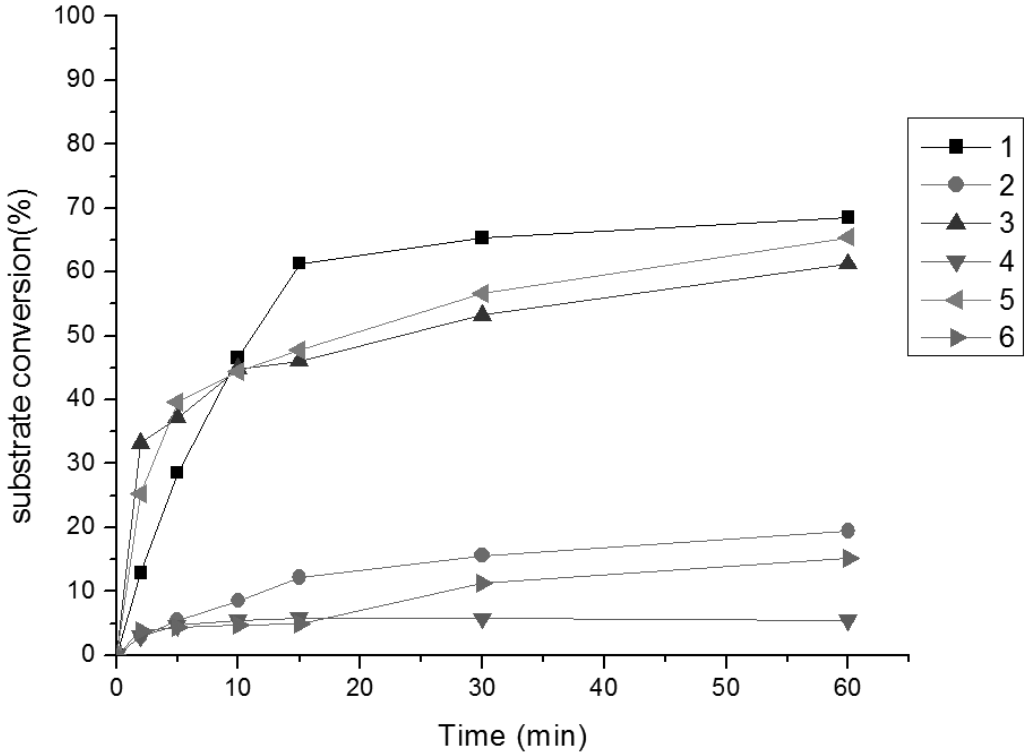


Figure 2.4 Time Course for UDP-GlcA and UDP-GalA reactions

Reactions contains 1 mM UTP, 40 ng of At USP or BiUSP, and: 1, AtUSP and 0.05 mM GlcA-1-P; 2, AtUSP and 0.8 mM GlcA-1-P; 3, AtUSP and 0.05 mM GalA-1-P; 4, AtUSP and 0.8 mM GalA-1-P; 5, BiUSP and 0.05 mM GlcA-1-P; 6, BiUSP and 0.8 mM GlcA-1-P.

2.2.4 Preparation and characterization of UDP-GlcA and UDP-GalA

Given the stability and efficiency, AtUSP was chosen for hundreds of milligrams scale synthesis of UDP-HexA. As shown in **Figure 5**, a one-pot three enzyme approach was employed. In the case of UDP-GlcA preparation, the monosaccharide GlcA was first converted into GlcA-1-P catalyzed by AtGlcAK, the glucuronokinase from *A. thaliana*.[40] The formation of UDP-GlcA was then achieved by AtUSP catalyzed reaction in the presence of UTP, and Mg^{2+}. Since UDP-sugar pyrophosphorylases catalyzed relations are usually reversible,[20] a

pyrophosphatase from *E. coli* (EcPpA) was added to digest the diphosphate byproduct. The same as previously proved,[37] addition of EcPpA greatly improved the final yield (207 mg pure UDP-GlcA in a yield of 89%) by driving the reaction forward. The synthesis of UDP-GalA was performed following the same route, except that AtGlcAK was substituted by BiGalK, a promiscuous galactokinase exhibiting phosphorylation activity towards GalA.[44] The total yield of UDP-GalA after P2 purification was 70% (162 mg).

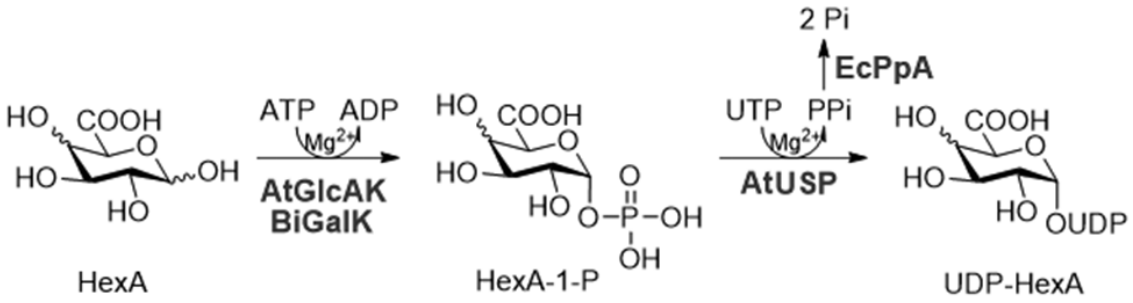
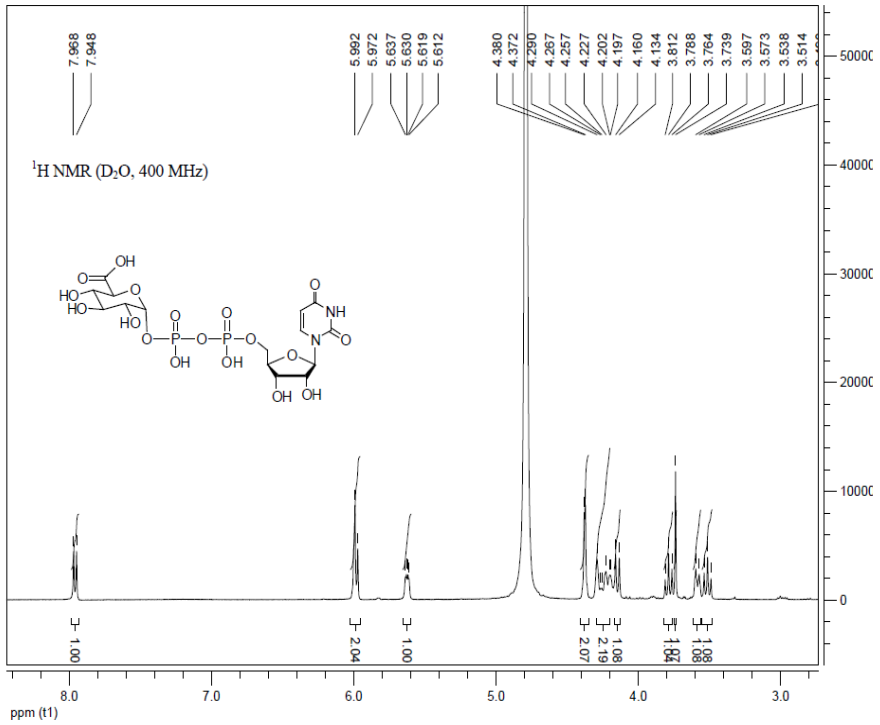


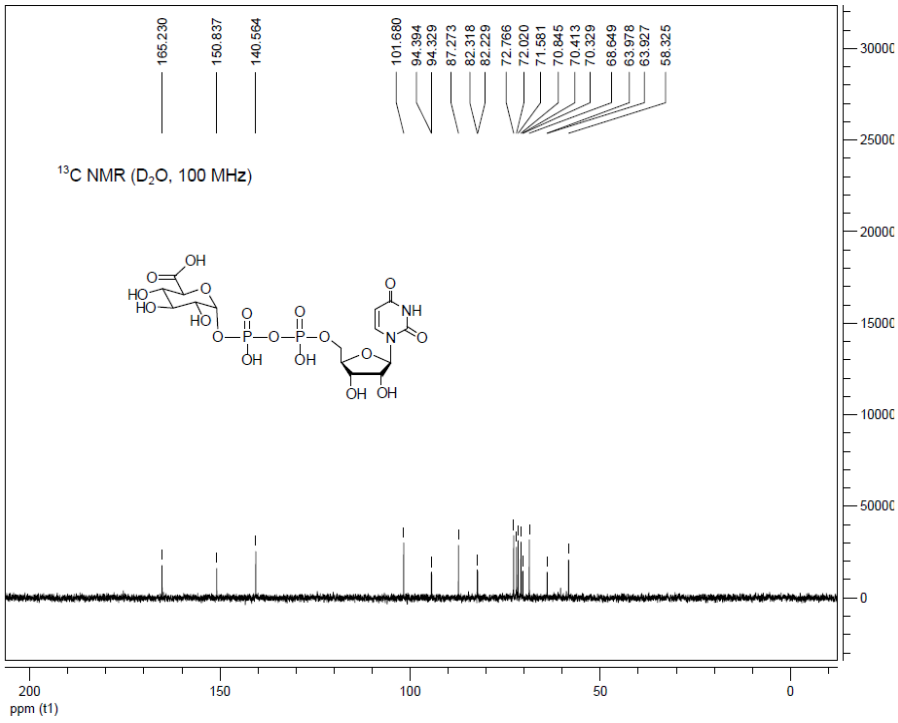
Figure 2.5 One-pot three-enzyme synthesis of UDP-GlcA and UDP-GalA
Enzymes used: AtGlcAK: Glucuronokinase from A. thaliana; BiGalK, Galactokinase from B. infantis ATCC15697; AtUSP, UDP-sugar pyrophosphorylase from A. thaliana; EcPpA, Pyrophosphatase from E. coli.

2.2.4.1 Spectroscopic data of Uridine 5'-diphosphate glucuronic acid

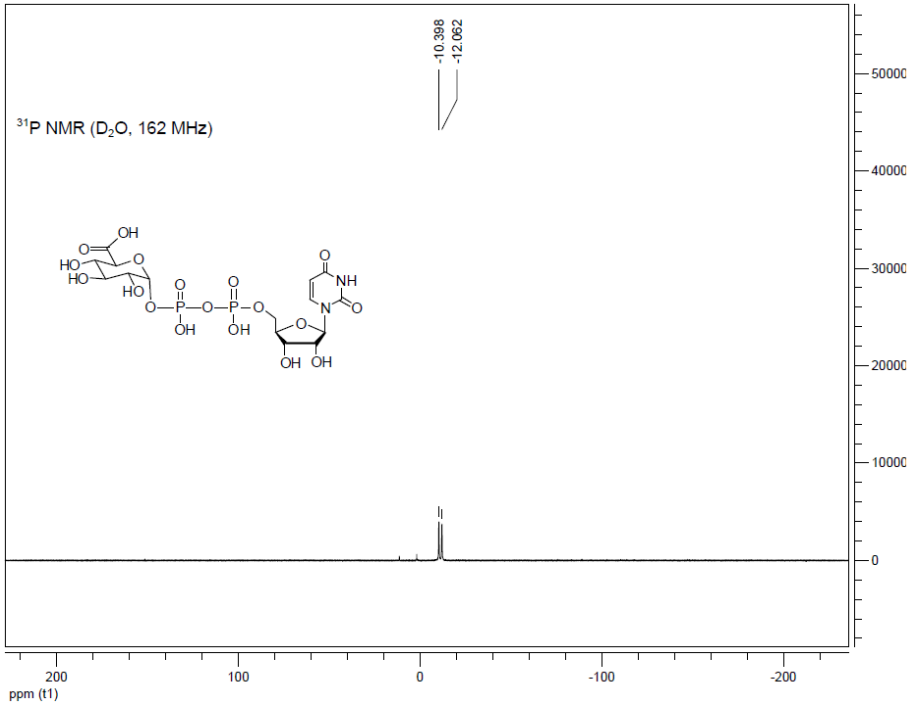
^{1}H NMR Spectrum of UDP-GlcA



^{13}C NMR Spectrum of UDP-GlcA



^{31}P NMR Spectrum of UDP-GlcA

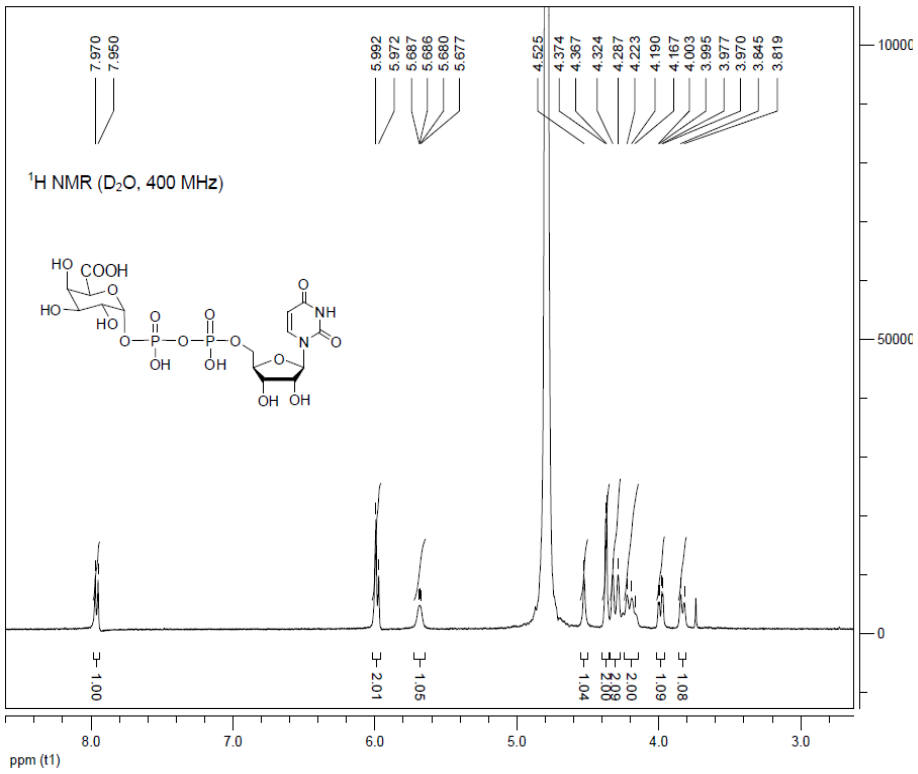


HRMS (ESI): Calcd 580.0343 for $C_{15}H_{22}N_2O_{18}P_2$; found: 579. 0256 $[M\text{-}H^+]^-$;

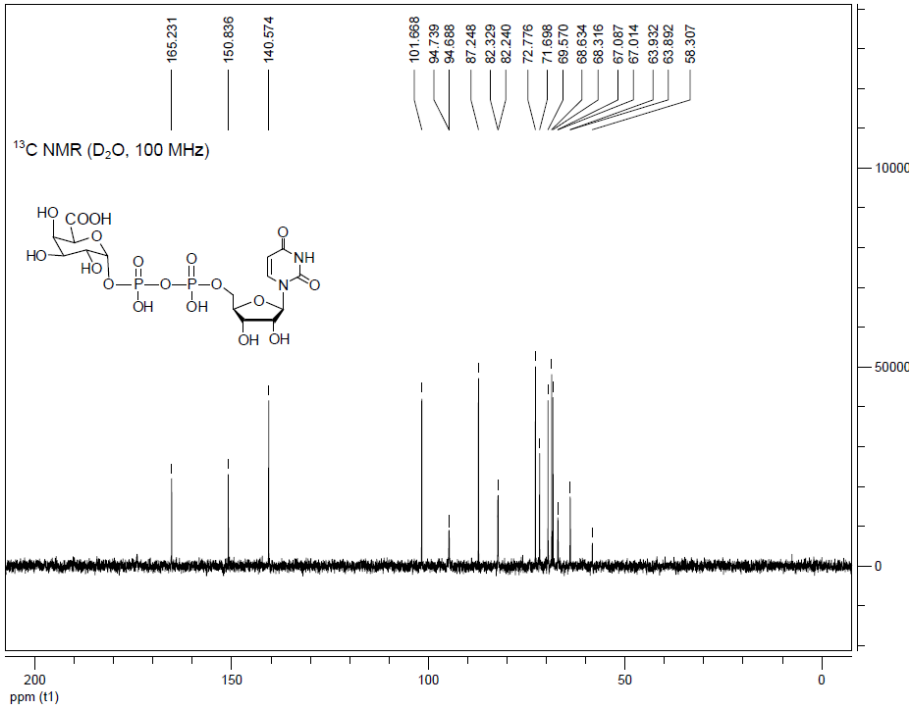
^{1}H NMR (400 MHz, D_2O): δ 3.51 (t, *J* = 9.6 Hz, 1 H), 3.57-3.60 (m,1 H), 3.74 (s,1 H), 3.79 (t, *J* = 9.6 Hz, 1H), 4.14 (d, *J* = 10.4 Hz, 1H), 4.20-4.29 (m,2 H), 4.37-4.38 (m,2 H), 5.62 (dd, *J* = 2.8 Hz, *J* = 7.2 Hz,1 H), 5.97-5.99 (m,2 H), 7.95 (d, *J* = 8.0 Hz, 1 H); ^{13}C NMR (100 MHz, D_2O): δ 58.33, 63.95 ($J_{C\text{-}P}$= 20.4 Hz), 68.65, 70.37 ($J_{C\text{-}P}$= 33.6 Hz), 70.85, 71.58, 72.02, 72.77, 82.27 ($J_{C\text{-}P}$= 35.6 Hz), 87.27, 94.36 ($J_{C\text{-}P}$= 26.0 Hz), 101.68, 140.56, 150.84, 165.23; ^{31}P NMR (162 MHz, D_2O): δ -12.06, -10.40;

2.2.4.2 Spectroscopic data of Uridine 5'-diphosphate galacturonic acid

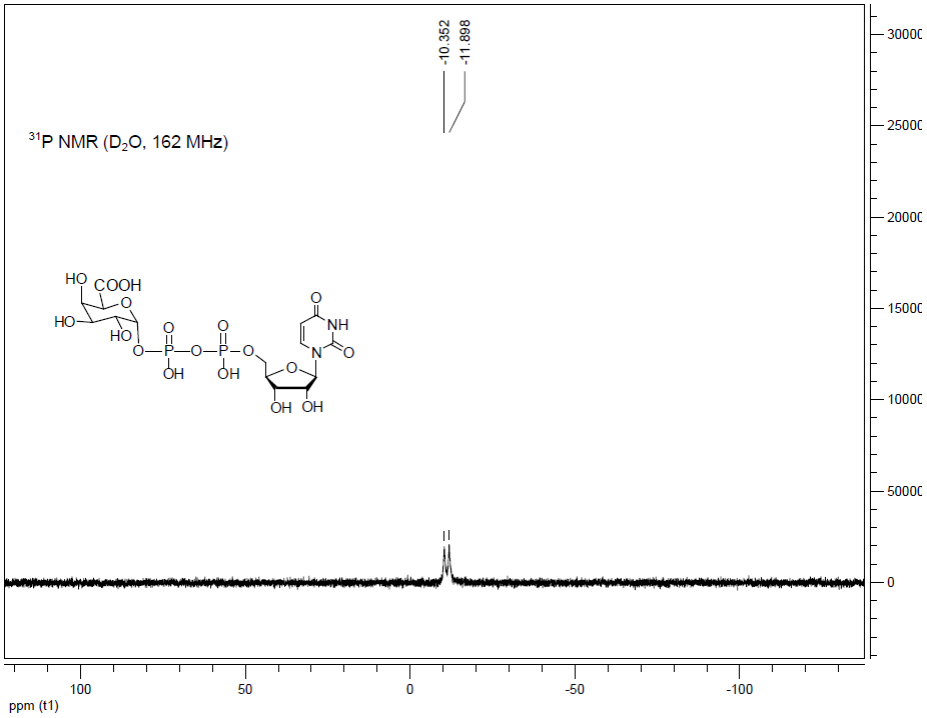
^{1}H NMR Spectrum of UDP-GalA



^{13}C NMR Spectrum of UDP-GalA



^{31}P NMR Spectrum of UDP-GlcA



HRMS (ESI): Calcd 580.0343 for $C_{15}H_{22}N_2O_{18}P_2$; found: 579. 0258 $[M\text{-}H^+]^-$;

^{1}H NMR (400 MHz, D_2O): δ 3.83 (d, *J* = 10.4 Hz, 1 H), 3.98 (dd, *J* = 3.2 Hz, *J* = 10.4 Hz,1 H), 4.17-4.22 (m,2 H), 4.29-4.32 (m,2 H), 4.36-4.37 (m,2 H), 4.53 (s,1 H), 5.68-5.69 (m,1 H), 5.97-5.99 (m,2 H), 7.96 (d, *J* = 8.0 Hz, 1 H); ^{13}C NMR (100 MHz, D_2O): δ 58.31, 63.91 ($J_{C\text{-}P}$= 16.0 Hz), 67.50 ($J_{C\text{-}P}$= 29.2 Hz), 68.32, 68.63, 69.57, 71.70, 72.78, 82.28 ($J_{C\text{-}P}$= 35.6 Hz), 87.25, 94.71 ($J_{C\text{-}P}$= 20.4 Hz), 101.67, 140.57, 150.84, 165.23; ^{31}P NMR (162 MHz, D_2O): δ -11.90, -10.35; HRMS (ESI):

2.3 Conclusion

In summary, we have biochemically characterized two highly efficient UDP-sugar

pyrophosphorylases (AtUSP and BiUSP) for the purpose of large scale UDP-HexA preparation. Genes or expression conditions were optimized to obtain high yields of enzymes (40-90 mg per L culture). Both enzymes showed comparable activities towards GlcA-1-P, whereas only AtUSP exhibited activity towards GalA-1-P. Specific activity study revealed that both USPs possessed broad substrate specificity, but towards partially different sugar-1-phosphates. In addition, we have developed a one-pot three-enzyme approach for efficient and rapid preparation of UDP-GlcA and UDP-GalA starting from inexpensive monosaccharides and nucleotides. Using such an approach, 207 and 162 mgs of UDP-GlcA and UDP-GalA were prepared within one week.

2.4 Experimental section

2.4.1 Materials

All chemical reagents were purchased from Sigma unless otherwise noted. Ni-NTA agarose was purchased from Qiagen. Talose-1-P,[37] D-Fucose-1-P,[44] 2-deoxy-Gal-1-P,[44] 2-deoxy-Glc-1-P [37] were prepared as previously described. The preparation of 6-deoxy-6-azido-Gal-1-P will be reported subsequently. Codon optimized gene of AtGlcAK, AtUSP were synthesized by Genscript (Piscataway, NJ). Genomic DNA of *B. infantis* ATCC 15697 was purchase from ATCC. *E. coli* DH5α and BL21 (DE3), as well as vector pET22b were purchased from EMD Millipore (Bilerica, MA). Vector pQE80Lwas purchased from Qiagen (Germantown, MD). BiGalK expression *E. coli* strain was kept by our group.

2.4.2 Molecular cloning Protein expression and purification

The glucuronokinase gene (*AtGlcAK*) and UDP-sugar pyrophosphorylase gene (*AtUSP*) from *A. thaliana* was codon optimized and synthesized with restriction sites (*Sac*I and *Hind*III)

(see supporting information for optimized DNA sequences). The genes were then cleaved by *Sal*I and *Hind*III, and inserted into pQE80L pretreated with corresponding restriction enzymes. The UDP-sugar pyrophosphorylase gene (*BiUSP, Blon_1169*) from *B. infantis* was amplified by PCR using *B. infantis* ATCC15697 genomic DNA as templates. Primers used are, Blon_1169F: GAGCATATGACCGAAATAAACGATAAGGCC and Blon_1169R: TATCTCGAGCACCCAATCGTCCGGTTCGAT. Yielded PCR fragments were digested with *Nde*I and *Hind*III and subsequently inserted into pET22b pretreated with corresponding enzymes. The recombinant plasmids were verified by DNA sequencing and transformed into *E. coli* BL21 (DE3) for protein expression.

E. coli BL21(DE3) cells harboring the above recombinant plasmids were grown in LB medium at 37 °C with shaking (20 rpm) till OD_{600} reached 0.6-0.8, followed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. After 20 h induction at 16 °C the cells were harvested by brief centrifuging, and stored at -80 °C until used. Protein purification was performed utilizing a Ni-NTA resin (Qiagen) column according to the manufacture instructions. The purified proteins were desalted by PD-10 column (GE life science, USA) against 50 mM Tris-HCl (pH 8.0) and 20% of glycerol. Enzymes were kept at -20 °C until used.

2.4.3 Activity assays of AtUSP and BiUSP towards GlcA-1-P and GalA-1-P

A 50 μL reaction system in 20 mM Tris-HCl (pH 8.0) consisted of 0.8 mM GlcA-1-P or GalA-1-P, 1 mM UTP, 2 mM $MgCl_2$, and varying amount of purified AtUSP or BiUSP. Reactions were allowed to proceed at 37 °C for 20 min and quenched by the addition of equal volumes of cold ethanol. The formation of UDP-HexA and consumption of UTP was analyzed

by Capillary Electrophoresis (Beckman Coulter) using the following conditions: 75 μm i.d. capillary (Beckman Coulter), 25 KV/140 μA, 5 s vacuum injection, 50 mM sodium tetraborate running buffer, pH 9.4., monitored at 254 nm.

2.4.4 Determination of kinetic parameters

Kinetic parameters of both AtUSP and BiUSP towards Glc-1-P and Gal-1-P were measured in 50 μL reactions in 20 mM Tris-HCl (pH 8.0). The reaction mixtures contain varying concentrations of GlcA-1-P or GalA-1-P (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 mM), 1 mM UTP, 2 mM $MgCl_2$. Reactions were initiated by the addition of 40 ng of AtUSP or BiUSP, performed at 37 °C for 5 min, and quenched by addition of equal volumes of cold ethanol. The sugar-1-P substrate concentrations used in the time course studies were 0.05 and 0.8 mM. The reactions were quenched at 0, 2, 5, 10, 15, 30, and 60 min. Analysis were performed by CE as described above, and the average value of triplicate assays was employed to generate Line-weaver-Burk plot, and K_m, V_{max}, k_{cat} values were calculated accordingly. Kinetic parameters of AtUSP and BiUSP towards UDP-GlcA and UDP-GalA were measured similarly, by replacing GlcA-1-P or GalA-1-P into UDP-GlcA or UDP-GalA, and replacing UTP into pyrophosphate.

2.4.5 Substrate specificity of AtUSP and BiUSP towards various sugar-1-phosphate

The reaction mixtures (50 μL total volume) contain 0.8 mM of different sugar-1-phosphates (**Table 2**), 1 mM UTP and 2 mM $MgCl_2$. Reactions were initiated by the addition of varying amounts (0.4-40 μg, to make sure the reactions convert no more than 25%) of AtUSP or BiUSP, performed at 37 °C for 10 min, and quenched by the addition of equal volumes of cold ethanol.

Analysis were performed by CE as described above, and the average value of triplicate assays was employed to generate specific activities towards each sugar-1-phosphate.

2.4.6 One pot three enzyme synthesis of UDP-GlcA and UDP-GalA

Preparative-scale synthesis of UDP-GlcA and UDP-GalA were performed in a 20 mL reaction system containing 50 mM pH 8.0 Tris-HCl buffer, 20 mM GlcA or GalA, 22 mM ATP, 22 mM UTP, 20 mM $MgCl_2$, and 0.1 to 2 mg of different enzymes. For the synthesis of UDP-GlcA, 0.6 mg of AtGlcAK, 0.4 mg of AtUSP, and 0.1 mg of EcPpA[37] were employed; for the synthesis of UDP-GalA, 2 mg of BiGalK, 0.4 mg of AtUSP, and 0.1 mg of EcPpA were employed. The reaction mixtures were incubated at 37 °C for 4 to 12 hours with gentle shaking. The generation of UDP-sugars were monitored by TLC (Isopropanol: NH_4OH: H_2O = 7: 3: 2). After 90% of monosaccharides converted into UDP-sugar, reactions were quenched by boiling for 5 min, centrifuged to remove precipitants, and concentrated for separation. The separation of UDP-sugars from the reaction mixtures were performed by gel filtration (Bio-Gel P2, Bio-Rad), fractions containing products were pooled and lyophilized. Digestion of nucleotides by alkaline phosphatase and further P2 purification were applied to achieve minimum 95% purity. The final products were lyophilized and characterized by HR-MS and NMR.

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3 CHEMOENZYMATIC SYNTHESIS OF A LIBRARY OF HUMAN MILK OLIGOSACCHARIDES

3.1 Introduction

Human milk, as the sole dietary source, contains all the necessary nutrients for the infant to thrive in the first few months. More importantly, human milk can also provide ingredients with health benefits which traditional food could not do. Human milk oligosaccharides (HMOs) are the third major component in human milk, only after lactose and lipid.[45-47] Concentrations and components of HMOs vary depending on the stages of lactation.[48-49] Particularly, one liter mature human milk contains about 12-14 grams HMOs.[50-56] The structures of about 130 discovered HMOs have been elucidated.[57-59] The major building blocks of HMOs are 5 monosaccharides, including D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-Fucose (Fuc), and N-acetyl neuraminic acid (Neu5Ac). Even though HMOs were first discovered and confirmed in 1950s, a comprehensive understanding of their functions is still out of reach, due to their inherit diversity and complexity. Increasing evidences show that HMOs can provide significant beneficial effects to the health of breast-fed infants through several mechanisms. For instance, HMOs could serve as prebiotics to promote the growth of desired bacteria in infant's intestine.[47-48, 60-63] Besides, HMOs are antiadhesive antimicrobials by serving as a receptor to prevent pathogen attachment to infant mucosal surfaces.[64-68] In addition, evidence has demonstrated that HMOs can modulate epithelial and immune cell responses, and reduce excessive mucosal leukocyte infiltration and activation, which in turn decreases the risk of necrotizing enterocolitis (NEC), one of the most common fatal disorders in preterm infants.[69-70] Furthermore, sialylated HMOs may also provide necessary nutrients for the development of brain and cognition of infants.[71-74]

Even though the general functions of HMOs has been explored and discovered, the functional roles of individual HMOs are far less clear because of very limited accesses towards sufficient amount of structurally defined HMOs. To date, only a handful of short-chain HMOs can be produced in large scale and the supply of more complicated and branched HMOs is highly demanded.

Until now, only a few approaches has been developed for the synthesis of a small number of well-defined HMOs.[48, 75-84] For example, Schmidt developed strategies to synthesize some highly branched HMOs by solution-phase and solid-phase synthesis.[78, 80-81] Enzymatic methods have also been employed to achieve relatively simple structures.[77] One of the biggest roadblocks in previous synthesis remains to be small quantity and limited variety of HMOs needed for biofunctional studies. Recently, we have developed an efficient Core Synthesis/Enzymatic Extension (CSEE) strategy for rapid preparation of N-glycan libraries.[85] In this study, similar strategy was successfully applied for HMOs synthesis. Briefly, 3 core oligosaccharides with one or two GlcNAc residue(s) at the non-reducing end were first synthesized by convergent assembly of 3 simple building blocks followed by extension of the cores by 4 robust glycosyltransferases to produce a library of 31 HMOs in my first project (**Figure.3.1**). After that we utilize the same CSEE strategy to expand the synthesis targets to around 69 HMOs based on the new core structure by 10 robust glycotransferases. (**Figure.3.2**).

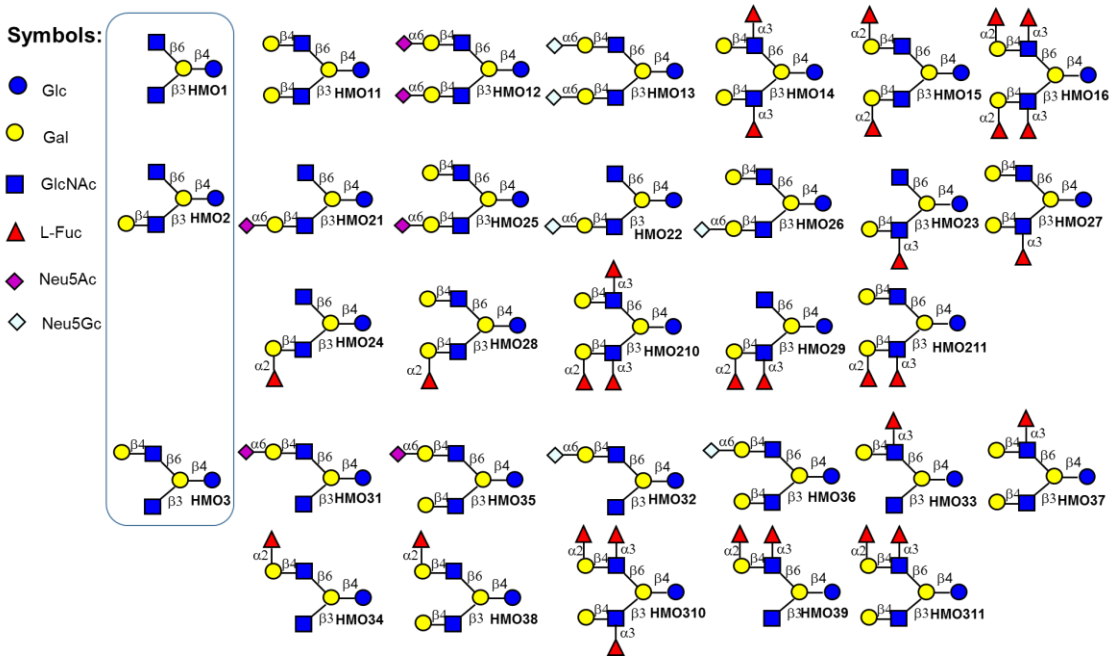


Figure 3.1 HMOs synthesized by the Core Synthesis/Enzymatic Extension (CSEE) strategy starting with 3 chemically prepared core structures (boxed)

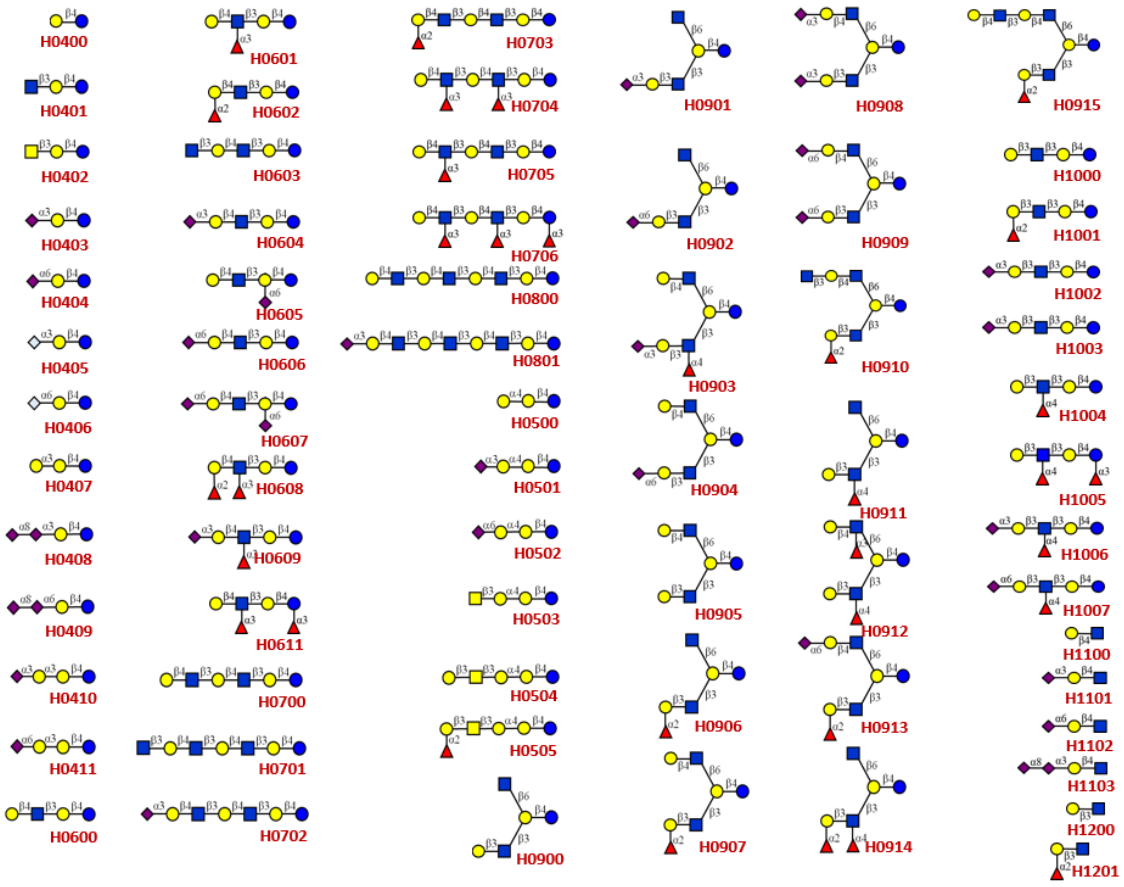


Figure 3.2 68 HMOs synthesized by the Core Synthesis/Enzymatic Extension (CSEE) strategy starting with new core structure

3.2 Results and Discussion

3.2.1 Convergent core synthesis

Previous studies highlighted the complexity and challenges associated with synthesizing HMOs via block coupling strategy. Schmidt developed sequential synthesis of lactose-containing oligosaccharides, including HMO lacto-N-tetraoside based on Solid-Phase Synthesis concept.[80] Madsen used one-pot glycosylations to achieve several human milk oligosaccharides.[79] Both of the methods can synthesize linear and simple oligosaccharides with obvious limitations in achieving more complex HMOs, especially highly branched ones. In this study, we developed an efficient and versatile methodology that utilized oligosaccharyl thioethers and oligosaccharyl

bromides as convergent donors for glycosylation, enabling branching assembly in one or two steps of glycosylations with excellent stereoselectivity and yields.

We envisaged that protected lactose **1 (Figure 2)** would be a versatile precursor for the synthesis of core structures, including symmetric and asymmetric ones, as C4, C6-hydroxyl groups (OH) on Gal are protected by benzylidene and the C3-OH is unprotected for chemical glycosylation. In order to achieve the selective protection of building block **1**, C3-OH group was selectively protected by 4-Methoxybenzyl ether (PMB) using standard condition, followed by C4, C6-OH protection with benzylidene group. In order to furnish the target cores, two oligosaccharyl thioethers and oligosaccharyl bromide were prepared **(Figure 2)**.

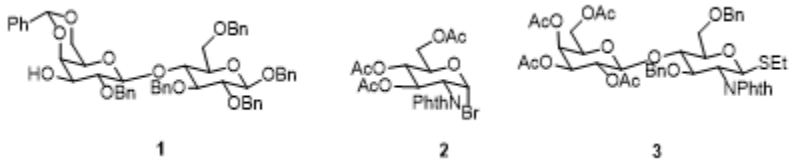


Figure 3.3 The three versatile building blocks for the assembly of the three core structures

3.2.2 Synthesis of building blocks

The synthesis of precursor **1** began with lactose peracetate, which was converted to the β-lactoside **4** by reaction with $BF_3{\cdot}Et_2O$, followed by deacetylation with NaOMe/MeOH condition to furnish compound **5**. Then **5** was treated with dibutyltin oxide, followed by the reaction with 4-Methoxybenzyl chloride to provide selective 3'-O-PMB protected lactoside with fair yield, which has been extensively studied.[83, 86] The following Benzylidene protection on 4', 6'-OH was conducted with Benzaldehyde dimethyl acetal, catalyzed by Camphorsulfonic acid (CSA), to get compound **6.** The perbenzylation of the remaining hydroxyls of **6** was performed by using sodium hydride and benzyl bromide in anhydrous DMF to give

compound **7**. After removing PMB protecting group of **7** treated with 2, 3-Dichloro-5, 6-dicyano-1, 4-benzoquinone (DDQ), building block **1** was achieved (**Scheme 1**).

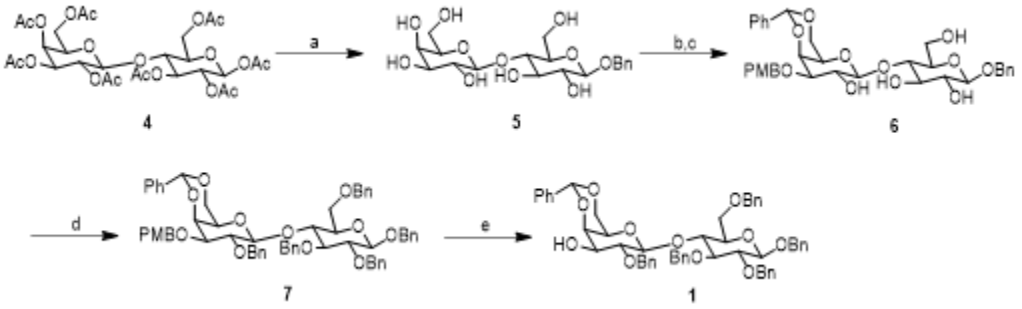


Figure 3.4 Synthesis of Building Block 1. Reagents and conditions

(a) (i) $BF_3{\cdot}Et_2O$, BnOH, DCM, 4 Å MS; (ii) NaOMe, MeOH, 50% over two steps; (b) (i) n-Bu_2SnO, MeOH, reflux; (ii) PMBCl, TBAI, Toluene, 4 Å MS reflux, 40%; (c) $PhCH(OMe)_2$, DMF, CSA, 75%; (d) BnBr, NaH, DMF, 95%; (e) DDQ, DCM/PBS Buffer=9:1, 90%.

Lactosamine building block **3** was initially envisioned to be synthesized using a straightforward fashion by coupling monosaccharide **9** and **10 (Scheme 2A)**. Unfortunately, only minor desired product was isolated by silica gel chromatography, and substantial amount of byproduct **11** was generated through a thioether migration reaction.[87] Therefore, a Koenigs–Knorr reaction coupling approach was carried out by installing C4 hydroxy group of glucosamine with high yield **(Scheme 2B)**. Building block **2** would be conveniently used in the synthesis of two asymmetric core oligosaccharides.

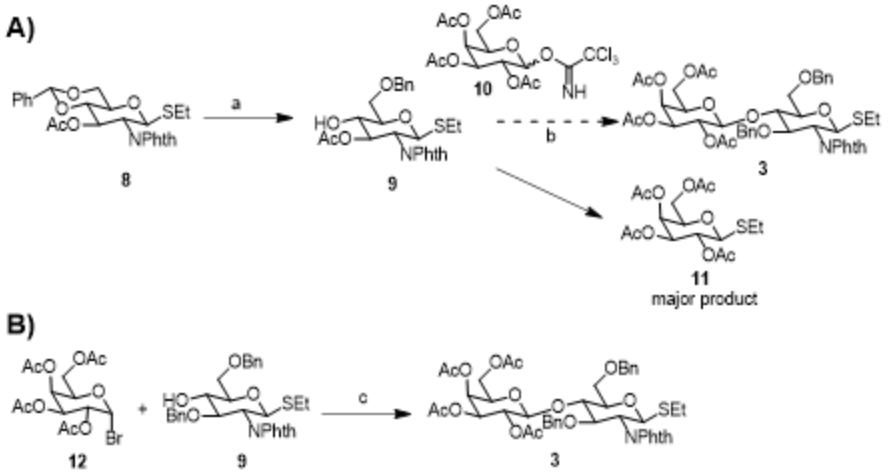


Figure 3.5 Synthesis of Building Block 3. (A) Failed reaction; (B) Successful reaction
Reagents and conditions: (a) Et_3SiH, TfOH, 4Å MS, -78°C, 85%; (b) TMSOTf, DCM, 4Å MS,-20°C; (c) AgOTf, DCM/Toluene,70%.

3.2.3 Synthesis of core oligosaccharides

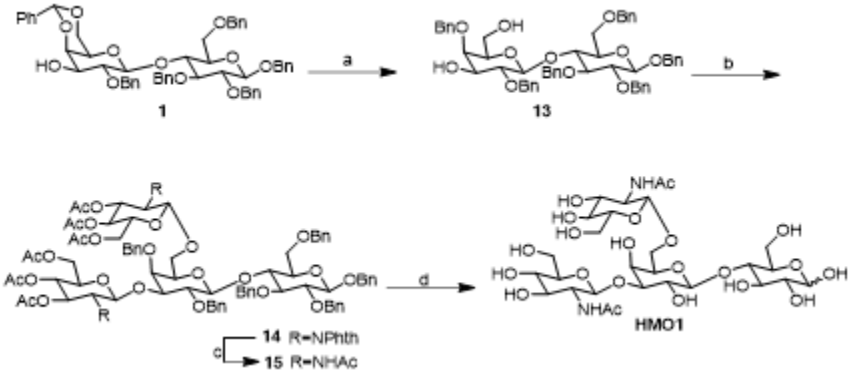


Figure 3.6 Synthesis of HMO1
Reagents and conditions: (a) Et_3SiH, $PhBCl_2$, 4 Å MS, DCM, -78°C, 85%; (b) **2**, AgOTf, 2,4,6-Collidine, -20 °C, 85%; (c) (i) Ethanediamine, *n*-BuOH; (ii) Ac_2O, Pyridine, DMAP; (d) (i) NaOMe/MeOH 75% over two steps; (ii) H_2, $Pd(OH)_2/C$, 80% over two steps.

Glycosylation of 3'-O-unprotected acceptor **1** with donor **3** proceeded at -20°C under AgOTf/NIS conditions to furnish the desired tetrasaccharide **16** in 85% yield. Then selective opening of the benzylidene ring at C6 of **16** using $Et_3SiH/PhBCl_2$ condition provided 6'-O-

unprotected acceptor **17** in 80% yield. The fully protected pentasaccharide was initially attempted to be synthesized by a convergent glycosylation of acceptor **17** and thiol donor **18**. Unfortunately, the desired product was not detected by TLC and ESI mass spectrometry analysis (**Scheme 4A**). Several other donors including oligosaccharyl trichloroacetimidate and thioethers donors were tried to install the pentasaccharide, but no product was detected. The big challenge should be caused by the bulky benzyl group on 4'-OH position, which has very large steric hindrance and stops the glycosylation on 6'-OH position, even though primary alcohol is very nucleophilic. Therefore, 4', 6'-O-unprotected lacto-N-tetraose **19** was proposed as acceptor for the glycosylation. Removal of the 4', 6'-O-benzylidene of **16** by treatment with ethanethiol in the presence of TsOH afforded acceptor 19. Glycosylation of acceptor **19** with glycosyl bromide **2** to achieve the protected target pentasaccharide **20** proceeded smoothly and regioselectively by use of AgOTf as Lewis acid at -20 °C in an excellent yield (85%). The two phthalimides of **20** were then converted into acetamides **21**, followed by the global deprotection of Ac and Bn groups. The core oligosaccharide **HMO2** was produced in a total yield of 53% over the four steps (**Figure 3.7B**).

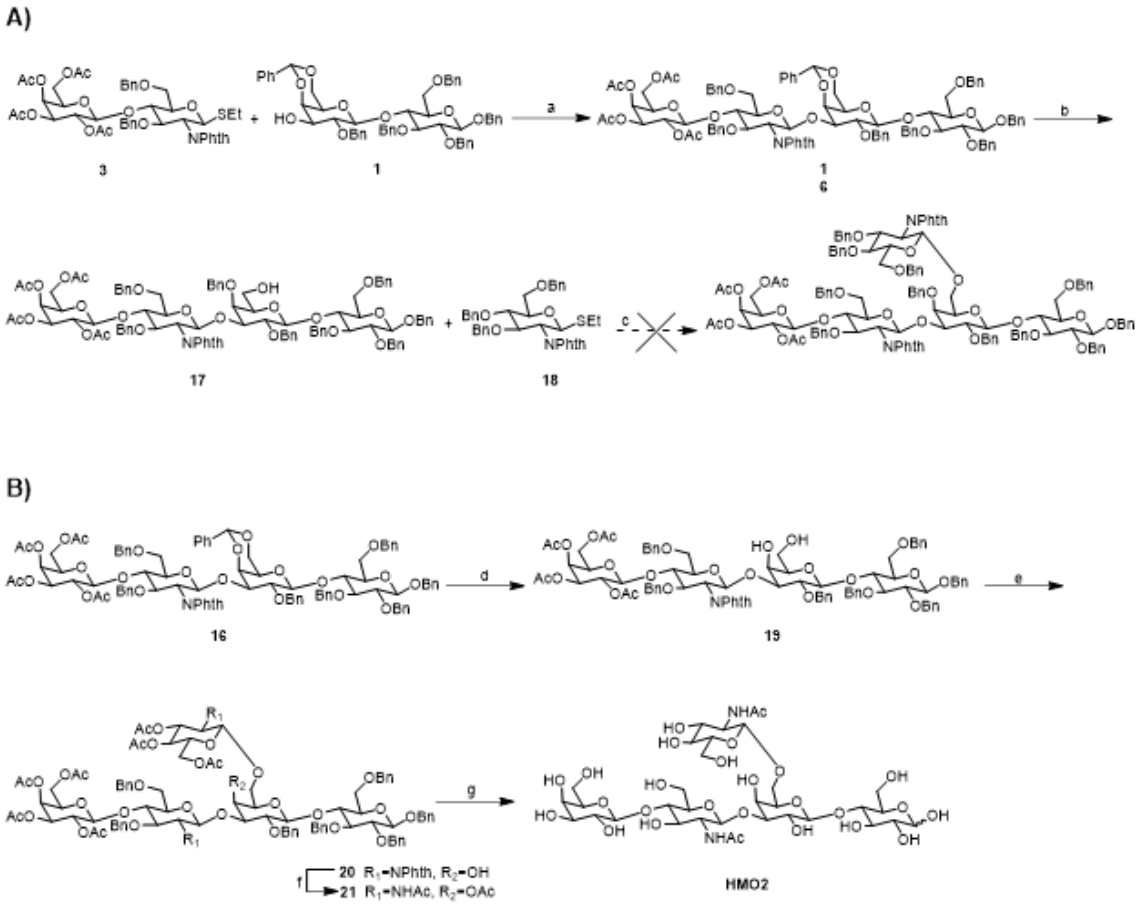


Figure 3.7 Synthesis of HMO2

(A) Failed reaction; (B) Successful reaction. Reagents and conditions: (a) NIS, TfOH, DCM, 4 Å MS,-20°C, 85%; (b) Et_3SiH, $PhBCl_2$, DCM, -78 °C. 80%; (c) NIS, AgOTf, DCM, 4 Å MS,-20°C; (d) TsOH, EtSH, DCM, 90%; (e) **2**, AgOTf, 2,4,6-Collidine, DCM, 4 Å MS, -20°C 85%; (f) (i) Ethanediamine, *n*-BuOH; (ii) Ac_2O, Pyridine, DMAP 62% over two steps; (g) (i) NaOMe/MeOH; (ii) H_2, $Pd(OH)_2/C$, 80% over two steps.

AgOTf promoted Glycosylation of 3'-O-unprotected acceptor **1** with donor **2** proceeded at -20°C to furnish the trisaccharide **22** in a good yield of 85%. Then deprotection of the 4', 6'-O-benzylidene of **22** by treatment of EtSH/TsOH provided the dialcohol **23.** Glycosylation of acceptor **23** with thiol donor **3** by treatment of AgOTf/NIS condition at -20°C to achieve the protected target pentasaccharide **24** in 70% yield. The two phthalimides of **24** were then converted into acetamides **25**. Complete deprotection of **25** was achieved by hydrogenolytic debenzylation

($Pd(OH)_2/C$, H_2) and complete de-O-acetylation using sodium methoxide in methanol, resulting in core oligosaccharide **HMO3** in a total yield of 67% over the four steps (**Figure 3.8**).

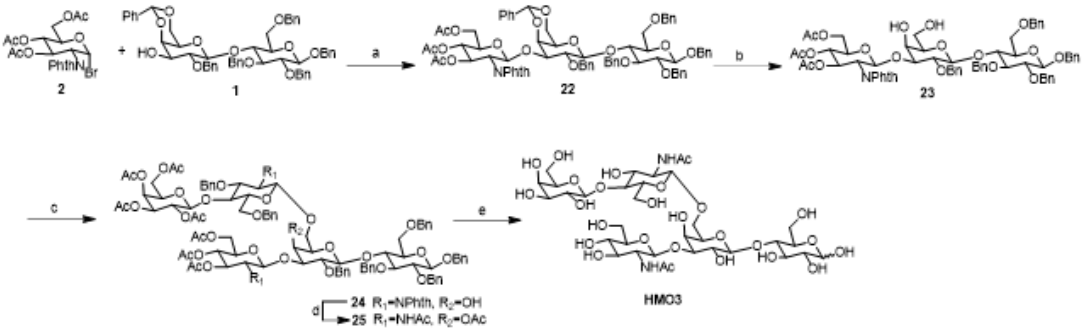


Figure 3.8 Synthesis of HMO3

Reagents and conditions: (a) AgOTf, 2, 4, 6-Collidine, DCM, 4Å MS, 85%; (b) TsOH, EtSH, DCM, 80%; (c) **3**, NIS, AgOTf, DCM, 4Å MS, -20°C,70%; (d) (i) Ethanediamine, *n*-Butanol; (ii) Ac_2O, Pyridine, DMAP; 70% over two steps; (e) (i) NaOMe, MeOH; (ii) $Pd(OH)_2$, H_2. 95% over two steps

3.2.4 Enzymatic extension of core structures

A total of 31 HMOs were enzymatically synthesized starting from the 3 core structures (**HMO1**, **HMO2**, **HMO3**) via an enzymatic extension approach using 4 robust GTs: β1,4 galactosyl-transferase from *Neisseria meningitidis* (LgtB),[88] α2,6-sialyltransferase from *Photobacterium damselae* (Pd2,6ST),[89] C-terminal 66 amino acids truncated α1,3-fucosyltransferase from Helicobacter pylori (Hpα1,3FT),[90] and □1,2-fucosyltransferase from *Helicobacter mustelae* (Hmα1,2FT).[91] All GTs were from bacteria and had high expression levels in *Escherichia coli*, high activity, and relatively relaxed substrate tolerance. As shown in **Figure 3A**, glycans **HMO11**-**HMO16** were prepared starting with the chemically prepared core **HMO1**. Briefly, in a 2 mL reaction system, 30 mg of **HMO1** (20 mM) was incubated with Gal (20 mM), $MgCl_2$ (20 mM), ATP (20mM), UTP (20mM) and variant amount of BiGalK, BiUSP and LgtB.[92] (**Figure 3D**) After overnight reaction, the mixture was terminated by boiling for 10min and

analyzed by MAlDI-MS which shows a single peak at m/z 1095.748, corresponding to **HMO11** $[M+Na]^+$. Meanwhile, on the HPLC-ELSD (Evaporative Light Scattering Detector) profile, a new peak (TR= 11.946 min) was observed. The reaction mixture was purified by HPLC using a water/acetonitrile gradient elution, yielding 40mg of **HMO11** (93% yield). The purified **HMO11** (99% pure) was then utilized for the syntheses of **HMO12-HMO16** (**Figure 3A**) catalyzed by Pd2,6ST, and □1,2FT, □1,2FT respectively (see ESI for details). Interestingly □1,3FT can specifically distinguish the GlcNAc from terminal Gal□□□□GlcNAc and □1,2FT preferentially attach to terminal Gal. We basically use this feature to biosynthesize Lewis X (Le^x) and Lewis Y (Le^y). In addition, difucosylated LacNAc motif [Fuc□1, 2-Gal-□1, 4-(Fuc□1, 3-)GlcNAc] was also generated while using Hm□1,2FT. The synthesis of asymmetric bi-antennary **HMO2x** and **HMO3x** (**Figure 3B**&**C**) were carried out by enzymatic extension of core **HMO2** and **HMO3**. Asymmetric Core **HMO2** and **HMO3** can more efficiently take advantage of different substrate specificities of GTs over symmetric **HMO1** via coupling a Gal to the terminal GlcNAc of one antennary. For example, to obtain **HMO311**, the Gal from □□GlcNAc branch was sequentially extended by Hm□1,2FT, Hp□1,3FT and LgtB . (**Figure 3C**) In contrast, **HMO310** were sequentially synthesized by Hm□1,2FT, LgtB and Hp□1,3FT (**Figure 3C**). Such synthetic routes were designed according to the substrate specificities of GTs to avoid undesirable glycosylation.

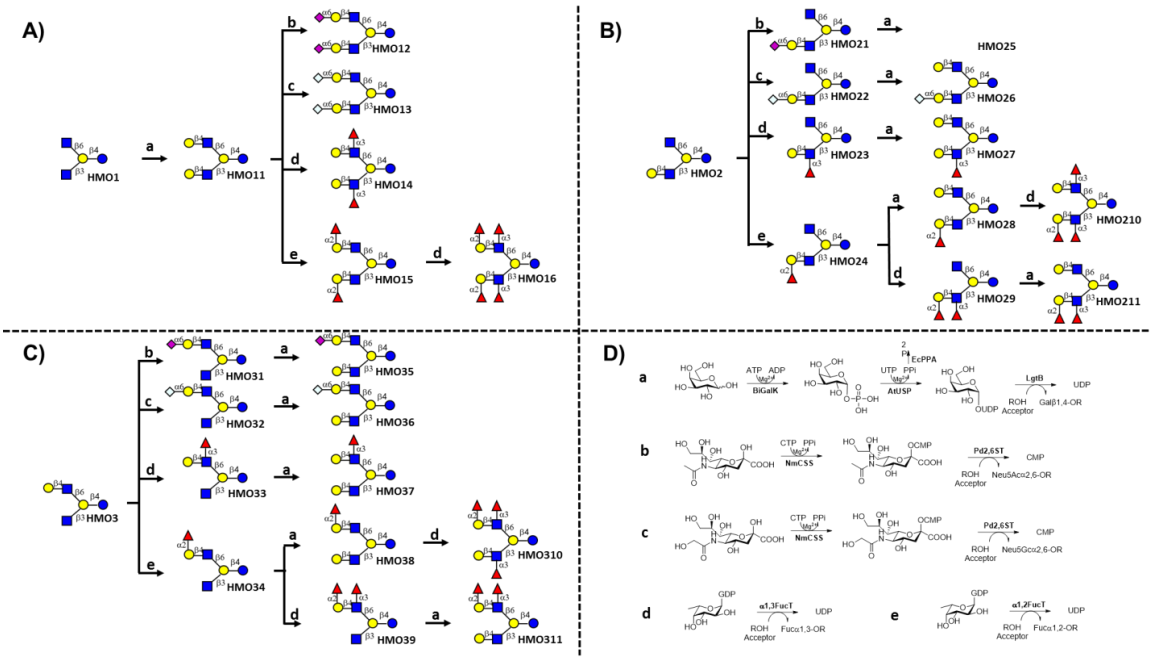


Figure 3.9 Enzymatic extension of human milk oligosaccharides

(a) LgtB, BiGalK, AtUSP, Gal, ATP, UTP, Mg^{2+}; (b) Pd2,6ST, NmCSS, Neu5Ac, CTP, Mg^{2+}; (c) Pd2,6ST, NmCSS, Neu5Gc, CTP, Mg^{2+}; (d) Hp□1,3FT, GDP-Fuc, Mn^{2+}; (e) Hm□1,2FT, GDP-Fuc, Mn^{2+}. LgtB, *Neisseria meningitidis* β1,4 galactosyltransferase; BiGalk, *Bifidobacterium infantis* galactokinase; AtUSP, *Arabidopsis thaliana* pyrophosphorylase; Pd2,6ST, *Photobacterium damselae* α2,6-sialyltrans-ferase; NmCSS, *Neisseria meningitidis* CMP-sialic acid synthetase; Hp α1,3FT, C-terminal 66 amino acids truncated *Helicobacter pylori* α1,3-fucosyltransferase; Hm α1,2FT, *Helicobacter mustelae* α1,2-fucosyltransferase.

After we published the first library of human milk oligosaccharides. We continue to expand our second library of HMOs based on eight core structures which were synthesized by either enzymatic or chemical approach. A total 69 HMOs were enzymatically synthesized using 10 robust GTs: β1,4 galactosyl-transferase from *Neisseria meningitidis* (LgtB),[88] α2,3-sialyltransferase from *Pasteurella multocida* (PmST1m) with reduced α2,3-sialidase activity,[93] β1,3-N-acetyulglucosaminyltransferase from *Helicobacter pylori* (LgtA),[94] α2,6-sialyltransferase from *Photobacterium damselae* (Pd2,6ST),[89] C-terminal 66 amino acids truncated α1,3-fucosyltransferase from *Helicobacter pylori* (Hp α1,3FT),[90] α1,2-fucosyltransferase from *Helicobacter mustelae* (Hm α1,2FT),[91] α1,4-galactosyltransferase from *Neisseria meningitidis*

(LgtC),[95] β1,3-N-acetyulgalactosaminyltransferase from *Haemophilus influenza* (LgtD),[96] α1,3-galactosyltransferase from bovine (α1,3GalT), [97] and α1,3/4-fucosyltransferase from *Helicobacter pylori*(α1,3/4 FucT). [98] All these glycotransferases were expressed in *E.Coli* system and have relatively high substrate tolerance. The 9 new core structures, either chemically or enzymatically synthesized, were utilized to expand the whole human milk oligosaccharides library.

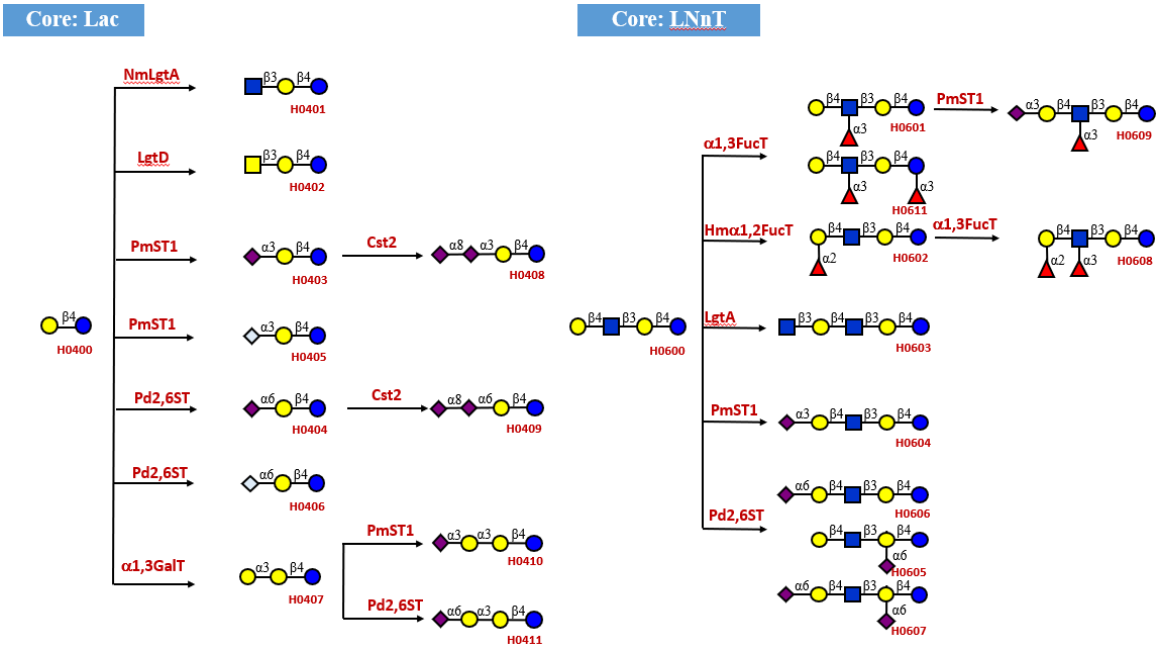


Figure 3.10 Enzymatic extension of Core Lac and LNnTof human milk oligosaccharides

As shown in **Figure 3.4**, glycans **H0401-H0411** were prepared starting with core **H0400**. Briefly, in enzymatic reaction system, **H0411** (20 mM) was incubated with sugar nucleotides (20 mM), $MnCl_2$ (20 mM) and variant amount of corresponding glycotransferases. After that, **H0408-H0411** were obtained by extending **H0403, H0404, H0407** via CstII, PmST1 and Pd2,6ST. **H0601-H0607** were prepared starting with core **H0600** via α1,3FucT, Hm α1,2FucT, LgtA,

PmST1 and Pd2,6ST. **H0608, H0609** were achieved by further extending **H0601** and **H0602** respectively via PmST1 and α1,3FucT.

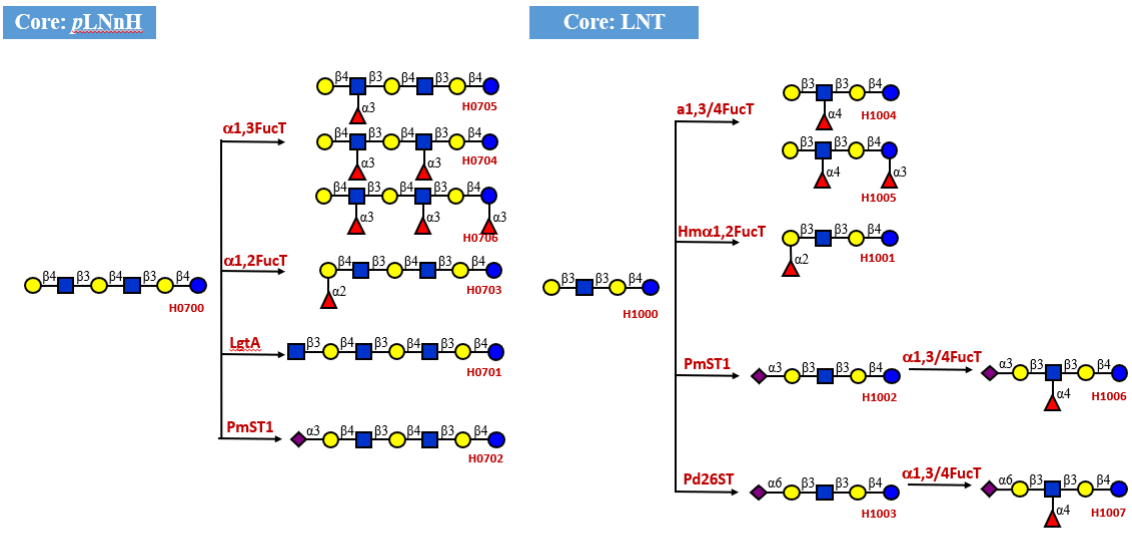


Figure 3.11 Enzymatic extension of Core pLNnH and LNT

As shown in **Figure 3.5**, glycans **H0701-H0706** were prepared starting with core **H0700** via α1,3FucT, Hm α1,2FucT, LgtA, PmST1. **H1001-H1005** were obtained by extending core **H1000** via α1,3/4FucT, Hm α1,2FucT, PmST1, Pd26ST**, H1006, H1007** were obtained by extending **H1002, H1003** respectively via α1,3/4FucT.

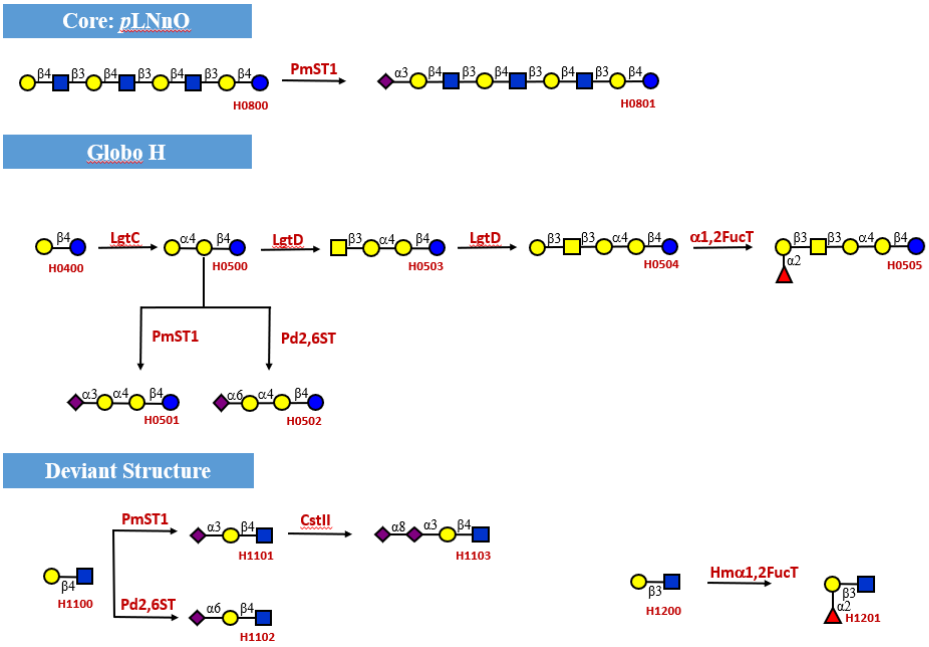


Figure 3.12 Enzymatic extension of Core pLNnO, GloboH, and Deviant Structure

As shown in **Figure 3.6**, glycans **H0801** were prepared starting with core **H0800** via PmST1. Globo H series was starting from **H0400**. We stepwisely use LgtC, LgtD, LgtD, Hm α1,2FucT to get **H0500**, **H0503**, **H0504**, **H0505**. **H0501**, **H0502** were obtain by using PmsT1 and Pd26ST starting from **H0500**. **H1101** and **H1102** were prepared by PmsT1 and Pd26ST starting frm **H1100**. CstII further transfer a Neu5Ac to **H1101** to form **H1103**. Hm α1,2FucT transfer fucose to **H1200** to form **H1201**.

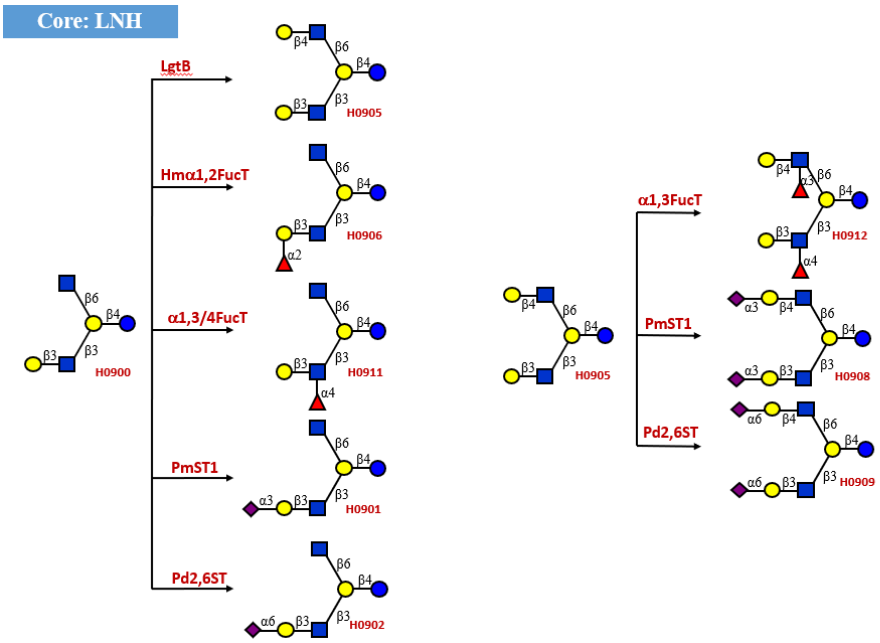


Figure 3.13 Enzymatic extension of Core LNH

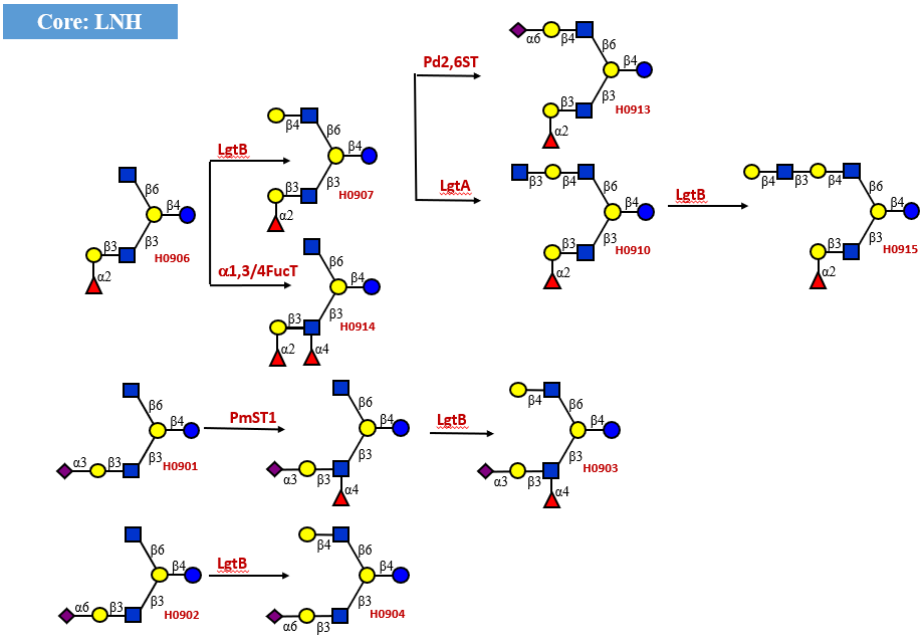


Figure 3.14 Enzymatic extension of Core LNH

As shown in **Figure 3.7, Figure 3.8**, glycans **H0901**, **H0902**, **H0905**, **H0906**, **H0911** were prepared starting with core **H0900** via LgtB, Hm α1,2FucT, α1,3/4FucT, PmST1, Pd26ST. After

that we branch these 5 structures to get another 11 HMOs (**H0912**, **H0908**, **H0909**, **H0907**, **H0914**, **H0913**, **H0910**, **H0915**, **H0903**, **H0904**. We transfer either fucose or Sialic acid to the terminal Gal of asymmetric bi-antennary core **H0900** to protect lower chain of core **H0900**. Therefore, we utilize different glycotransferases to sequentially extend upper chain of core **H0900**. According to this strategy, we synthesized a 16 HMOs based on **H0900**.

3.3 Conclusion

In summary, we have utilized our well-developed CSEE strategy for efficient synthesis of a library of structure-defined HMOs, which was assisted with rapid HPLC purification. The combination of CSEE and HPLC purification allows us to deliver diverse and high purity of 31 homogenous HMOs. These HMOs are valuable materials for bioactivity evaluation as well as glycan analysis. In this work, oligosaccharyl thioethers and oligosaccharyl bromide were consistently utilized as chemical glycosylation donors for the convergent installation of branched lactose-terminated antennae. This general and efficient method furnished 3 core oligosaccharides with high stereoselectivity and excellent yields. This work further confirmed that any GlcNAc-terminated glycans could be extended to 5 or more glycans, including Le^X and SLe^X, which are very important epitopes in glycobiology. The CSEE demonstrated a practical way to harvest diverse and complex HMOs with defined structures for various applications. The "mass" production of more homogenous HMOs and bioactivity evaluation are underway.

After completion of first version of library of HMOs. We chemoenzymatically synthesize another 69 HMOs via 10 robust glycotransferases based on 9 core structures with reasonable yield and high purity in milligram scale. The same rapid HPLC separation method was applied on second version of library of HMOs. The CSEE and HPLC method provided an efficient and practical way

to diversify the complicated HMOs in a semi-preparative scale without tedious, complicated and time-consuming purification process comparing to chemical synthesis.

3.4 Experimental Sections

General Methods: All chemicals were purchased as reagent grade and used without further purification. Anhydrous dichloromethane (CH_2Cl_2), acetonitrile (CH_3CN), tetrahydrofuran (THF), N, N-dimethyl formamide (DMF), toluene, and methanol (MeOH) were purchased from a commercial source without further distillation. Pulverized Molecular Sieves MS-4 Å (Aldrich) for glycosylation was activated by heating at 350 °C for 3 h. All reactions were performed with dry solvents and under nitrogen unless otherwise stated. Thin-layer chromatography (TLC) with 60 F_{254} silica gel plastic plates were detected visualized under UV (254 nm) and/or by staining with a solution of 10 ml anisaldehyde and 10 ml 95% H_2SO_4 in 400 mL Ethanol, followed by heating on a hot plate. Column chromatography was carried out on silica gel (EMD 230-400 mesh ASTM) and P2 gel (Bio-rad). Optical rotation values were measured using a PerkinElmer Model 343 polarimeter at the ambient temperature in specified solvents. ^{1}H NMR spectra were recorded on a Bruker AVANCE 400 (400 MHz) or Bruker AVANCE 500 (500 MHz) spectrometer at 25 °C. All ^{1}H Chemical shifts (in ppm) were assigned according to $CDCl_3$ (δ= 7.24 ppm) or D_2O (δ = 4.79 ppm). ^{13}C NMR spectra were obtained with Bruker AVANCE 400 spectrometer and calibrated with $CDCl_3$ (δ = 77.00 ppm). Coupling constants (*J*) are reported in hertz (Hz). Splitting patterns are described using the following abbreviations: s, singlet; brs, broad singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; m, multiplet. 1H NMR spectra are reported in the following order: chemical shift, multiplicity, coupling constant(s), and number(s) of protons. All NMR signals were assigned on the basis of ^{1}H NMR and ^{13}C NMR experiments. High resolution MALDI mass spectra were recorded on a Bruker Ultraflextreme spectrometer.

Neu5Ac and Neu5Gc were purchased from Carbosynth Limited. ATP and CTP were purchased from Sigma. Thermosensitive Alkaline Phosphatase from shrimp (FastAP) was purchased from Thermo Scientific. Other enzymes including *Neisseria meningitidis* □1,4-galactosyltransferase (NmLgtB), □2,6-sialyltransferase from *Photobacterium damslae* (Pd2,6ST) , C-terminal 66 amino acids truncated *Helicobacter pylori* □1,3-fucosyltransferase (Hp□1,3FT) , *Helicobacter mustelae* □1,2-fucosyltransferase (Hmα1,2FT), CMP-sialic acid synthetase from *N. meningitides* (NmCSS) were expressed and purified as previously described. Enzymes were then desalted against 50 mM Tris-HCl, 100 mM NaCl, and 50% glycerol, and stored at -20 °C for long term use. Sugar nucleotides uridine 5'-diphospho-galactose (UDP-Gal), cytidine 5'-monophospho- N-acetylneuraminic acid (CMP-Neu5Ac), cytidine 5'-monophospho- N-Glycolylneuraminic acid (CMP-Neu5Gc) and guanoside 5'-diphospho-L-fucose (GDP-Fuc) were prepared as described below.

General Procedures

A) Transformation of N-Phth to NHAc procedure: A mixture of N-Phth protected oligosaccharide was dissolved in *n*-BuOH at room temperature, followed by addition of ethylenediamine (*n*-BuOH: ethylenediamine = 2:1). After being stirred at 90 °C for 12 h, the mixture was evaporated in vacuo to give a residue for the next step without further purification. To a solution of the residue in pyridine was added Ac_2O. After being stirred at room temperature for 12 h, the solution was diluted with EtOAc and washed with aqueous 1 M HCl, saturated aqueous $NaHCO_3$, and brine solution. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo to give a residue, which was purified by silica gel column chromatography to give NHAc compound.

B) Deacetylation procedure: Ac-protected oligosaccharide was dissolved in MeOH, and NaOMe in MeOH was added until pH was about 10. After stirring at room temperature for 12 h, the solution was neutralized with ion-exchange resin (H^+), and then filtered. The residue was concentrated under vacuo to afford the desired deacetylated product.

C) Deprotection of benzyl group: $Pd(OH)_2$ on carbon was added to a solution of protected oligosaccharide in MeOH/H_2O (10/1). The mixture was stirred under 1 atmosphere of hydrogen. After being stirred for 24 h, the mixture was filtered through a PTFE syringe filter and concentrated in vacuo. The residue was purified by Bio-Gel P-2 (BIO-RAD) column chromatography using water as eluent. The product was then lyophilized to get target compound as white powder.

D) Production of oligosaccharyl bromide: Add peracetylated oligosaccharide portionwise to a stirred solution of HBr (33%) in glacial acetic acid (20.0 ml) at 0 °C. After all the sugar has been added, the reaction mixture was stirred at room temperature for 45 min. TLC analysis (Hexanes: Ethyl acetate=1:1) indicates formation of product and consumption of starting material. Then the reaction was quenched by ice water (200 ml) and then extract the product with DCM (2 × 200 ml). Wash the combined organic extracts with a solution of $NaHCO_3$ (aq., sat., 2 × 200 ml), dry with Na_2SO_4, filter and then concentrate in vacuo. The crude product was used without further purification.

E) General methods for enzyme treatment and HPLC purification In general, 31 HMOs were enzymatically synthesized by 4 glycosyltransferases (NmLgtB, Pd2,6ST, Hpα1,3FT, Hmα1,2FT) in the nearly same reaction condition. Another 69 HMOs were enzymatically synthesized by total 10 glycosyltransferases (LgtA, LgtB, LgtC, LgtD, Hpα1,3FT, Hpα1,3/4FT, Hmα1,2FT, PmST1, Pd26ST, CstII) Reactions contain 50 mM Tris-HCl (pH 8.0), 10 mM of acceptor HMOs, 12 mM

of sugar nucleotide (or its corresponding synthetase), 10 mM of $MnCl_2$, and varying amounts of Glycotransferases. FastAP (1 U/200 □L) was also added to digest the reaction byproduct UDP to drive reaction forward. Reactions incubated at 37 °C for overnight, and monitored by HILIC-ELSD (Waters XBridge BEH amide column, 130 Å, 4.6 mm × 250 mm under a gradient running condition (solvent A: 100 mM ammonium formate, pH 3.4; solvent B: acetonitrile; flow rate: 1 mL/min; B%: 65-50% within 25 min)). The desired products were detected by a high-efficient ELSD (evaporative light scattering detector) which increases the sample concentration to minimize the noise and deliver higher sensitivity. After over 90% acceptor converted, the reaction was quenched by boiling for 10 min, followed concentration by rotary evaporator. HPLC-A210nm was then used to purify target HMOs using a semi-preparative column (Waters XBridge BEH amide column, 130 Å, 5 □m, 10 mm × 250 mm) under a gradient running condition which are solvent A: 100 mM ammonium formate, pH 3.4; solvent B: acetonitrile; flow rate: 4 mL/min; B%: 65-50% within 25 min.[85] MS data for purified HMOs were obtained by ESI-MS and MALDI-MS.

Benzyl O-(4, 6-O-benzylidene-3-O-(4-methoxybenzyl)- β-D-galactopyranosyl)-(1→4)- β-D-glucopyranoside (6)

A suspension of benzyllactose **5** (12.0 g, 27.78 mmol) and Bu_2SnO (7.6 g, 30.54 mmol) in anhydrous MeOH (100 mL) was heated to reflux and stirred for 8 h. The solvent was removed in vacuo. Then the residue was dissolved in dry toluene (100 mL). *p*-Methoxybenzyl chloride (3.76 mL, 20.37 mmol), tetrabutylammonium iodide (2.05 g, 11.10 mmol), and 4 Å molecular sieves (5 g) were added. The resulting mixture was heated to reflux for another 8 h and then cooled to room temperature. The suspension was filtered through a Celite pad and the filtrate was concentrated

and chromatographied (dichloromethane-methanol=6:1) to afford 9.2 g of crude product (60% yield).

Benzaldehyde dimethyl acetal (2.75 mL, 18.33 mmol) was added to a solution of the above crude product (7.8 g, 14.10 mmol) in anhydrous Dimethylformamide (100 ml), then Camphorsulfonic acid was added to adjust the PH to about 2.0-3.0. The reaction mixture was stirred overnight and then quenched with triethylamine. The mixture was concentrated in vacuum. The residue was purified by flash column chromatography (dichloromethane-methanol=10:1) to give **6** as a white solid (8.47 g, 87.0%). $[\alpha]_D^{20}$ = +6.7 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.48-7.51 (dd, 2H), 7.28-7.38 (m, 10 H), 6.85-6.87 (m, 2 H), 5.34 (s, 1 H), 4.87 (d, J = 11.95 Hz, 1 H), 4.59-4.63 (m, 4 H), 4.45 (d, J = 7.8 Hz, 1 H), 4.36 (d, J = 8.1 Hz, 1 H), 4.21 (d, J = 14.0 Hz, 1 H), 4.10 (s, 1 H), 3.92-4.00 (m, 3 H), 3.79-3.89 (m, 2 H), 3.78 (s, 3 H), 3.60-3.69 (m, 3 H), 3.40-3.50 (m, 2 H), 3.27-3.40 (m, 4 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 137.6,137.2, 130.0,129.5, 128.5, 128.3, 128.2, 127.9, 126.3, 113.9, 103.6, 101.8, 101.1, 78.8, 74.9,74.7, 73.5, 72.7, 71.3, 71.2, 69.1, 69.0, 66.9, 61.9, 55.3. HRMS: $[M + Na]^+$ $C_{34}H_{40}NaO_{12}$ calcd for 663.2417, found 663.2420.

Benzyl O-(2-O-Benzyl-4, 6-O-benzylidene-3-O-(4-methoxybenzyl)- β-D-galactopyranos-yl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (7)

60% NaH (2.25 g, 56.25 mmol) and BnBr (6.66 ml, 56.25 mmol) were added to a stirred solution of **6** (6.0g, 9.38 mmol) in DMF (60 mL) cooled at 0°C. The color of the solution changed to light yellow. The reaction mixture was maintained at room temperature for 4 h. Then the solution was quenched with MeOH. The mixture was diluted with EtOAc and washed with water. The organic layer was dried with Na_2SO_4 and concentrated. The residue was purified on a silica gel column (Hexanes: EtOAc = 6:1) to afford the product **7** (1.85 g, 92.5%) as white powder. $[\alpha]_D^{20}$ =

+7.4 (c 1.0, CH_2Cl_2). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.57-7.59 (m, 2 H), 7.51-7.54 (m, 2 H), 7.42-7.45 (m, 2 H), 7.31-7.38 (m,23 H), 7.23-7.24 (m, 2 H), 6.90-6.92 (m, 2 H), 5.51 (s, 1H), 5.25 (d, *J* = 11.0 Hz, 1 H), 4.98-5.03 (m, 2 H), 4.90 (d, *J* = 11.1 Hz, 1 H), 4.79-4.85 (m, 3 H),4.71-4.74 (m, 3 H), 4.63 (d, *J* = 12.0 Hz, 1 H), 4.52-4.55 (m, 2 H), 4.43 (d, *J* = 12.0 Hz, 1 H), 4.26 (dd, *J* = 1.4 Hz, 12.4 Hz, 1 H), 4.04-4.08 (m, 2 H), 3.96 (dd, *J* = 4.2, 11.3 Hz, 1 H), 3.90 (dd, *J* = 1.8, 12.5 Hz, 1 H), 3.85 (s, 3 H), 3.79-3.83 (m, 2 H), 3.69 (t, *J* = 8.8 Hz, 1 H), 3.56-3.60 (m, 1 H), 3.40-3.46 (m, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 159.3, 139.0, 139.0, 138.7, 138.6, 138.2, 137.6, 130.5, 129.4, 128.9, 128.7, 128.4, 128.3, 128.1, 128.0, 127.8, 127.8, 127.6, 127.6, 127.5, 127.4, 127.3, 126.6, 113.8, 103.0, 102.6, 101.4, 83.1, 81.9, 79.4, 78.9, 77.7, 75.9, 75.3, 75.2, 75.1, 73.8, 73.0, 71.37, 71.1, 69.0, 68.3, 66.4, 55.3. HRMS: $[M + Na]^+$ $C_{62}H_{64}NaO_{12}$ calcd for 1023.4295, found 1023.4285.

Benzyl O-(2-O-Benzyl-4, 6-O-benzylidene- β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (1)

2, 3-Dichloro-5, 6-dicyano-1, 4-benzoquinone (2.75 g, 12.10 mmol) was added to a solution of **7** (6.0 g, 6.05 mmol) in 9:1 CH_2Cl_2– Phosphate-buffered saline (200 mL) was added. The solution was stirred for 1.5h at room temperature and diluted with CH_2Cl_2. The solution was washed with aqueous $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The residue was was purified on a silica gel column (Hexanes: EtOAc = 5:1) to afford the product **1** (5.02 g, 95%) as white powder. $[\alpha]_D^{20}$ = +15.4 (c 1.0, CH_2Cl_2). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.52-7.55 (m, 4 H), 7.20-7.45 (m, 26 H), 5.56 (s, 1 H), 5.21 (d, *J* = 8.0 Hz 1 H), 4.97-5.02 (m, 2 H), 4.78-4.83 (m, 4 H), 4.65-4.73 (m, 2 H), 4.51-4.54 (m, 2 H), 4.46 (d, *J* = 12.3 Hz, 1 H), 4.30 (d, *J* = 12.3 Hz, 1 H), 4.06-4.13 (m, 2 H), 3.93-3.97 (m, 2 H), 3.79 (dd, *J* = 1.3, 11.0 Hz, 1 H), 3.68 (m, 1 H), 3.55-3.60 (m, 3 H), 3.38-3.41 (m, 1 H), 3.14 (s, 1 H) ; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.9, 138.7, 138.6,

138.5, 137.9, 137.5, 129.2, 128.8, 128.4, 128.2, 128.0, 127.8, 127.6, 127.4, 126.5, 102.8, 102.6, 101.5, 83.1, 81.9, 80.2, 77.6, 75.9, 75.7, 75.2, 75.1, 73.1, 72.9, 71.0, 68.9, 68.2, 66.5. HRMS: $[M + Na]^+$ $C_{54}H_{56}NaO_{11}$ calcd for 903.3720, found 903.3725.

Ethyl 3, 6-di-O-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (9)

A mixture of compound **8** (2 g, 4.09 mmol) and 4 Å molecular sieves (2 g) in dry CH_2Cl_2 was stirred at room temperature under nitrogen for 2 h. Triethylsilane (2.1 mL, 13.1 mmol) and TfOH (1.05 mL, 11.9 mmol) were sequentially added at - 78 °C. The reaction mixture was stirred at -78 °C for 2 h and then quenched with MeOH (2 mL) and Et_3N (2 mL). The resulting mixture was filtered. The filtrate was diluted with CH_2Cl_2 and washed with aqueous $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The residue was purified on a silica gel column (Hexanes: EtOAc = 5:1) to afford the product **9** (1.85 g, 92.5%) as white powder. $[\alpha]_D^{20}$ = +91.0 (c 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.82 (d, J = 5.9 Hz, 1 H), 7.66-7.72 (m, 3 H), 7.28-7.42 (m, 5 H), 7.03-7.10 (m, 2 H), 6.92-7.00 (m, 3 H), 5.32 (d, J = 9.9 Hz, 1 H), 4.80 (d, J = 12.0 Hz, 1 H), 4.54-4.70 (m, 3 H), 4.25-4.34 (m, 2 H), 3.80-3.89 (m, 3 H), 3.70-3.73 (m, 1 H), 2.59-2.72 (m, 2 H), 1.19 (t, J = 7.3 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 168.1, 167.6, 138.2, 137.7, 134.0, 133.9, 131.6, 128.5, 127.9, 127.9, 127.8, 127.5, 123.5, 123.3, 81.2, 79.7, 78.0, 74.5, 74.2, 73.78, 70.7, 54.5, 24.0, 15.0. HRMS: $[M + Na]^+$ $C_{30}H_{31}NNaO_6S$ calcd for 556.1770, found 556.1760.

Ethyl 2, 3, 4, 6-tetra-O-acetyl- β-D-glucopyranosyl-(1→4)-3, 6-di-O-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (3)

2, 3, 4, 6-Tetra-O-acetyl-β-D-galactosyl bromide (3.35 g, 8.17 mmol) was prepared by following general procedure (D). Then the bromide donor (3.35 g, 8.17 mmol) and 3, 6-di-O-benzyl-2-deoxy- 2-phthalimido-1-thio-β-D-glucopyranoside **9** (2.64 g. 5.43 mmol) were dissolved in a mixture of dry toluene and CH_2Cl_2 (1:1, 30 mL). Powdered molecular sieves (4 Å) were added

and the mixture was stirred under nitrogen for 1 h. The flask was wrapped in aluminum foil and cooled to −45°C. AgOTf (2.79 g, 10.86 mmol) dissolved in dry toluene (20 mL) was added during 1 h under the exclusion of light. After additional stirring for 30 min at −45°C, the reaction mixture was quenched by aqueous $Na_2S_2O_3$. The mixture transferred to a separatory funnel via a Celite-packed glass filter funnel. The organic phase was separated, dried with Na_2SO_4, filtered, and concentrated. Purification of the residue by silica gel column chromatography (Hexanes:EtOAc=4:1) gave compound **3** (5.81 g, 80%). $[\alpha]_D^{20}$ = +31.0 (c 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.77 (d, J = 6.3 Hz, 1 H), 7.62-7.66 (m, 3 H), 7.29-743(m, 5 H), 7.01(d, J = 7.0 H), 6.82-6.92 (m, 3 H), 5.13-5.28 (m, 3 H), 4.78-4.87 (m, 3 H), 4.62 (d, J = 7.9 Hz, 1 H), 4.43-4.51 (m, 2 H), 4.20-4.28 (m, 2 H), 4.09 (t, J = 9.5 Hz, 1 H), 3.89-4.02 (m, 2 H), 3.79 (s, 2 H), 3.64 (t, J = 6.8 Hz, 1 H), 3.56 (d, J = 10.0 Hz, 1 H), 2.55-2.75 (m, 2 H), 2.06 (s, 3 H), 2.02 (s, 6 H), 1.97 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.3, 170.2, 170.0, 169.2, 167.9, 167.4, 138.5, 137.9, 133.9, 133.7, 131.6, 128.6, 128.0, 127.9, 127.9, 127.1, 123.4, 123.3, 100.3, 81.1, 79.1, 77.8, 77.6, 74.5, 73.6, 70.4, 69.5, 67.7, 66.9, 60.7, 54.7, 23.9, 20.8, 20.7, 20.6, 20.6, 14.9. HRMS: $[M + Na]^+$ $C_{44}H_{49}NNaO_{15}S$ calcd for 886.2721, found 886.2729.

Benzyl O-(2, 4-di-O-Benzyl-β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (13)

A mixture of compound **1** (2 g, 2.27 mmol) and 4 Å molecular sieves (2 g) in dry CH_2Cl_2 was stirred at room temperature under nitrogen for 2 h. Triethylsilane (0.69 mL, 4.34 mmol) and $PhBCl_2$ (0.56 mL, 4.34 mmol) were sequentially added at - 78 °C. The reaction mixture was stirred at -78 °C for 2 h and then quenched by the addition of MeOH (2 mL) and Et_3N (2 mL). The resulting mixture was filtered. The filtrate was diluted with CH_2Cl_2 and washed with aqueous $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The residue was purified on a silica gel

column (Hexanes: EtOAc = 5:1) to afford the product **13** (1.70 g, 85%) as white powder. $[\alpha]_D^{20}$ = +8.9 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.35-7.52 (m, 27 H), 7.26-7.34 (m, 3 H), 5.03-5.16 (m, 3 H), 4.78-4.97 (m, 6 H), 4.70-4.75 (m, 2 H), 4.59-4.65 (m, 2 H), 4.52 (d, *J* = 7.0 Hz, 1 H), 4.08 (t, *J* = 8.7 Hz, 1 H), 3.92 (d, *J* = 2.6 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.9, 138.7 (2 C), 138.5, 137.6, 128.6, 128.6, 128.5, 128.5, 128.4, 128.1, 127.9, 127.8, 127.7, 127.6, 102.8, 102.6, 82.8, 81.9, 80.5, 77.0, 75.8, 75.5, 75.3, 75.2 (2 C), 75.1, 74.4, 73.4, 71.1, 68.4, 61.7. HRMS: $[M + Na]^+$ $C_{54}H_{58}NaO_{11}$ calcd for 905.3877, found 905.3867.

Benzyl O-(3, 4, 6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→3)-[3, 4, 6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl]-(1→6)-(2, 4-di-O-benzyl-β-D-galac-topyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (14)

3, 4, 6-Tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucosyl bromide was prepared by following the general procedure (D). Powdered molecular sieves (4 Å) (3.0 g) was added to a solution of above bromide donor (4.80 g, 5.44 mmol) and **13** (800 mg, 0.907 mmol) in anhydrous dichloromethane (20 mL). The suspension was stirred under nitrogen for 1.5 h at room temperature and then cooled to -30°C. Then 2, 4, 6-collidine (0.72 mL, 5.44 mmol), and freshly dried AgOTf (1.40 g, 5.44 mmol) was sequentially added to the reaction mixture. After stirring for 2 h at -30°C, the mixture was allowed to warm up to rt overnight, diluted with CH_2Cl_2, filtered through Celite. The filtrate was diluted with CH_2Cl_2 and washed with aqueous $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The residue was purified on a silica gel column (Hexanes: EtOAc = 5:1) to afford the product **14** (1.33 g, 85%) as white powder. $[\alpha]_D^{20}$ = +6.4 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.85 (t, *J* = 4.01 Hz, 1 H), 7.72-7.74 (m, 2 H), 7.43-7.56 (m, 2 H), 7.18-7.40 (m, 27 H), 7.08-7.16 (m, 3 H), 6.97 (m, 2 H), 5.76-5.87 (m, 2 H), 5.62 (d, *J* = 8.24 Hz, 1 H), 5.39(d, *J* = 8.43, 1H), 5.16-5.24 (m, 2 H), 4.85-4.93 (m, 4 H), 4.75 (d, *J* = 10.6 Hz, 1 H), 4.55-4.66 (m, 3

H), 4.35-4.44 (m, 5 H), 4.19-4.33 (m, 5 H), 4.12 (d, *J* = 12.7 Hz, 1 H), 3.91 (t, *J* = 10.1 Hz, 1 H), 3.71-3.86 (m, 5 H), 3.47-3.59 (m, 5 H), 3.33-3.47 (m, 2 H), 3.21 (t, *J* = 5.9 Hz, 1 H), 3.05(dd, *J* = 3.2, 9.7 Hz, 1 H), 2.09 (s, 3 H), 2.09 (s, 3 H), 2.02 (s, 3 H), 1.98 (s, 3 H), 1.87(d, 6 H); ^{13}C NMR ($CDCl_3$, 100 MHz): *δ* 170.7, 170.6, 170.1, 170.0, 169.5, 169.5, 139.1, 139.0, 138.6, 138.5, 138.4, 137.7, 134.4, 134.0, 128.4, 128.3, 128.2, 128.2, 128.1, 128.0, 127.8, 127.6, 127.5, 127.4, 127.4, 127.1, 127.0, 126.9, 126.5, 102.4, 102.3, 99.0, 97.5, 83.1, 81.7, 81.7, 78.6, 76.2, 76.1, 75.6, 75.2, 75.0, 74.9, 74.1, 73.1, 72.8 (2 C), 71.7, 71.6, 70.8, 70.8 (2 C), 70.5, 68.9, 68.8, 68.0, 66.8, 61.7, 61.6, 55.1, 54.7, 20.8, 20.7, 20.7, 20.6, 20.5, 20.4. HRMS: $[M + Na]^+$ $C_{94}H_{96}N_2NaO_{29}$ calcd for 1739.5996, found 1739.5980.

Benzyl O-(3, 4, 6-tri-O-acetyl-2-deoxy-acetamido-β-D-glucopyranosyl)-(1→3)-[3, 4, 6-tri-O-acetyl-2-deoxy-acetamido-β-D-glucopyranosyl]-(1→6)-(2,4-di-O-benzyl-β-D-galactopyr-anosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (15)

Following the general procedure (A) compound **14** (1.16 g, 0.66 mmol) yielded the compound **15** (762mg, 75% over two steps). $[\alpha]_D^{20}$ = -1.9 (c 0.4, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): *δ* 7.25-7.48 (m, 27 H), 7.10-7.18 (m, 3 H), 5.78 (d, *J* = 9.5 Hz, 1 H), 5.01-5.16 (m, 6 H), 4.90-5.00 (m, 3 H), 4.75-4.91 (m, 4 H), 4.60-4.71 (m, 3 H), 4.41-4.55 (m, 4 H), 4.29 (d, *J* = 3.5 Hz, 1 H), 3.96-4.15 (m, 5 H), 3.64-3.86 (m, 8 H), 3.52-3.64 (m, 4 H), 3.45-3.51 (m, 1 H), 3.23 (d, *J* = 9.7 Hz, 1 H), 2.07 (s, 3 H), 2.06 (s, 3 H), 2.05 (s, 3 H), 2.00 (s, 3 H), 1.99 (s, 3 H), 1.98 (s, 3 H), 1.51 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): *δ* 170.9, 170.8, 170.6, 170.5, 170.3, 169.8, 169.3, 169.2, 139.4, 138.9, 138.8, 138.6, 137.9, 137.4, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 126.2, 102.6, 102.5, 102.4, 101.4, 84.3, 82.4, 82.1, 79.5, 76.7, 76.4, 76.2, 75.2, 74.9, 74.7, 74.5, 74.4, 73.3, 73.2, 72.7, 72.0, 70.9, 70.6, 68.8, 68.5, 67.9,

67.5, 62.0, 61.6, 54.4, 53.7, 23.6, 22.8, 20.8, 20.8, 20.7, 20.6 (3 C). HRMS: $[M + Na]^+$ $C_{82}H_{96}N_2NaO_{27}$ calcd for 1563.6098, found 1563.6126.

2-deoxy-acetamido-β-D-glucopyranosyl-(1→3)-[2-deoxy-acetamido-β-D-glucopyranos-yl]-(1→6)- β-D-galactopyranosyl-(1→4)-α,β-D-glucopyranose (HMO1)

Following the general procedure (B) and (C), compound **15** (400 mg, 0.26 mmol) yielded the compound **HMO1** (152 mg, 80% over two steps). ^{1}H NMR (D_2O, 400 MHz): δ 5.15 (d, J = 3.6 Hz, 0.55 H, Glc-1 H-1 of α form), 4.53-4.62 (m, overlap with D_2O, 2.45 H, GlcNAc-1 H-1, GlcNAc-2 H-1 and Glc-1 H-1 of β form), 4.36 (d, J = 7.7 Hz, 1 H, Gal-1 H-1), 4.07(d, J = 2.4 Hz, 1 H), 3.58-3.95 (m, 13 H), 3.45-3.58 (m, 5 H), 3.32-3.45 (m, 4 H), 3.16-3.26 (m, 1 H), 1.99 (s, 3 H), 1.96 (s, 3 H); ^{13}C NMR (D_2O, 100 MHz): δ 174.9, 174.6, 102.9, 102.8 (GlcNAc-1, GlcNAc-2, C-1),101.1 (Gal-1, C-1), 95.7 (Glc-1, C-1 of β form), 91.8 (Glc-1, C-1 of α form), 81.7, 78.8, 75.8 (2 C), 75.6, 73.8, 74.7, 74.3, 73.8, 73.5, 73.4, 69.86, 69.8, 69.6, 68.7, 68.4, 60.7, 60.5, 55.6, 55.5, 22.4, 22.2. HRMS: $[M + Na]^+$ $C_{28}H_{48}N_2NaO_{21}$ calcd for 771.2647, found 771.2520.

Benzyl O-(2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→4)- 3, 6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-(2-O-benzyl-4, 6-O-benzylidene-β-D-galac-topyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (16)

Powdered molecular sieves (4 Å) (3.0 g) was added to a solution of Compound **3** (871 mg, 0.96 mmol) and **1** (652 mg, 0.74 mmol) in anhydrous dichloromethane (20 mL). The suspension was stirred under nitrogen for 1.5 h at room temperature and then cooled to -30°C. Then NIS (260mg, 1.15mmol), and TMSOTf (35 µl, 0.19 mmol) was sequentially added to the reaction mixture. After stirring for 2 h at -30°C, the mixture was allowed to warm up to rt. overnight, diluted with CH_2Cl_2, filtered through Celite. The filtrate was diluted with CH_2Cl_2 and washed with aqueous $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The residue was

purified on a silica gel column (Hexanes: EtOAc = 5:1) to afford the product **16** (1.04 g, 86%) as white powder. $[\alpha]_D^{20}$ = +12.9 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.53-7.55 (m, 2 H), 7.45-7.50 (m, 2 H), 7.27-7.45 (m, 27 H), 7.19 (t, *J* = 3.6 Hz, 2 H), 7.09-7.16 (m, 3 H), 7.02-7.06 (m, 2 H), 6.84-6.94 (m, 5 H), 5.49 (s, 1 H), 5.47 (d, *J* = 7.4 Hz, 1 H), 5.35 (d, *J* = 3.2 Hz, 1 H), 5.25 (dd, *J* = 7.9, 10.4 Hz, 1 H), 5.09 (d, *J* = 10.7 Hz, 1 H), 4.88-4.99 (m, 3 H), 4.85 (d, *J* = 12.0 Hz, 1 H), 4.65-4.78 (m, 4 H), 4.61 (d, *J* = 12.0 Hz, 1 H), 4.44-4.55 (m, 3 H), 4.30-4.39 (m, 4 H), 4.18-4.28 (m, 5 H), 4.09 (t, *J* = 9.3 Hz, 1 H), 3.98-4.04 (m, 2 H), 3.89-3.96 (m, 1 H), 3.80-3.89 (m 2 H), 3.66-3.79 (m, 3 H), 3.53-3.64 (m, 2 H), 3.44-3.51 (m, 3 H), 3.38 (d, *J* = 10.1 Hz, 1 H), 2.93-3.02 (m, 2 H), 2.12 (s, 3 H), 2.10 (s, 3 H), 2.05 (s, 3 H), 2.03 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.3, 170.2, 170.1, 169.2, 139.0, 138.6 (2 C), 138.5, 138.3, 137.8, 137.6, 133.5, 131.3, 128.7, 128.6 (2 C), 128.4, 128.3 (2 C), 128.2, 128.1 (2 C), 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.2, 127.1, 126.7, 126.5, 126.3, 123.1, 102.4 (2 C), 100.7, 100.6, 99.6, 83.1, 81.8, 80.9, 78.4, 77.7, 76.0, 75.7, 75.0, 74.8, 74.7, 74.5, 74.3, 73.8 (2 C), 73.0 (2 C), 71.1, 70.9 (2 C), 70.6, 69.7, 68.8, 68.7, 67.9, 67.0, 66.4, 60.8, 55.8, 20.9, 20.7 (2 C), 20.6. HRMS: $[M + Na]^+$ $C_{96}H_{99}NNaO_{26}$ calcd for 1704.6353, found 1704.6383.

Benzyl O-(2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→4)- 3, 6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-(2-O-benzyl-β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (19)

To a solution of compound **16** (800 mg, 0.49 mmol) in anhydrous MeOH (10 mL) was added TsOH (8.4 mg) and EtSH (0.21 ml, 2.93 mmol). The reaction mixture was stirred at rt. for 6 h and then quenched with triethylamine and evaporated under reduced pressure. The mixture was purified with silica column (Hexanes: Acetone = 5:1) to get white power compound **19** (702 mg, 90%). $[\alpha]_D^{20}$ = +18.4 (c 0.5, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.38-7.52 (m, 11 H),

7.25-7.36 (m, 16 H), 7.22-7.26 (m, 2 H), 7.03-7.14 (m, 3 H), 6.99-7.03 (m, 2 H), 6.85-6.92 (m, 2 H), 6.78 (d, *J* = 7.1 Hz, 2 H), 5.29-5.31 (m, 2 H), 5.19-5.25 (m, 1 H), 4.86-4.98 (m, 4 H), 4.83 (d, *J* = 12.2 Hz, 1 H), 4.68-4.77 (m, 3 H), 4.65 (d, *J* = 8.3 Hz, 1 H), 4.57-4.62 (m, 2 H), 4.46 (t, *J* = 12.4 Hz, 2 H), 4.34-4.40 (m, 1 H), 4.26-4.34 (m, 3 H), 4.17-4.26 (m, 3 H), 3.95-4.08 (m, 3 H), 3.81-3.88 (m, 1 H), 3.72-3.79 (m, 3 H), 3.64-3.72 (m, 2 H), 3.47-3.55 (m, 2 H), 3.34-3.46 (m, 5 H), 3.17-3.23 (m, 1 H), 3.00-3.06 (m, 1 H), 2.87 (s, 1 H), 2.11 (s, 3 H), 2.08 (s, 3 H), 2.05 (s, 3 H), 2.02 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.3, 170.2, 170.1, 169.2, 138.9, 138.4, 138.3 (2 C), 137.6, 137.5, 133.6, 131.1, 128.8, 128.4, 128.3 (3 C), 128.2 (2 C), 128.1, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.2, 126.7, 126.4, 123.2, 102.4, 102.1, 100.6, 99.0, 83.8, 82.8, 81.5, 78.3, 77.9, 76.6, 76.4, 75.6, 74.9, 74.6, 74.3, 73.8, 73.7, 73.1, 71.0, 70.9, 70.7, 69.6, 68.3, 67.9, 67.7, 66.9, 62.2, 60.8, 55.6, 20.8, 20.68, 20.6, 20.6. HRMS: $[M + Na]^+$ $C_{89}H_{95}NNaO_{26}$ calcd for 1616.6040, found 1616.6065.

Benzyl O-(2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→4)- 3, 6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3))-[3, 4, 6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl]-(1→6)-(2-O-benzyl-β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (20)

3, 4, 6-Tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucosyl bromide was prepared by following the general procedure (D). Powdered molecular sieves (4 Å) (3.0 g) was added to a solution of **19** (460 mg, 0.29 mmol)**,** 2, 4, 6-collidine (76 µL, 0.58 mmol), and freshly dried AgOTf (150 mg, 0.58 mmol) in anhydrous dichloromethane (20 mL). The suspension was stirred under nitrogen for 1.5 h at room temperature and then cooled to -30°C. Then a solution of above bromide donor (460 mg, 0.29 mmol) in dichloromethane (5.0 mL) was added dropwise during 30 min to the reaction mixture. After stirring for 2 h at -30°C, the mixture was allowed to warm up to rt.

overnight, diluted with CH_2Cl_2, filtered through Celite. The filtrate was diluted with CH_2Cl_2 and washed with aqueous $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The residue was purified on a silica gel column (Hexanes: EtOAc = 5:1) to afford the product **20** (495 mg, 85%) as white powder. $[\alpha]_D^{20}$ = +11.7 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.76-7.91 (m, 4 H), 7.49-7.56 (m, 2 H), 7.41-7.49 (m, 8 H), 7.14-7.20 (m, 2 H), 7.05-7.11 (m, 1 H), 6.97-7.04 (m, 4 H), 6.85-6.94 (m, 3 H), 6.75 (d, *J* = 7.4 Hz, 2 H), 5.73 (dd, *J* = 9.1, 10.61 Hz, 1 H), 5.43 (d, *J* = 8.3 Hz, 1 H), 5.33 (d, *J* = 3.5 Hz, 1 H), 5.12-5.25 (m, 2 H), 4.99 (d, *J* = 8.9 Hz, 1 H), 4.77-4.95 (m, 3 H), 4.72-4.84 (m, 3 H), 4.61-4.71 (m, 2 H), 4.48-4.58 (m, 2 H), 4.33-4.47 (m, 3 H), 4.09-4.31 (m, 8 H), 3.80-4.06 (m, 6 H), 3.65-3.80 (m, 5 H), 3.62 (d, *J* = 10.0 Hz, 1 H), 3.50-3.56 (m, 2 H), 3.36-3.45 (m, 3 H), 3.20-3.27 (m, 2 H), 3.09-3.17 (m, 2 H), 2.87 (s, 1 H), 2.14 (s, 3 H), 2.11 (s, 3 H), 2.09 (s, 3 H), 2.04 (s, 3 H), 2.02 (s, 3 H), 1.99 (s, 3 H), 1.87(s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.7, 170.3, 170.2, 170.0 (2 C), 169.5, 169.5, 139.1, 138.7, 138.4, 138.3 (2 C), 137.7, 137.6, 131.2, 128.8, 128.4, 128.3 (3 C), 128.2, 128.1, 127.9 (2 C), 127.8, 127.7, 127.6 (2 C), 127.4, 127.2, 126.5, 123.2, 102.4, 101.9, 100.7, 98.5, 97.6, 83.8, 83.1, 81.9, 78.4, 77.8, 76.5, 76.3, 75.7, 75.1, 74.9, 74.5, 74.4, 74.4, 73.9, 72.9 (2 C), 72.0, 71.7, 71.1, 71.0, 70.8, 70.6, 69.5, 68.8, 68.1, 67.6, 66.9, 66.6, 61.7, 60.8, 55.4, 54.7, 20.9, 20.8, 20.67, 20.6 (3 C), 20.5. HRMS: $[M + Na]^+$ $C_{109}H_{114}N_2NaO_{35}$ calcd for 2033.7100, found 2033.7089.

Benzyl O-(2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→4)- 3, 6-di-O-benzyl-2-deoxy-acetamido-β-D-glucopyranosyl-(1→3)-[3, 4, 6-tri-O-acetyl-2-deoxy-acetamido-β-D-glucopy-ranosyl]-(1→6)-(4-O-acetyl-2-O-benzyl-β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (21)

Following the general procedure (A), compound **20** (400 mg, 0.20 mmol) yielded the compound **21** (232 mg, 62% over two steps). $[\alpha]_D^{20}$ = -1.0 (c 1.2, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400

MHz): δ 7.51 (m, 2H), 7.21-7.43 (m, 33 H), 5.89 (d, J = 9.5 Hz , 1 H), 5.43 (d, J = 8.4 Hz, 1 H), 5.33 (d, J = 2.8 Hz, 1 H), 4.33-5.24 (m, 23 H), 4.24 (d, J = 8.2 Hz, 1 H), 3.94-4.10 (m, 6 H), 3.45-3.86 (m, 15 H), 3.23 (d, J = 9.7 Hz, 1 H), 2.12 (s, 3 H), 2.11 (s, 3 H), 2.05 (s, 3 H), 2.04 (s, 3 H), 2.01 (s, 3 H), 2.00 (s, 3 H), 1.99 (s, 3 H), 1.97 (s, 3 H), 1.94 (s, 3 H), 1.54 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz):δ 170.8, 170.7, 170.4, 170.3, 170.2, 170.0, 170.0 (2 C), 169.7, 169.2, 139.4, 138.7, 138.6 (2 C), 138.0, 137.7, 137.4, 128.7, 128.6, 128.4, 128.4 (2 C), 128.3, 128.2, 128.1, 128.0, 127.9 (2 C), 127.8, 127.7, 127.6, 127.0, 102.6, 102.3, 101.1, 100.7, 100.0, 83.8, 82.4, 79.5, 78.3, 77.9, 76.2 (2 C), 75.6, 75.0, 74.9, 74.6, 74.4, 73.9, 73.5, 73.3, 73.2 (2 C), 73.2, 70.9, 70.8, 70.7, 70.6, 70.0, 69.4, 68.3, 68.0, 67.9, 67.9, 67.3, 66.9, 61.5, 60.8, 54.5, 53.6, 20.9, 20.8, 20.7 (3 C), 20.6 (3 C), 20.5 (2 C). HRMS: $[M + Na]^+$ $C_{99}H_{116}N_2NaO_{34}$ calcd for 1899.7307, found 1899.7287.

β-D-galactopyranosyl-(1→4)-2-deoxy-acetamido-β-D-glucopyranosyl-(1→3)-[2-deoxy-acetamido-β-D-glucopyranosyl]-(1→6)- β-D-galactopyranosyl-(1→4)-α,β-D-glucopyranose (HMO2)

Following the general procedure (B) and (C), compound **21** (200 mg, 0.11 mmol) yielded the compound **HMO2** (87 mg, 90%). ^{1}H NMR (D_2O, 400 MHz): δ 5.11 (d, J = 3.6 Hz, 0.42 H, Glc-1 H-1 of α form), 4.53-4.62 (m, 1.58 H, GlcNAc-1 H-1, Glc-1 H-1 of β form), 4.50 (d, J = 8.4 Hz, 1 H, GlcNHAC-2 H-1), 4.36 (d, J = 7.9 Hz, 1 H, Gal-1 H-1), 4.31 (d, J = 8.0 Hz, 1 H, Gal-2 H-1), 4.03 (d, J = 3.0 Hz, 1 H), 3.77-3.92 (m, 5 H), 3.40-3.77 (m, 21 H), 3.30-3.38 (m, 2 H), 3.17 (t, J = 8.9 Hz, 1 H), 1.95 (s, 3 H), 1.92 (s, 3 H); ^{13}C NMR (D_2O, 100 MHz): δ 174.9, 174.5, 103.0, 102.8, 102.7 (GlcNAc-1, GlcNAc-2, Gal-2, C-1), 101.0 (Gal-1, C-1), 95.7 (Glc-1, C-1 of β form), 91.8 (Glc-1, C-1 of α form), 81.8, 78.9, 78.8, 78.1, 75.8, 75.3, 74.7, 74.5, 74.3,

73.8, 73.4, 72.5, 72.1, 71.4, 71.2, 70.9, 69.6, 68.5, 68.4, 61.0, 60.1, 60.0, 59.8, 55.5, 55.2, 22.4, 22.2. HRMS: $[M + Na]^+$ $C_{34}H_{58}N_2NaO_{26}$ calcd for 933.3175, found 933.3195.

Benzyl O-(3, 4, 6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→3)-(2-O-benzyl-4, 6-O-benzylidene-β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopy-ranoside (22)

3, 4, 6-Tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucosyl bromide was prepared by following the general procedure (D). Powdered molecular sieves (4 Å) (2.0 g) was added to a solution of above bromide donor **2** (1.2 g, 2.43 mmol) and **1** (500 mg, 0.61 mmol) in anhydrous dichloromethane (20 mL). The suspension was stirred under nitrogen for 1.5 h at room temperature and then cooled to -30°C. Then 2, 4, 6-collidine (0.32 mL, 2.43 mmol), and freshly dried AgOTf (624 mg, 2.43 mmol) was sequentially added to the reaction mixture. After stirring for 2 h at -30°C, the mixture was allowed to warm up to rt overnight, diluted with CH_2Cl_2, filtered through Celite. The filtrate was diluted with CH_2Cl_2 and washed with aqueous $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The residue was purified on a silica gel column (Hexanes: Acetone = 6:1) to afford the product **22** (626 mg, 85%) as white powder. $[\alpha]_D^{20}$ = +12.6 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.30-7.63 (m, 26 H), 7.22-7.27 (m, 3 H), 7.16-7.21 (m, 3 H), 6.89-6.96 (m, 2 H), 5.90 (dd, *J* = 9.4, 10.65 Hz, 1 H), 5.76 (d, *J* = 8.3 Hz, 1 H), 5.59 (s, 1 H), 5.29 (t, *J* = 9.7 Hz, 1 H), 5.17 (d, *J* = 10.6 Hz, 1 H), 4.92-4.99 (m, 2 H), 4.72-4.82 (m, 2 H), 4.63 (t, *J* = 11.6 Hz, 1 H), 4.54 (dd, *J* = 8.3, 10.8 Hz, 1 H), 4.25-4.47 (m, 9 H), 3.93-4.05 (m, 3 H), 3.61-3.73 (m, 2 H), 3.49-3.60 (m, 3 H), 3.44 (d, *J* = 11.3 Hz, 1 H), 3.14 (s, 1 H), 2.98-3.04 (m, 1 H), 2.13 (s, 3 H), 2.12 (s, 3 H), 1.90 (s, 3 H); ^{13}C NMR (D_2O, 100 MHz): δ 170.5, 170.2, 169.6, 139.0, 138.7, 138.6, 138.5, 138.3, 137.6, 134.2, 128.7, 128.4, 128.4, 128.3, 128.2, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 127.3, 126.9, 126.4, 123.4, 102.4 (2 C), 100.9, 99.3, 83.1, 81.8, 81.3, 76.9, 75.9, 75.8, 75.1,

74.8, 74.3, 73.1, 71.9, 71.0, 70.8, 69.0, 68.9, 67.9, 66.4, 62.2, 54.8, 53.7, 20.9, 20.8, 20.5. HRMS: $[M + Na]^+$ $C_{74}H_{75}NNaO_{20}$ calcd for 1320.4780, found 1320.4806.

Benzyl O-(3, 4, 6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→3)-(2-O-benzyl-β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (23)

To a solution of compound **22** (602 mg, 0.46 mmol) in anhydrous MeOH (10 mL) was added TsOH (16.0 mg) and EtSH (0.21 mL, 2.93 mmol). The reaction mixture was stirred at rt. for 6 hours then quenched with triethylamine and evaporated under reduced pressure. The mixture was purified with silica column (Hexanes: Acetone=5:1) to get white power compound **23** (445 mg, 80%). $[\alpha]_D^{20}$ = +17.7 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.51-7.66 (m, 4 H), 7.41-7.45 (m, 2 H), 7.25-7.40 (m, 18 H), 7.06-7.17 (m, 3 H), 6.83 (d, *J* = 6.7 Hz, 2 H), 5.86 (dd, *J* = 9.1, 10.6 Hz, 1 H), 5.65 (d, *J* = 8.5 Hz, 1 H), 5.18 (t, *J* = 9.4 Hz, 1 H), 4.88-4.96 (m, 3 H), 4.69-4.76 (m, 2 H), 4.62 (d, *J* = 12.0 Hz, 1 H), 4.43-4.56 (m, 2 H), 4.37-4.41 (m, 1 H), 4.23-4.32 (m, 9 H), 3.95-4.03 (m, 3 H), 3.87-3.93 (m, 1 H), 3.68-3.75 (m, 1 H), 3.52-3.66 (m, 2 H), 3.38-3.50 (m, 5 H), 3.23-3.29 (m, 1 H), 3.03-3.08 (m, 1 H), 2.83 (d, J=1.5 Hz, 1 H), 2.15 (s, 3 H), 2.09 (s, 3 H), 1.88 (s, 3 H); ^{13}C NMR (D_2O, 100 MHz): δ 170.8, 170.1, 169.5, 138.8, 138.6, 138.4, 138.3, 134.3, 128.4 (2 C), 128.3, 128.2, 128.2, 128.0, 127.9, 127.8, 127.7 (2 C), 127.6, 127.5, 126.8, 126.3, 123.5, 102.4, 102.1, 98.7, 84.3, 82.8, 81.7, 77.8, 76.3, 75.7, 75.0, 74.7, 74.3, 73.9, 73.1, 72.1, 70.9, 70.5, 69.0, 68.0, 67.7, 62.2, 61.9, 54.6, 20.8, 20.7, 20.4. HRMS: $[M + Na]^+$ $C_{67}H_{71}NNaO_{20}$ calcd for 1232.4467, found 1232.4490.

Benzyl O-(3, 4, 6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→3)-[2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→4)-3, 6-di-O-benzyl-2-deoxy-acetamido-β-D-glucopyranosyl]-(1→6)-(2-O-benzyl-β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl-β-D-glu-copyranoside (24)

Powdered molecular sieves (4 Å) (1.0 g) was added to a solution of Compound **3** (230 mg, 0.27mmol) and **23** (261 mg, 0.21 mmol) in anhydrous dichloromethane (10 mL). The suspension was stirred under nitrogen for 1.5 hours at room temperature and then cooled to -30°C. Then NIS (61 mg, 0.27 mmol), and AgOTf (35 mg, 0.105 mmol) was sequentially added to the reaction mixture. After stirring for 2 h at -30°C, the mixture was allowed to warm up to rt. overnight, diluted with CH_2Cl_2, filtered through Celite. The filtrate was diluted with CH_2Cl_2 and washed with aqueous $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The residue was purified on a silica gel column (Hexanes: Acetone = 5:1) to afford the product **24** (295 mg, 70%) as white powder. $[\alpha]_D^{20}$ = +13.0 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.76-8.08 (m, 5 H), 7.49-7.72 (m, 3 H), 7.17-7.48 (m, 25 H), 6.96-7.11 (m, 5 H), 6.87-6.95 (m, 3 H), 6.74 (d, *J* = 7.3 Hz, 2 H), 5.55-5.61 (dd, *J* = 9.3, 10.37 Hz, 1 H), 5.30 (d, *J* = 3.3 Hz, 1 H), 5.11-5.20 (m, 2 H), 5.04 (d, *J* = 8.5 Hz, 1 H), 4.69-4.95 (m, 9 H), 4.42-4.66 (m, 6 H), 4.21-4.39 (m, 6 H), 4.03-4.15 (m, 5 H), 3.91-4.02 (m, 3 H), 3.70-3.88 (m, 4 H), 3.50-3.68 (m, 4 H), 3.23-3.46 (m, 8 H), 3.00-3.07 (m, 1 H), 2.60-2.69 (m, 1 H), 2.11 (s, 3 H), 2.09 (s, 3 H), 2.08 (s, 3 H), 2.07 (s, 3 H), 2.03 (s, 3 H), 2.00 (s, 3 H), 1.85 (s, 3 H); ^{13}C NMR (D_2O, 100 MHz): δ 171.2, 170.4, 170.3, 170.1, 170.0, 169.2, 169.2, 138.8, 138.7, 138.5, 138.3, 138.2, 137.7, 137.6, 128.7, 128.5, 128.4, 128.3, 128.2 (2 C), 128.0 (2 C), 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 126.6, 126.2, 102.5, 101.8, 100.3, 97.8, 97.4, 84.6, 82.9, 81.8, 77.9 (2 C), 77.3, 76.5, 76.1, 75.7, 75.0, 74.8, 74.7, 74.5, 74.4, 73.7, 73.1, 71.4, 71.0, 70.9, 70.4, 70.1, 70.0, 69.5, 69.4, 69.2, 67.8, 67.2, 66.9, 65.5, 64.5, 62.2, 60.7, 55.6, 54.1, 20.9, 20.7, 20.7, 20.6 (3 C), 20.4. HRMS: $[M + Na]^+$ $C_{109}H_{114}N_2NaO_{35}$ calcd for 2033.7100, found 2033.7129.

Benzyl O-(3, 4, 6-tri-O-acetyl-2-deoxy-acetamido-β-D-glucopyranosyl)-(1→3)-[2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→4)-3, 6-di-O-benzyl-2-deoxy-acetamido-β-

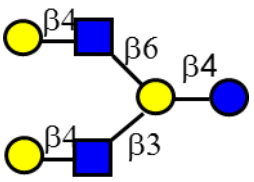
D-gluc-opyranosyl]-(1→6)-(4-O-acetyl-2-O-benzyl-β-D-galactopyranosyl)-(1→4)-2, 3, 6-tri-O-benzyl- β-D-glucopyranoside (25)

Following the general procedure (A), compound **24** (400 mg, 0.20 mmol) yielded the compound **25** (183 mg, 70% over two steps). $[\alpha]_D^{20}$ = -2.0 (c 1.0, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.16-7.51 (m, 35 H), 5.20-5.36 (m, 2 H), 4.97-5.20 (m, 6 H), 4.89-4.97 (m, 2 H), 4.69-4.89 (m, 8 H), 4.56-4.69 (m, 4 H), 4.29-4.56 (m, 7 H), 4.08-4.26 (m, 2 H), 3.41-4.04 (m, 20 H), 3.32-3.40 (m, 1 H), 3.23-3.31 (m, 1 H), 3.08-3.19 (d, J = 8.6 Hz, 1 H), 2.10 (s, 3 H), 2.09 (s, 3 H), 2.08 (s, 3 H), 2.04 (s, 3 H), 2.01 (s, 3 H), 1.98 (s, 9 H), 1.92 (s, 3 H), 1.57 (s, 3 H); ^{13}C NMR (D_2O, 100 MHz): δ 171.0, 170.9, 170.6 (2 C), 170.2, 170.0, 169.8, 169.7, 169.3 (2 C), 139.1, 139.0, 138.7, 138.5, 138.2, 137.9, 137.4, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7 (2 C), 127.6, 127.5, 126.6, 102.4, 102.0, 101.1, 100.0, 99.8, 82.9, 82.0, 79.9, 78.4, 78.2, 77.4, 76.4, 75.5, 75.0, 74.8, 74.1, 73.7, 73.6, 73.3, 72.7, 72.3, 71.7, 71.0, 70.9, 70.4, 69.5, 68.3, 67.9, 66.9, 66.7, 61.5, 60.8, 55.2, 54.3, 53.9, 20.9, 20.8 (2 C), 20.7 (2 C), 20.6 (3 C), 20.50 (2 C). HRMS: $[M + Na]^+$ $C_{99}H_{116}N_2NaO_{34}$ calcd for 1899.7307, found 1899.7357.

2-deoxy-acetamido-β-D-glucopyranosyl (1→3)-[β-D-galactopyranosyl-(1→4)-2-deoxy-acetamido-β-D-glucopyranosyl]-(1→6)-β-D-galactopyranosyl-(1→4)-α,β-D-glucopyranose (HMO3)

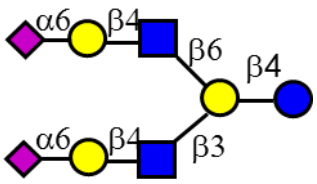
Following the general procedure (B) and (C), compound **25** (183 mg, 0.098 mmol) yielded the compound **HMO3** (85 mg, 95%). ^{1}H NMR (D_2O, 400 MHz): δ 5.14 (d, J = 3.8 Hz, 0.54 H, Glc-1 H-1 of α form), 4.54-4.63 (m, overlap with D_2O, 2.46 H, GlcNAc-1, GlcNAc-2, H-1, Glc-1 H-1 of β form), 4.39 (d, J = 7.8 Hz, Gal-1, 1 H), 4.35 (d, J = 7.8 Hz, Gal-1, 1 H), 4.06 (d, J = 3.1 Hz, 1 H), 3.82-3.95 (m, 4 H), 3.42-3.82(m, 22 H), 3.33-3.42 (m, 2 H), 3.21 (t, J = 8.39 Hz, 1 H), 1.98 (s, 3 H), 1.96 (s, 3 H); ^{13}C NMR (D_2O, 100 MHz): δ 174.9, 174.5, 103.0, 102.9,

102.8 (GlcNAc-1, GlcNAc-2, Gal-2, C-1), 101.0 (Gal-1, C-1), 95.7 (Glc-1, C-1 of β form), 91.8 (Glc-1, C-1 of α form), 81.7, 79.0, 78.9, 78.4, 75.7, 75.3, 74.7, 74.7, 74.4, 73.9, 73.6, 73.5, 72.5, 72.4, 71.0, 69.9, 69.7, 68.7, 68.6, 68.4, 61.0, 60.5, 60.0, 55.7, 55.0, 22.4, 22.2. HRMS: $[M + Na]^+$ $C_{34}H_{58}N_2NaO_{26}$ calcd for 933.3175, found 933.3161.



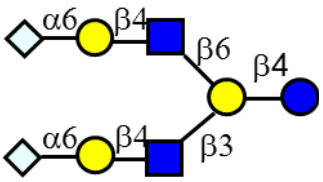
HMO11

^{1}H NMR (D_2O, 500 MHz): δ 5.10(d, J = 3.5 Hz, 0.61 H, Glc-1 H-1 of α form), 4.50-4.60 (m, 2.39 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.33-4.37 (m, 2 H, Gal-2 H-1, Gal-3 H-1), 4.31 (d, J = 7.9 Hz, 1 H, Gal-1 H-1), 4.02 (d, J = 3.1 Hz, 1 H), 3.78-3.79 (m, 6 H), 3.57-3.76 (m, 18 H), 3.53-3.57 (m, 2 H), 3.39-3.51 (m, 7 H), 3.17 (t, J = 8.3 Hz, 1 H), 1.94 (s, 3 H), 1.91 (s, 3 H). MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{31}$ calcd for 1095.370, found 1095.390.



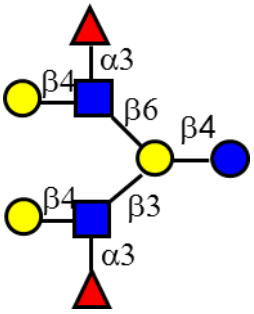
HMO12

^{1}H NMR (D_2O, 500 MHz): δ 5.10 (d, J = 3.5 Hz, 0.51 H, Glc-1 H-1 of α form), 4.50-4.58 (m, 2.49 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.36 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.03 (d, J = 3.0 Hz, 1 H), 3.38-3.91 (m, 47H), 3.17 (t, J = 8.3 Hz, 1 H), 2.52-2.57 (m, 2 H), 1.97 (s, 3 H), 1.93 (s, 3 H), 1.91 (s, 6 H), 1.55-1.63 (m, 2 H). ESI-MS: $[M - 2H]^{2-}$ $C_{62}H_{100}N_4O_{47}$ calcd for 826.2784, found 826.2760.



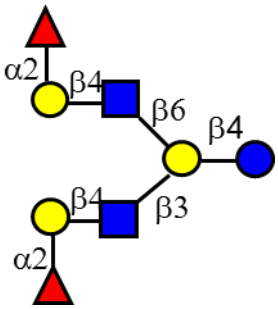
HMO13

^{1}H NMR (D_2O, 500 MHz): δ 5.09 (d, J = 3.7 Hz, 0.29 H, Glc-1 H-1 of α form), 4.50-4.63 (m, 2.71 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.36 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.02 (d, J = 3.0 Hz, 1 H), 3.99 (s, 4 H), 3.38-3.91 (m, 48 H), 3.18 (t, J = 8.3 Hz, 1 H), 2.52-2.60 (m, 2 H), 1.97 (s, 3 H), 1.93 (s, 3 H), 1.55-1.65 (m, 2 H). ESI-MS: $[M - 2H]^{2-}$ $C_{62}H_{100}N_4O_{49}$ calcd for 842.2734, found 842.2760.



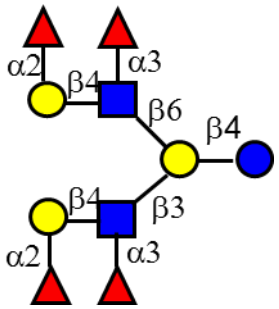
HMO14

^{1}H NMR (D_2O, 500 MHz): δ 5.10 (d, J = 3.5 Hz, 0.33 H, Glc-1 H-1 of α form), 4.96-5.02 (m, 2 H, Fuc-1 H-1, Fuc-2 H-1), 4.50-4.61 (m, 2.67 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.36 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.01 (d, J = 3.1 Hz, 1 H), 3.63-3.91 (m, 25 H), 3.40-3.63 (m, 21 H), 3.35-3.39 (m, 2 H), 3.17 (t, J = 8.4 Hz, 1 H), 1.90-1.93 (d, J = 6.5 Hz, 6 H). MALDI-MS: $[M + Na]^{+}$ $C_{52}H_{88}N_2NaO_{39}$ calcd for 1387.487, found 1387.486.



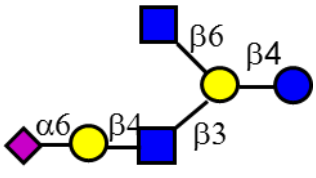
HMO15

^{1}H NMR (D_2O, 500 MHz): δ 5.15-5.22 (m, 2 H, Fuc-1 H-1, Fuc-2 H-1), 5.10(d, J = 3.5 Hz, 0.33 H, Glc-1 H-1 of α form), 4.45-4.61 (m, 2.67 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.39-4.45 (m, 2 H, Gal-2 H-1, Gal-3 H-1), 4.30 (d, J = 7.9 Hz, 1 H, Gal-1 H-1), 4.06-4.13 (m, 2 H), 4.01 (d, J = 3.1 Hz, 1 H), 3.41-3.91 (m, 44 H), 3.30-3.39 (m, 2 H), 3.16 (t, J = 8.4 Hz, 1 H), 1.93 (s, 3 H), 1.91 (s, 3 H), 1.09-1.11 (d, J = 6.7 Hz, 6 H). MALDI-MS: $[M + Na]^+$ $C_{52}H_{88}N_2NaO_{39}$ calcd for 1387.487, found 1387.490.



HMO16

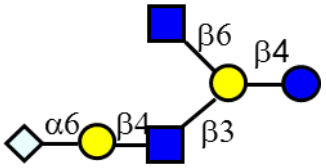
^{1}H NMR (D_2O, 500 MHz): δ 5.12-5.18(m, 2 H, Fuc-2 H-1, Fuc-4 H-1), 5.10 (d, J = 3.5 Hz, 0.40 H, Glc-1 H-1 of α form), 4.93-5.01 (m, 2 H, Fuc-1 H-1, Fuc-3 H-1), 4.70-4.78 (m, 2 H), 4.45-4.61 (m, 2.60 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.35-4.41 (m, 2 H, Gal-2 H-1, Gal-3 H-1), 4.31 (d, J = 7.9 Hz, 1 H, Gal-1 H-1), 4.09-4.16 (m, 2 H), 4.00 (d, J = 3.1 Hz, 1 H), 3.41-3.91 (m, 44 H), 3.28-3.38 (m, 2 H), 3.16 (t, J = 8.4 Hz, 1 H), 1.93 (s, 3 H), 1.90 (s, 3 H), 1.10-1.15 (m, 12 H). MALDI-MS: $[M + Na]^+$ $C_{64}H_{108}N_2NaO_{47}$ calcd for 1679.602, found 1679.607.



HMO21

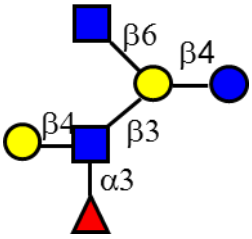
^{1}H NMR (D_2O, 500 MHz): δ 5.09 (d, J = 3.6 Hz, 0.22 H, Glc-1 H-1 of α form), 4.45-4.61 (m, 2.78 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.26-4.36 (m, 2 H, Gal-1

H-1, Gal-2 H-1), 4.17-4.22 (m, 1 H), 4.09-4.16 (m, 2 H), 4.00 (d, *J* = 3.1 Hz, 1 H), 3.36-3.90 (m, 32 H), 3.26-3.36 (m, 2 H), 3.16 (t, *J* = 8.4 Hz, 1 H), 2.50-2.56 (m, 1 H), 1.92 (s, 3 H), 1.91 (s, 3 H), 1.89 (s, 3 H), 1.58 (t, *J* = 12.3 Hz, 1 H). ESI-MS: $[M - H]^-$ $C_{45}H_{74}N_3O_{34}$ calcd for 1200.4159, found 1200.4190.



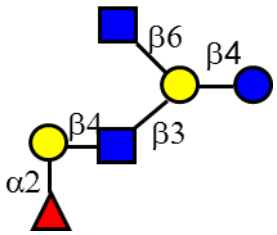
HMO22

^{1}H NMR (D_2O, 500 MHz): δ 5.09 (d, *J* = 3.6 Hz, 0.27 H, Glc-1 H-1 of α form), 4.47-4.62 (m, 2.73 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.35 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.06-4.16 (m, 1 H), 4.01 (d, *J* = 3.1 Hz, 1 H), 3.99 (s, 2 H), 3.38-3.90 (m, 33 H), 3.29-3.37 (m, 2 H), 3.16 (t, *J* = 8.4 Hz, 1 H), 2.53-2.58 (m, 1 H), 1.93 (s, 3 H), 1.92 (s, 3 H), 1.60 (t, *J* = 12.3 Hz, 1 H). ESI-MS: $[M - H]^-$ $C_{45}H_{74}N_3O_{35}$ calcd for 1216.4108, found 1216.4129.



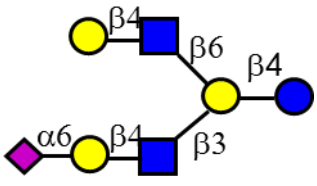
HMO23

^{1}H NMR (D_2O, 500 MHz): δ 5.09 (d, *J* = 3.6 Hz, 0.36 H, Glc-1 H-1 of α form), 5.00 (d, *J* = 3.9 Hz, 1 H, Fuc-1 H-1), 4.46-4.60 (m, 2.64 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.36 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.02 (d, *J* = 3.1 Hz, 1 H), 3.40-3.90 (m, 31 H), 3.29-3.40 (m, 2 H), 3.16 (t, *J* = 8.4 Hz, 1 H), 2.50-2.56 (m, 1 H), 1.94 (s, 3 H), 1.90 (s, 3 H), 1.05 (d, *J* = 6.7 Hz, 3 H). MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{30}$ calcd for 1079.376, found 1079.371.



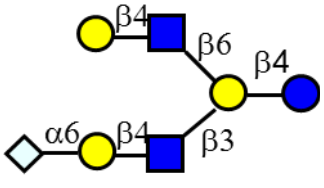
HMO24

^{1}H NMR (D_2O, 500 MHz): δ 5.19 (d, J = 2.6 Hz, 1 H, Fuc-1 H-1), 5.09 (d, J = 3.6 Hz, 0.50 H, Glc-1 H-1 of α form), 4.46-4.60 (m, 2.50 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.42 (d, J = 7.8 Hz, 1 H, Gal-2 H-1), 4.31 (d, J = 8.0 Hz, 1 H, Gal-1 H-1), 4.07-4.12 (m, 1 H), 4.01 (d, J = 3.1 Hz, 1 H), 3.41-3.90 (m, 28 H), 3.29-3.38 (m, 3 H), 3.16 (t, J = 8.4 Hz, 1 H), 1.94 (s, 3 H), 1.91 (s, 3 H), 1.10 (d, J = 6.5 Hz, 3 H). MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{30}$ calcd for 1079.376, found 1079.379.



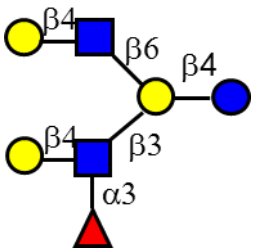
HMO25

^{1}H NMR (D_2O, 500 MHz): δ 5.09(d, J = 3.9 Hz, 0.28 H, Glc-1 H-1 of α form), 4.48-4.62 (m, 2.72 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.37 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.01 (d, J = 3.1 Hz, 1 H), 3.38-3.90 (m, 41 H), 3.17 (t, J = 8.4 Hz, 1 H), 2.50-2.57 (m, 1 H), 1.93 (s, 3 H), 1.92 (s, 3 H), 1.90 (s, 3 H), 1.59 (t, J = 11.9 Hz, 1 H). ESI-MS: $[M - H]^-$ $C_{51}H_{84}N_3O_{39}$ calcd for 1362.4687, found 1362.4759.



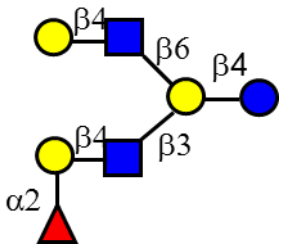
HMO26

^{1}H NMR (D_2O, 500 MHz): δ 5.10 (d, J = 3.7 Hz, 0.51 H, Glc-1 H-1 of α form), 4.50-4.63 (m, 2.49 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.29-4.37 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.02 (d, J = 2.9 Hz, 1 H), 3.99 (s, 2 H), 3.39-3.90 (m, 41 H), 3.17 (t, J = 8.3 Hz, 1 H), 2.54-2.59 (m, 1 H), 1.94 (s, 3 H), 1.93 (s, 3 H), 1.61 (t, J = 12.2 Hz, 1 H). ESI-MS: $[M - H]^-$ $C_{51}H_{84}N_3O_{39}$ calcd for 1378.4637, found 1378.4660.



HMO27

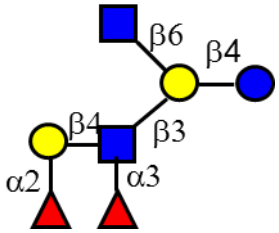
^{1}H NMR (D_2O, 500 MHz): δ 5.10 (d, J = 3.7 Hz, 0.55 H, Glc-1 H-1 of α form), 5.00 (d, J = 4.0 Hz, 1 H, Fuc-1 H-1), 4.69-4.74 (m, 1 H), 4.50-4.61 (m, 2.45 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.38 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.02 (d, J = 2.9 Hz, 1 H), 3.34-3.91 (m, 32 H), 3.17 (t, J = 8.3 Hz, 1 H), 2.54-2.59 (m, 1 H), 1.94 (s, 3 H), 1.90 (s, 3 H), 1.05 (d, J = 6.6 Hz, 3 H). MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{35}$ calcd for 1241.428, found 1241.432.



HMO28

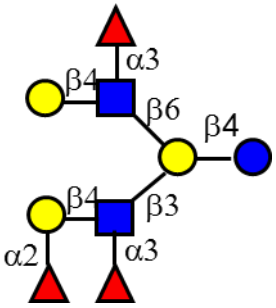
^{1}H NMR (D_2O, 500 MHz): δ 5.19 (d, J = 2.4 Hz, 1H, Fuc-1 H-1), 5.10 (d, J = 3.7 Hz, 0.42 H, Glc-1 H-1 of α form), 4.48-4.60 (m, 2.58 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.45 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.06-4.15 (m, 1 H), 4.01 (d, J = 2.9

Hz, 1 H), 3.38-3.90 (m, 31 H), 3.31-3.37 (m, 1 H), 3.17(t, *J* = 8.8 Hz, 1 H), 1.93 (s, 3 H), 1.91 (s, 3 H), 1.10 (d, *J* = 6.5 Hz, 3 H). MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{35}$ calcd for 1241.428, found 1241.423.



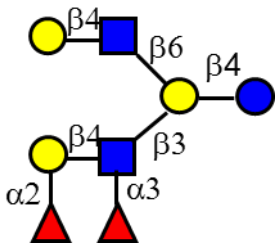
HMO29

^{1}H NMR (D_2O, 500 MHz): δ 5.15 (d, *J* = 2.6 Hz, 1 H, Fuc-2 H-1), 5.09(d, *J* = 3.7 Hz, 0.36 H, Glc-1 H-1 of α form), 4.99 (d, *J* = 3.7 Hz, 1 H, Fuc-1 H-1), 4.72-4.78 (m, 1 H), 4.46-4.61 (m, 2.64 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.42 (d, *J* = 7.8 Hz, 1 H, Gal-2 H-1), 4.31 (d, *J* = 8.0 Hz, 1 H, Gal-1 H-1), 4.10-4.16 (m, 1 H), 4.01 (d, *J* = 2.9 Hz, 1 H), 3.40-3.92 (m, 33 H), 3.29-3.37 (m, 3 H), 3.16 (t, *J* = 8.4 Hz, 1 H), 1.94 (s, 3 H), 1.90 (s, 3 H), 1.09-1.16 (m, 6 H). MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{34}$ calcd for 1225.433, found 1225.438.



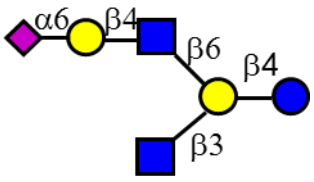
HMO210

^{1}H NMR (D_2O, 500 MHz): δ 5.16 (d, *J* = 2.6 Hz, 1 H, Fuc-2 H-1), 5.10 (d, *J* = 3.5 Hz, 0.40 H, Glc-1 H-1 of α form), 4.93-5.01 (m, 2 H, Fuc-1 H-1, Fuc-3 H-1), 4.67-4.79 (m, 2 H), 4.45-4.61 (m, 2.60 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.26-4.41 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.09-4.16 (m, 1 H), 4.00 (d, *J* = 3.1 Hz, 1 H), 3.41-3.91 (m, 44 H), 3.28-3.40 (m, 3 H), 3.16 (t, *J* = 8.4 Hz, 1 H), 1.93 (s, 3 H), 1.90 (s, 3 H), 1.02-1.17 (m, 9 H). MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{34}$ calcd for 1533.544, found 1533.538.



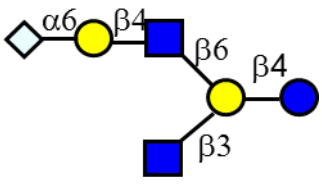
HMO211

^{1}H NMR (D_2O, 500 MHz): δ 5.16 (d, J = 2.7 Hz, 1 H, Fuc-2 H-1), 5.10 (d, J = 3.7 Hz, 0.47 H, Glc-1 H-1 of α form), 4.99 (d, J = 3.7 Hz, 1 H, Fuc-1 H-1), 4.72-4.78 (m, 1 H), 4.49-4.62 (m, 2.53 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.29-4.41 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.10-4.16 (m, 1 H), 4.01 (d, J = 3.2 Hz, 1 H), 3.77-3.91 (m, 8 H), 3.39-3.77 (m, 35 H), 3.30-3.36 (m, 1 H), 3.17 (t, J = 8.4 Hz, 1 H), 1.94 (s, 3 H), 1.90 (s, 3 H), 1.09-1.16 (m, 6 H). MALDI-MS: $[M + Na]^+$ $C_{52}H_{88}N_2NaO_{39}$ calcd for 1387.486, found 1387.490.



HMO31

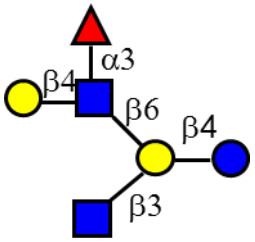
^{1}H NMR (D_2O, 500 MHz): δ 5.09(d, J = 3.7 Hz, 0.31 H, Glc-1 H-1 of α form), 4.47-4.58 (m, 2.69 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.34 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.06-4.16 (m, 1 H), 4.02 (d, J = 3.1 Hz, 1 H), 3.28-3.91 (m, 35 H), 3.26-3.36 (m, 2 H), 3.18 (t, J = 8.4 Hz, 1 H), 2.51-2.57 (m, 1 H), 1.96 (s, 3 H), 1.92 (s, 3 H), 1.91 (s, 3 H), 1.59 (t, J = 12.2 Hz, 1 H). ESI-MS: $[M - H]^-$ $C_{45}H_{74}N_3O_{34}$ calcd for 1200.4159, found 1200.4122.



HMO32

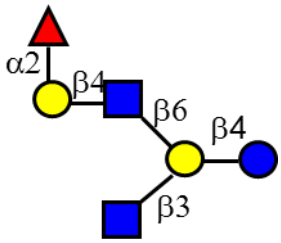
^{1}H NMR (D_2O, 500 MHz): δ 5.09 (d, J = 3.9 Hz, 0.28 H, Glc-1 H-1 of α form), 4.50-4.58 (m, 2.72 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.34 (m, 2 H, Gal-1

H-1, Gal-2 H-1), 4.02 (d, *J* = 3.2 Hz, 1 H), 3.28-3.91 (m, 35 H), 3.18 (t, *J* = 8.3 Hz, 1 H), 2.53-2.58 (m, 1 H), 1.96 (s, 3 H), 1.91 (s, 3 H), 1.59 (t, *J* = 12.2 Hz, 1 H). ESI-MS: $[M - H]^-$ $C_{45}H_{74}N_3O_{35}$ calcd for 1216.4108, found 1216.4139.



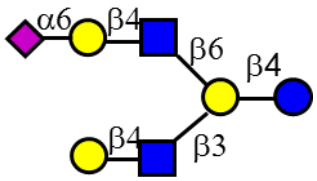
HMO33

^{1}H NMR (D_2O, 500 MHz): *δ* 5.09 (d, *J* = 3.7 Hz, 0.19 H, Glc-1 H-1 of α form), 4.98 (d, *J* = 3.9 Hz, 1 H, Fuc-1 H-1), 4.66-4.73 (m, 1 H), 4.48-4.58 (m, 2.81 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.27-4.35 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.02 (d, *J* = 3.1 Hz, 1 H), 3.28-3.90 (m, 38 H), 3.16 (t, *J* = 8.4 Hz, 1 H), 1.92 (s, 3 H), 1.90 (s, 3 H), 1.04 (d, *J* = 6.6 Hz, 3 H). MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{30}$ calcd for 1079.376, found 1079.380.



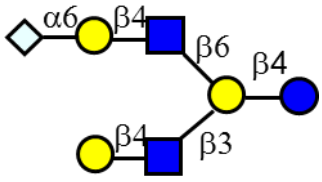
HMO34

^{1}H NMR (D_2O, 500 MHz): *δ* 5.18 (d, *J* = 2.6 Hz, 1 H, Fuc-1 H-1), 5.09(d, *J* = 3.9 Hz, 0.41 H, Glc-1 H-1 of α form), 4.66-4.73 (m, 1 H), 4.45-4.58 (m, 2.59 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.39-4.44 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.31 (d, *J* = 7.5 Hz, 1 H), 4.06-4.12 (m, 2 H), 4.02 (d, *J* = 3.1 Hz, 1 H), 3.39-3.90 (m, 28 H), 3.28-3.38 (m, 3 H), 3.16 (t, *J* = 8.4 Hz, 1 H), 1.94 (s, 3 H), 1.91 (s, 3 H), 1.11 (d, *J* = 6.7 Hz, 3 H). MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{30}$ calcd for 1079.376, found 1079.372.



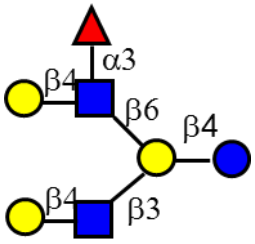
HMO35

^{1}H NMR (D_2O, 500 MHz): δ 5.09 (d, J = 3.8 Hz, 0.53 H, Glc-1 H-1 of α form), 4.51-4.60 (m, 2.47 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.37 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.02 (d, J = 3.0 Hz, 1 H), 3.38-3.91 (m, 41 H), 3.18 (t, J = 8.4 Hz, 1 H), 2.50-2.57 (m, 1 H), 1.96 (s, 3 H), 1.91 (s, 6 H), 1.59 (t, J = 12.0 Hz, 1 H). ESI-MS: $[M - H]^-$ $C_{51}H_{84}N_3O_{39}$ calcd for 1362.4687, found 1362.4799.



HMO36

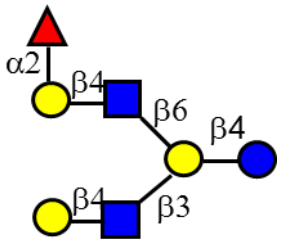
^{1}H NMR (D_2O, 500 MHz): δ 5.09 (d, J = 3.8 Hz, 0.52 H, Glc-1 H-1 of α form), 4.50-4.60 (m, 2.48 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.38 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.02 (d, J = 3.0 Hz, 1 H), 3.99 (s, 2 H), 3.38-3.91 (m, 41 H), 3.18 (t, J = 8.4 Hz, 1 H), 2.53-2.59 (m, 1 H), 1.96 (s, 3 H), 1.91 (s, 3 H), 1.59 (t, J = 12.0 Hz, 1 H). ESI-MS: $[M - H]^-$ $C_{51}H_{84}N_3O_{39}$ calcd for 1378.4637, found 1378.4609.



HMO37

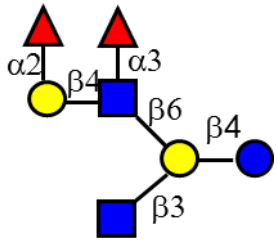
^{1}H NMR (D_2O, 500 MHz): δ 5.09 (d, J = 3.8 Hz, 0.34 H, Glc-1 H-1 of α form), 4.98 (d, J = 4.0 Hz, 1 H, Fuc-1 H-1), 4.67-4.74 (m, 1 H), 4.50-4.61 (m, 2.66 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.27-4.38 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.01 (d, J =

3.1 Hz, 1 H), 3.30-3.90 (m, 37 H), 3.16 (t, *J* = 8.6 Hz, 1 H), 1.92 (s, 3 H), 1.91 (s, 3 H), 1.05 (d, *J* = 6.6 Hz, 3 H). MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{35}$ calcd for 1241.428, found 1241.436.



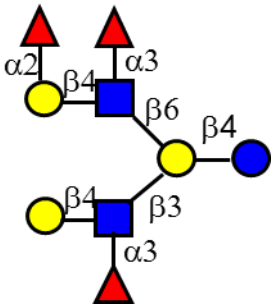
HMO38

^{1}H NMR (D_2O, 500 MHz): δ 5.18 (d, *J* = 2.6 Hz, 1 H, Fuc-1 H-1), 5.09 (d, *J* = 3.8 Hz, 0.69 H, Glc-1 H-1 of α form), 4.46-4.60 (m, 2.31 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.43 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.07-4.13 (m, 1 H), 4.02 (d, *J* = 3.2 Hz, 1 H), 3.39-3.89 (m, 36 H), 3.33-3.38 (m, 1 H), 3.17 (t, *J* = 8.4 Hz, 1 H), 1.94 (s, 3 H), 1.91 (s, 3 H), 1.11 (d, *J* = 6.5 Hz, 3 H). MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{35}$ calcd for 1241.428, found 1241.435.



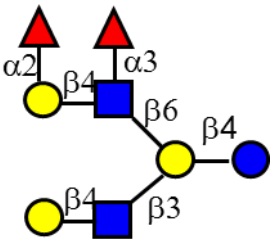
HMO39

^{1}H NMR (D_2O, 500 MHz): δ 5.16 (d, *J* = 2.9 Hz, 1 H, Fuc-2 H-1), 5.10(d, *J* = 3.7 Hz, 0.39 H, Glc-1 H-1 of α form), 4.97 (d, *J* = 4.0 Hz, 1 H, Fuc-1 H-1), 4.72-4.78 (m, 1 H), 4.45-4.58 (m, 2.61 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.40 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.10-4.16 (m, 1 H), 4.02 (d, *J* = 3.06 Hz, 1 H), 3.40-3.93 (m, 33 H), 3.29-3.38 (m, 3 H), 3.16(t, *J* = 8.4 Hz, 1 H), 1.93 (s, 3 H), 1.91 (s, 3 H), 1.08-1.17 (m, 6 H). MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{34}$ calcd for 1225.433, found 1225.430.



HMO310

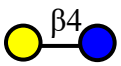
^{1}H NMR (D_2O, 500 MHz): δ 5.16 (d, J = 3.0 Hz, 1 H, Fuc-3 H-1), 5.09 (d, J = 3.7 Hz, 0.62 H, Glc-1 H-1 of α form), 4.96-5.01 (m, 2 H), 4.69-4.78 (m, overlap with D_2O, 2 H), 4.47-4.61 (m, 2.38 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.28-4.40 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.10-4.16 (m, 1 H), 4.02 (d, J = 3.2 Hz, 1 H), 3.32-3.93 (m, 43 H), 3.16 (t, J = 8.4 Hz, 1 H), 1.93 (s, 3 H), 1.90 (s, 3 H), 1.02-1.17 (m, 9 H). MALDI-MS: $[M + Na]^+$ $C_{58}H_{98}N_2NaO_{43}$ calcd for 1533.544, found 1533.550.

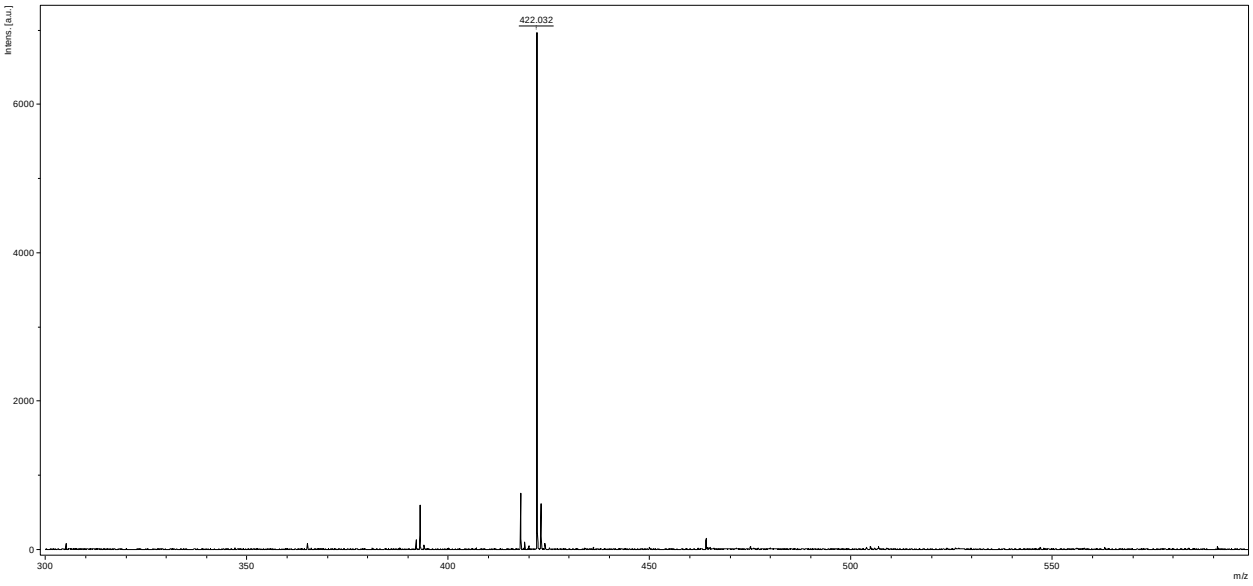


HMO311

^{1}H NMR (D_2O, 500 MHz): δ 5.16 (d, J = 3.2 Hz, 1 H, Fuc-2 H-1), 5.10 (d, J = 3.6 Hz, 0.67 H, Glc-1 H-1 of α form), 4.97 (d, J = 3.9 Hz, 1 H, Fuc-1 H-1), 4.72-4.77 (m, 1 H), 4.47-4.60 (m, 2.37 H, GlcNAc-1 H-1, GlcNAc-2 H-1, Glc-1 H-1 of β form), 4.29-4.39 (m, 3 H, Gal-1 H-1, Gal-2 H-1, Gal-3 H-1), 4.10-4.16 (m, 1 H), 4.02 (d, J = 3.1 Hz, 1 H), 3.39-3.93 (m, 41 H), 3.30-3.38 (m, 1 H), 3.16 (t, J = 8.4 Hz, 1 H), 1.94 (s, 3 H), 1.91 (s, 3 H), 1.09-1.17 (m, 6 H). MALDI-MS: $[M + Na]^+$ $C_{52}H_{88}N_2NaO_{39}$ calcd for 1387.486, found 1387.478.

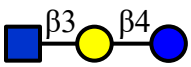
H0400

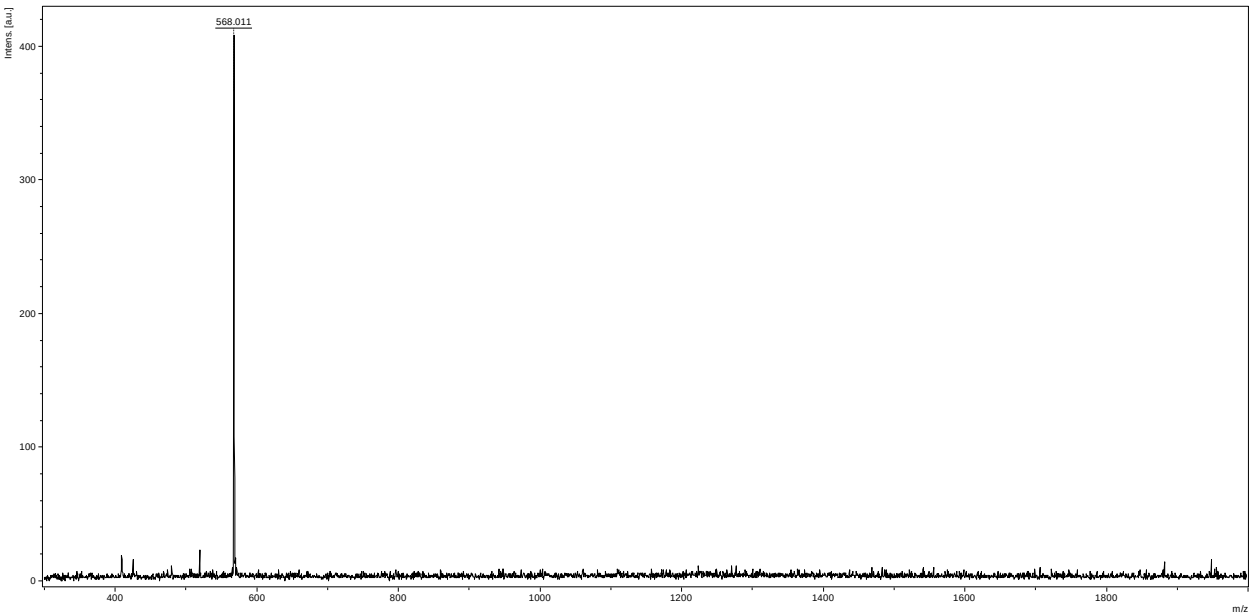




MALDI-MS: $[M + Na]^+$ $C_{15}H_{29}NNaO_{11}$ calcd for 422.1638, found 422.032.

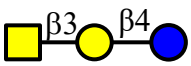
H0401

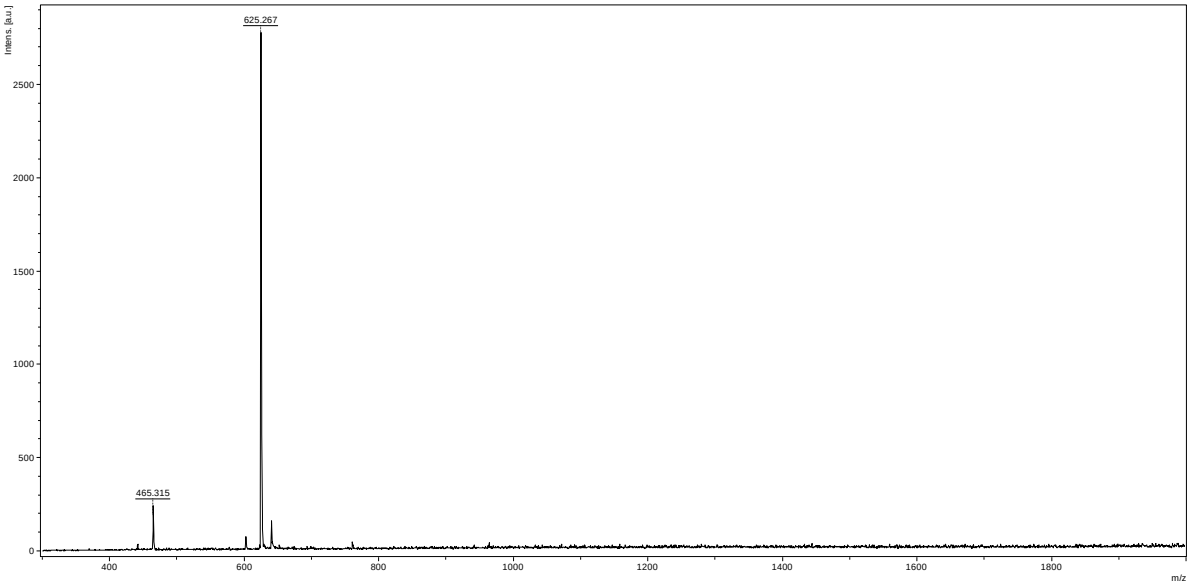




MALDI-MS: $[M + Na+H_2O]^+$ $C_{20}H_{35}NNaO_{16}$ calcd for 568.1854, found 568.011.

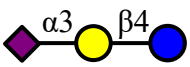
H0402

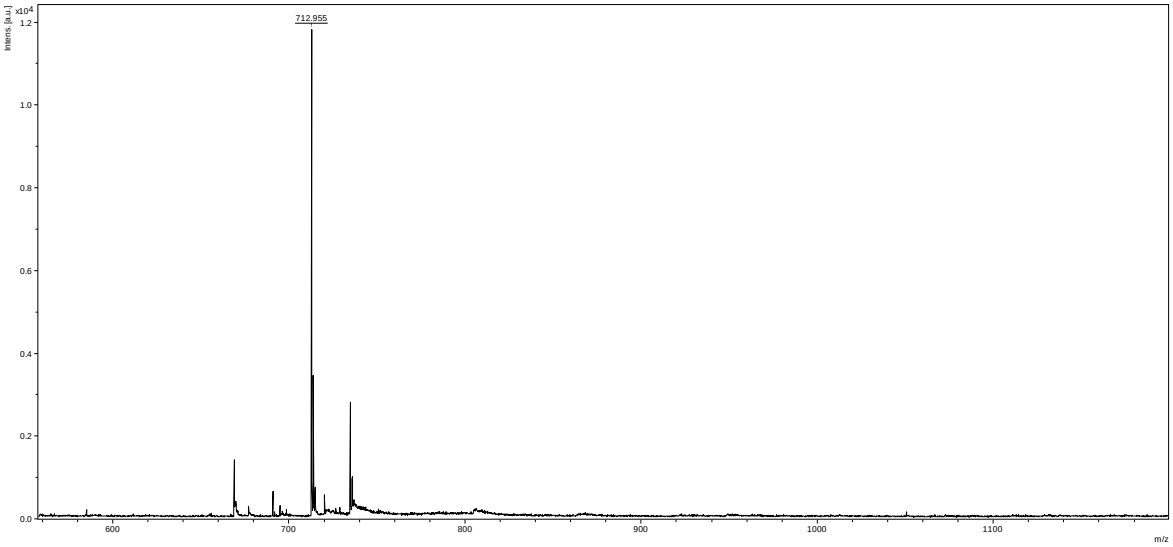




MALDI-MS: $[M + Na]^+$ $C_{23}H_{42}N_2NaO_{16}$ calcd for 625.2432, found 625.267.

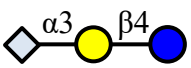
H0403

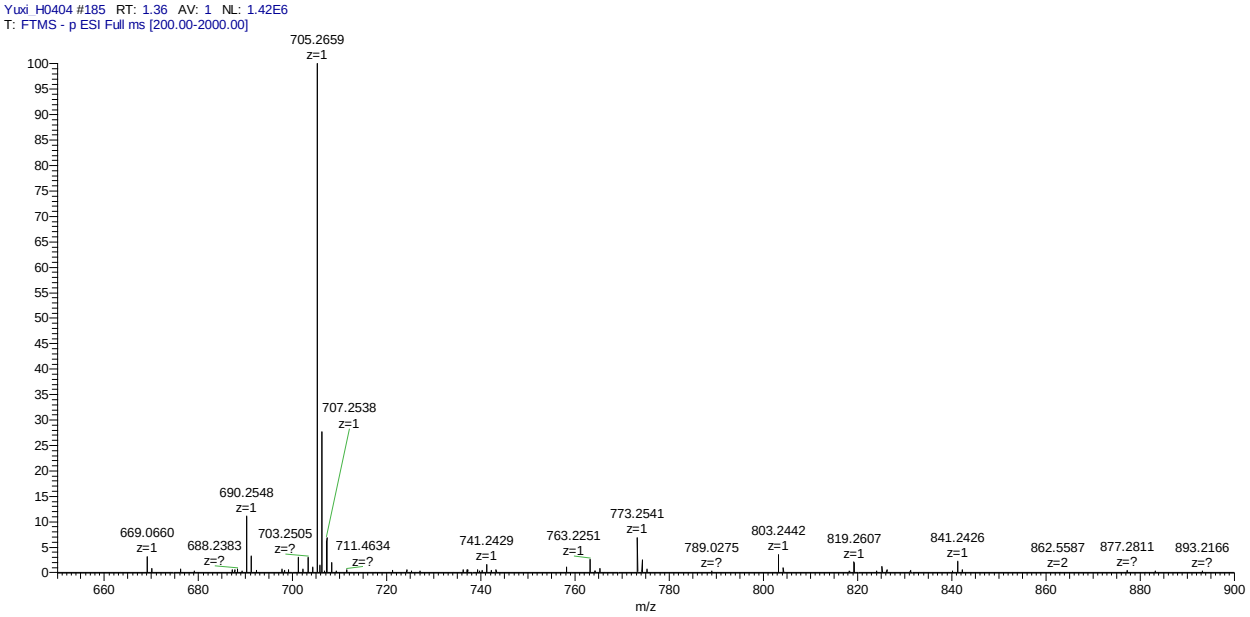




MALDI-MS: $[M + Na]^+$ $C_{26}H_{46}N_2NaO_{19}$ calcd for 713.2592, found 712.955.

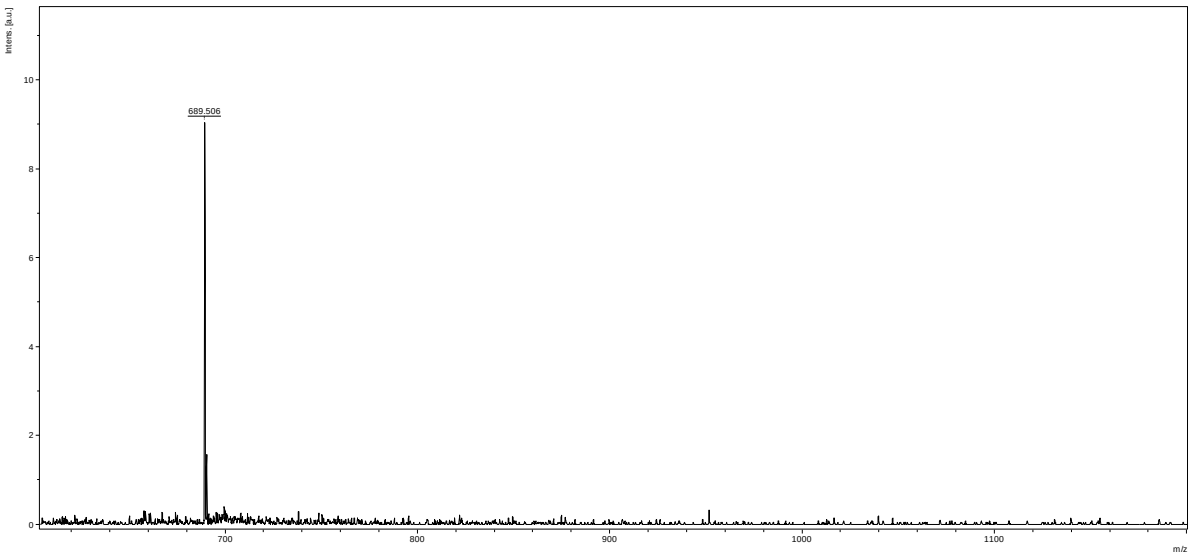
H0404





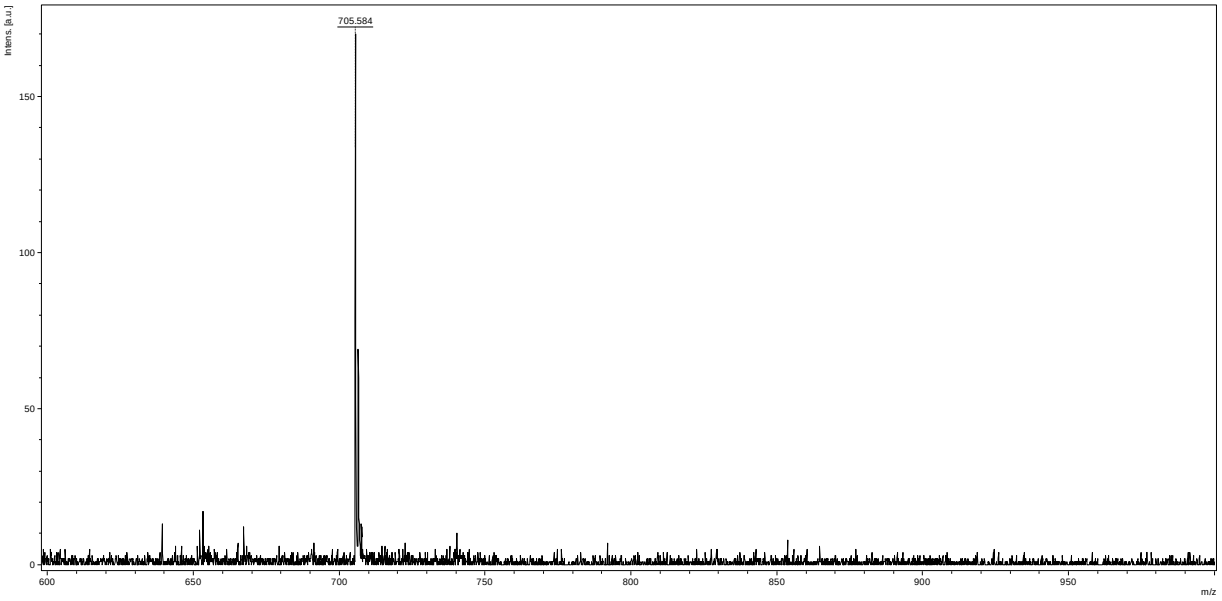
ESI-MS: $[M - H]^-$ $C_{26}H_{46}N_2O_{20}$ calcd for 705.2566, found 705.2659.

H0405



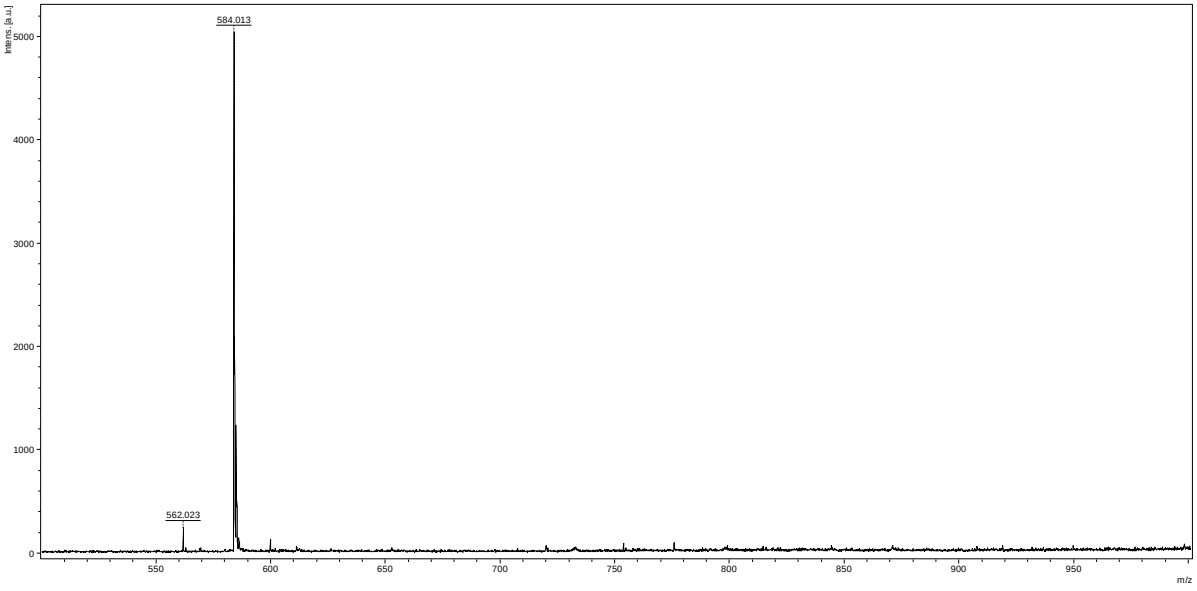
ESI-MS: $[M - H]^-$ $C_{26}H_{45}N_2O_{19}$ calcd for 689.2617, found 689.506.

H0406



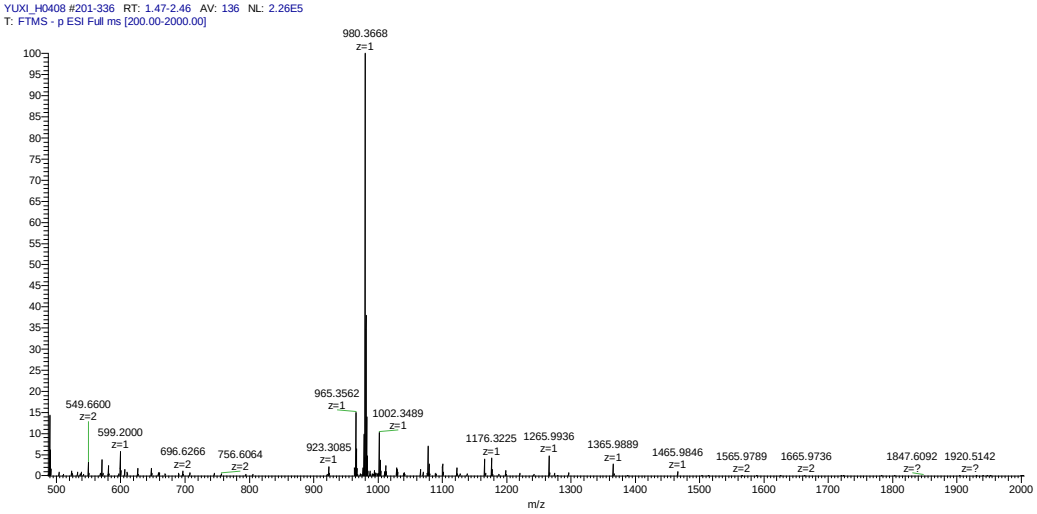
ESI-MS: $[M - H]^-$ $C_{26}H_{46}N_2O_{20}$ calcd for 705.2566, found 705.584.

H0407



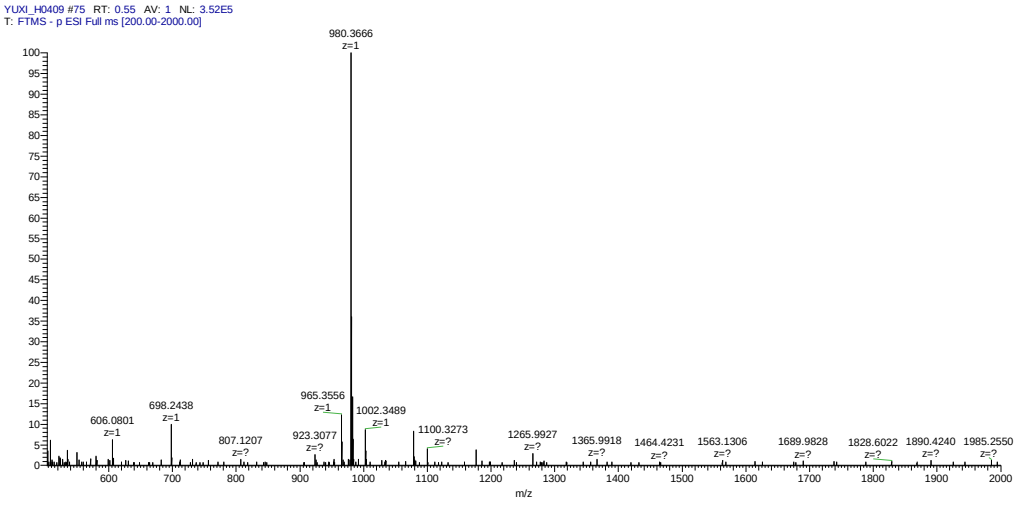
MALDI-MS: $[M + Na]^+$ $C_{21}H_{39}NNaO_{16}$ calcd for 584.2167, found 584.013.

H0408



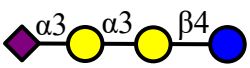
ESI-MS: $[M - H]^-$ $C_{37}H_{62}N_3O_{27}$ calcd for 980.3571, found 980.3668.

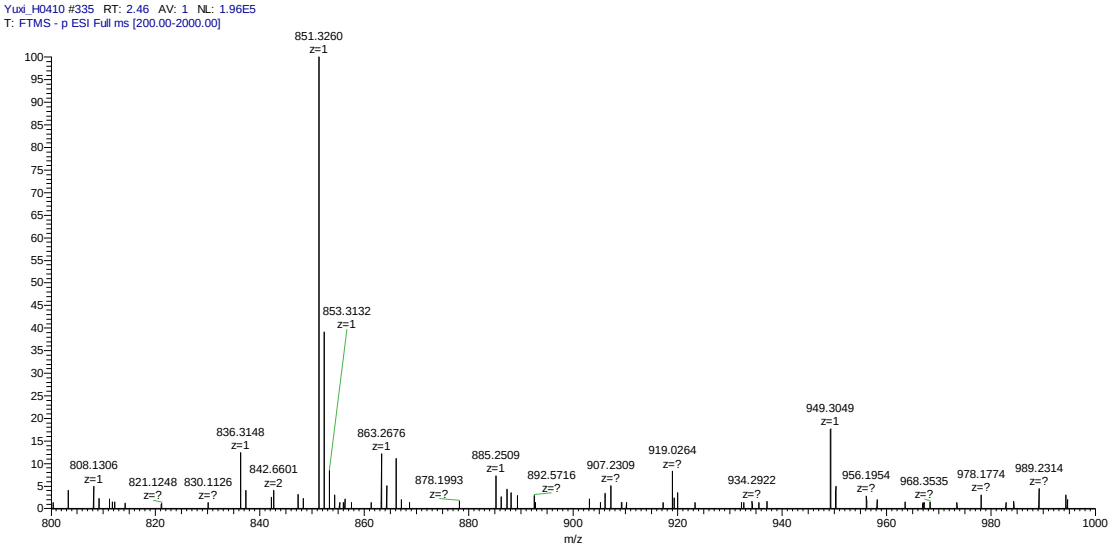
H0409



ESI-MS: $[M - H]^-$ $C_{37}H_{62}N_3O_{27}$ calcd for 980.3571, found 980.3666.

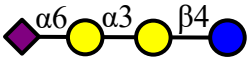
H0410

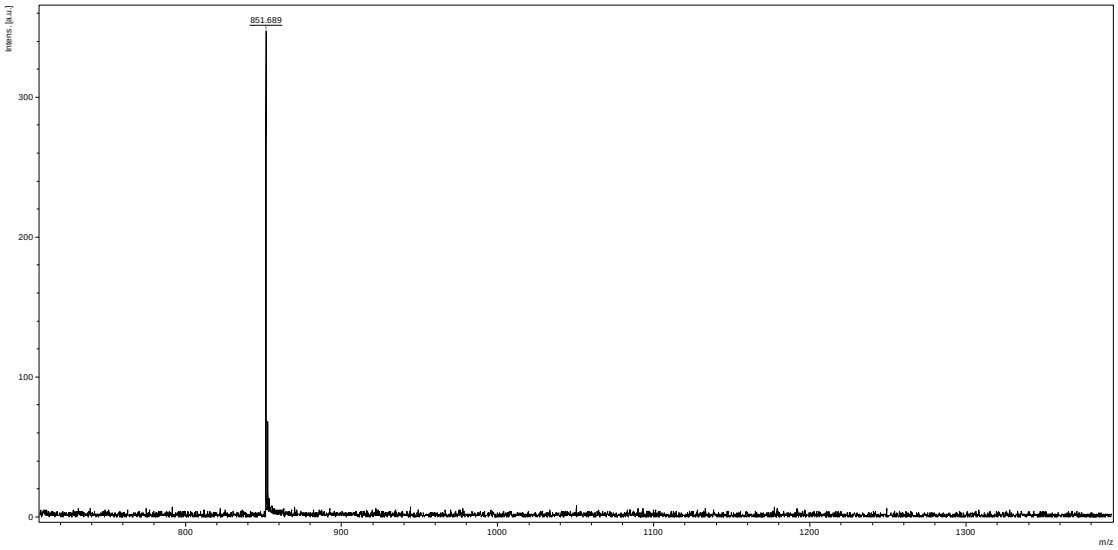




ESI-MS: $[M - H]^-$ $C_{32}H_{55}N_2O_{24}$ calcd for 851.3145, found 851.3260.

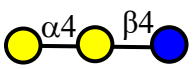
H0411

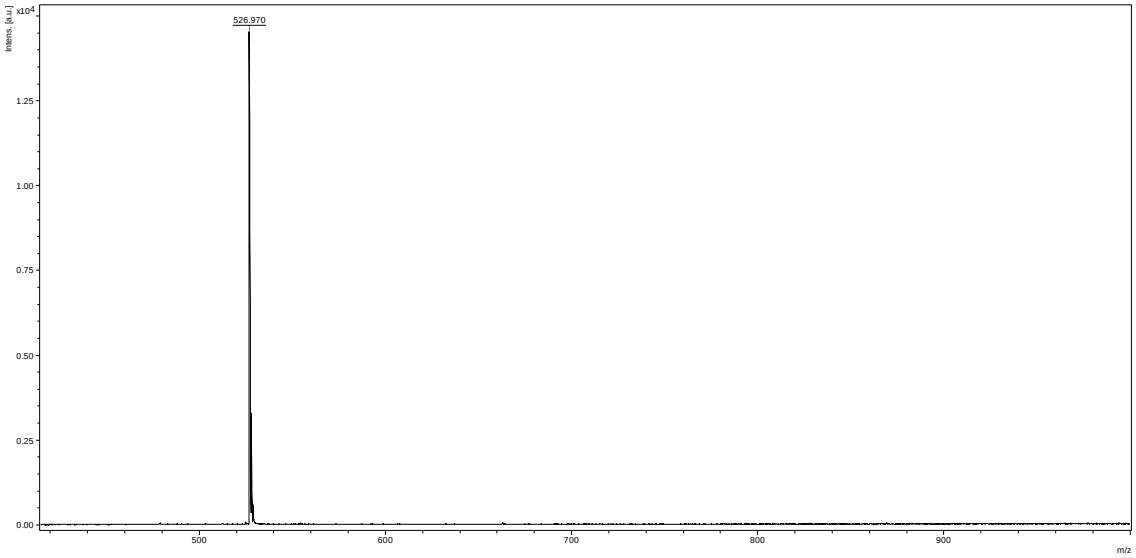




MALDI-MS: $[M - H]^-$ $C_{32}H_{55}N_2O_{24}$ calcd for 851.3145, found 851.689.

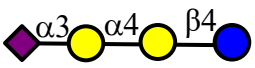
H0500

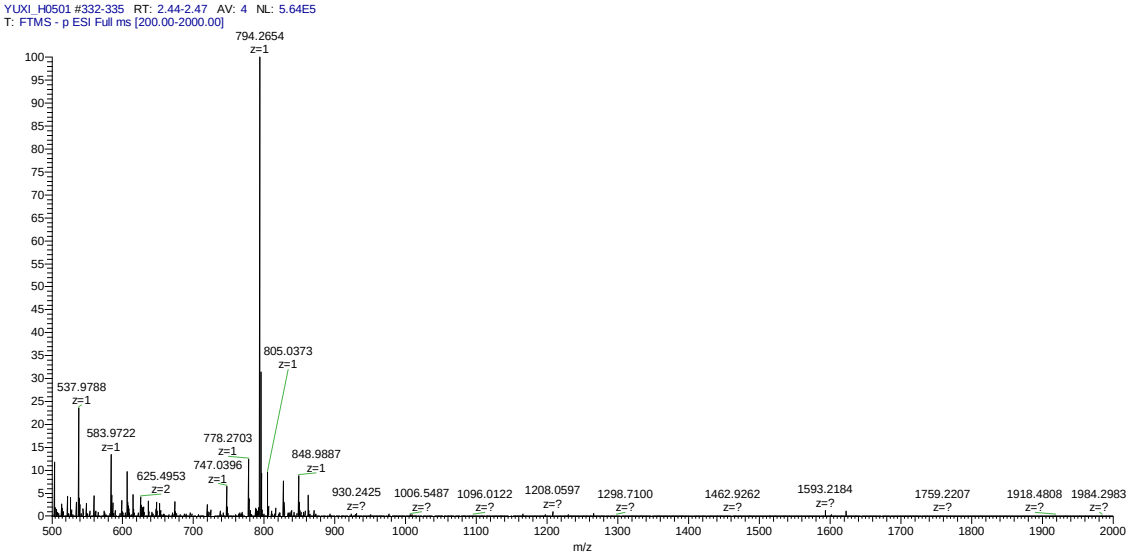




MALDI-MS: $[M + Na]^+$ $C_{18}H_{32}NaO_{16}$ calcd for 527.1588, found 526.970.

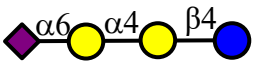
H0501

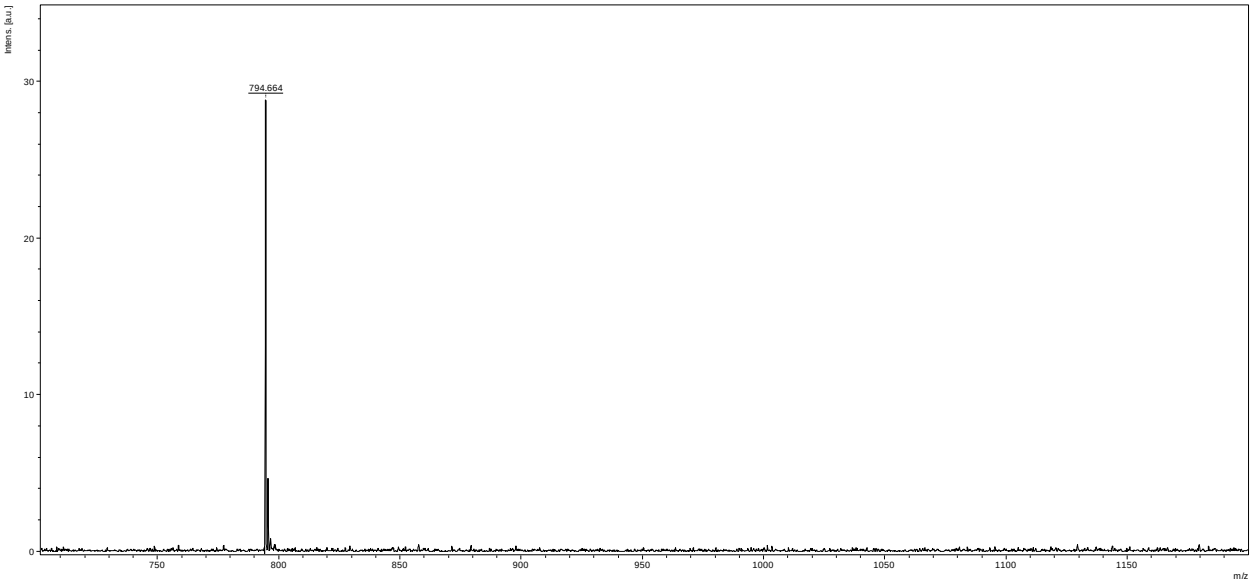




ESI-MS: $[M - H]^-$ $C_{29}H_{48}NO_{24}$ calcd for 794.2566, found 794.2654.

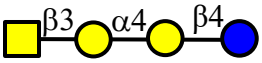
H0502

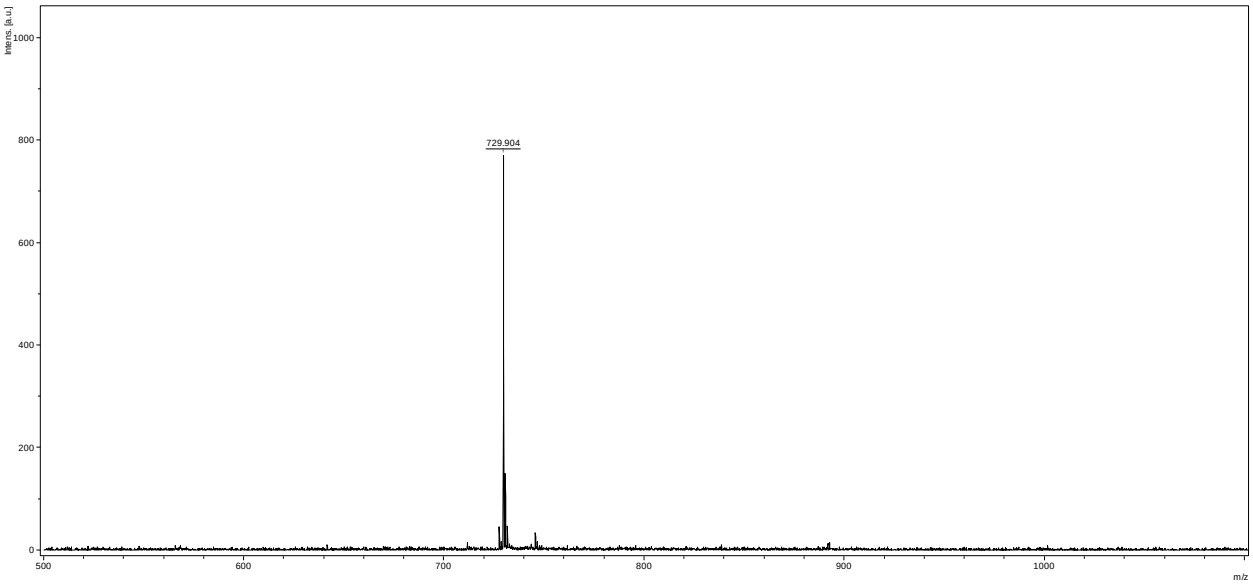




MALDI-MS: $[M - H]^-$ $C_{29}H_{48}NO_{24}$ calcd for 794.2566, found 794.664.

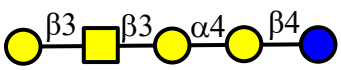
H0503

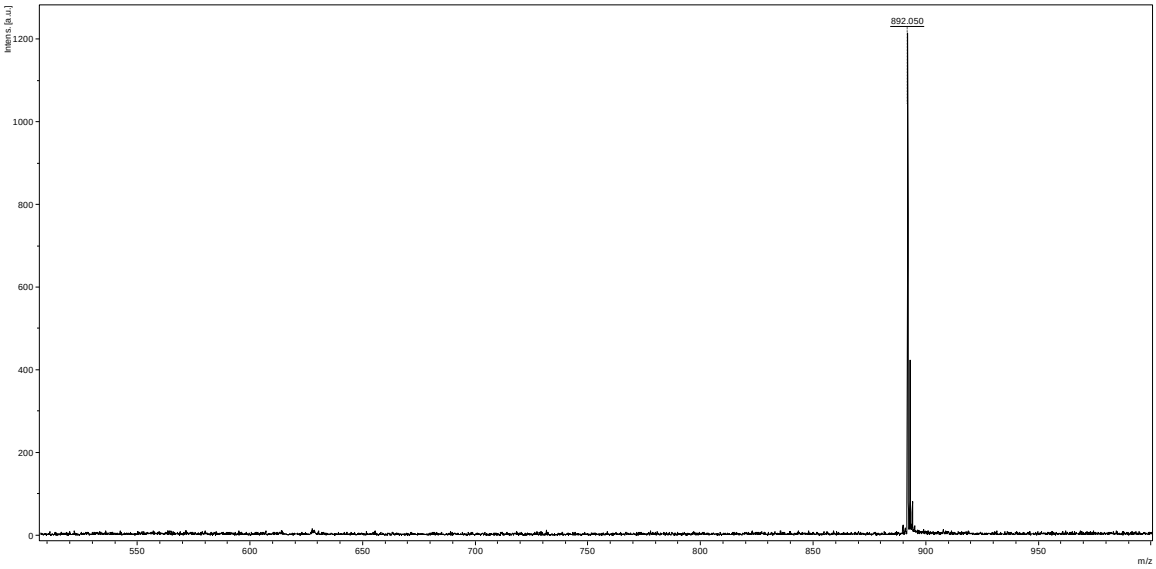




MALDI-MS: $[M + Na]^+$ $C_{26}H_{45}NNaO_{21}$ calcd for 730.2382, found 729.904.

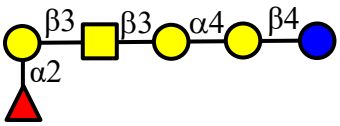
H0504

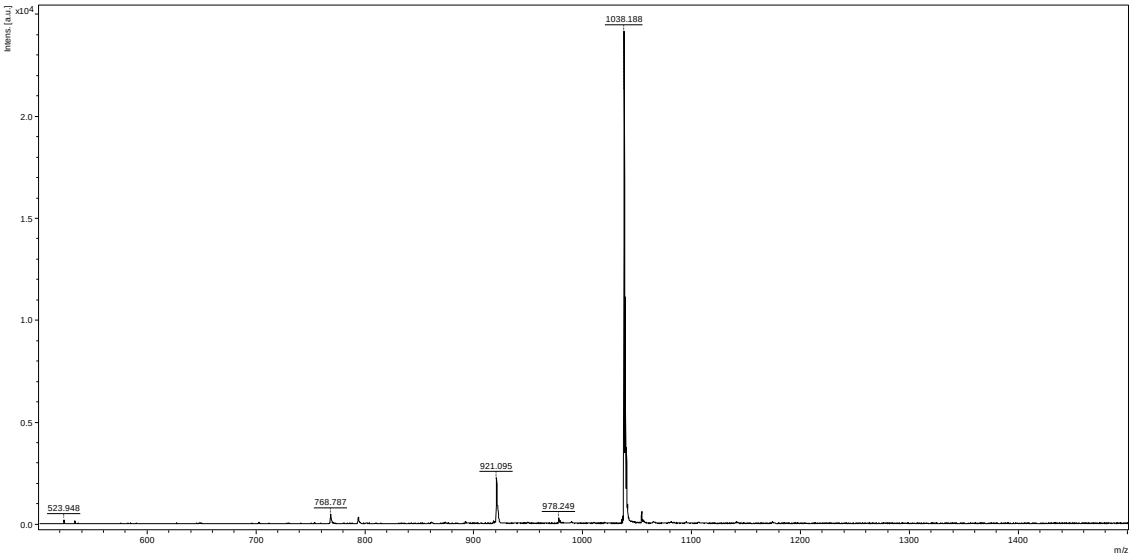




MALDI-MS: $[M + Na]^+$ $C_{32}H_{55}NNaO_{26}$ calcd for 892.2910, found 892.050.

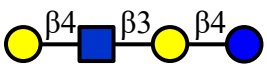
H0505

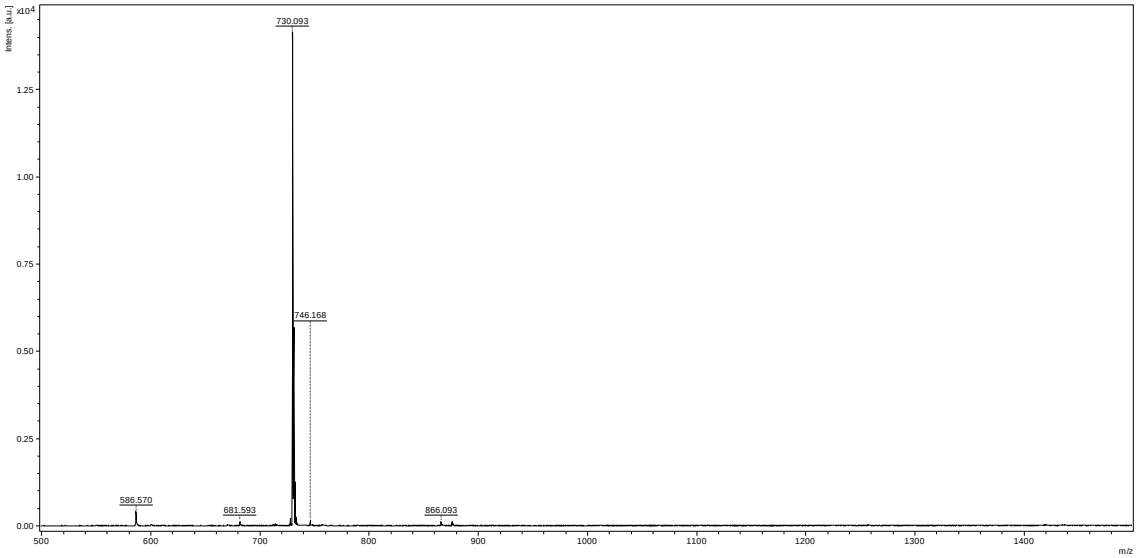




MALDI-MS: $[M + Na]^+$ $C_{38}H_{65}NNaO_{30}$ calcd for 1038.3489, found 1038.188.

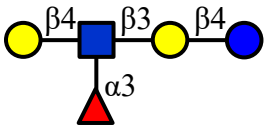
H0600

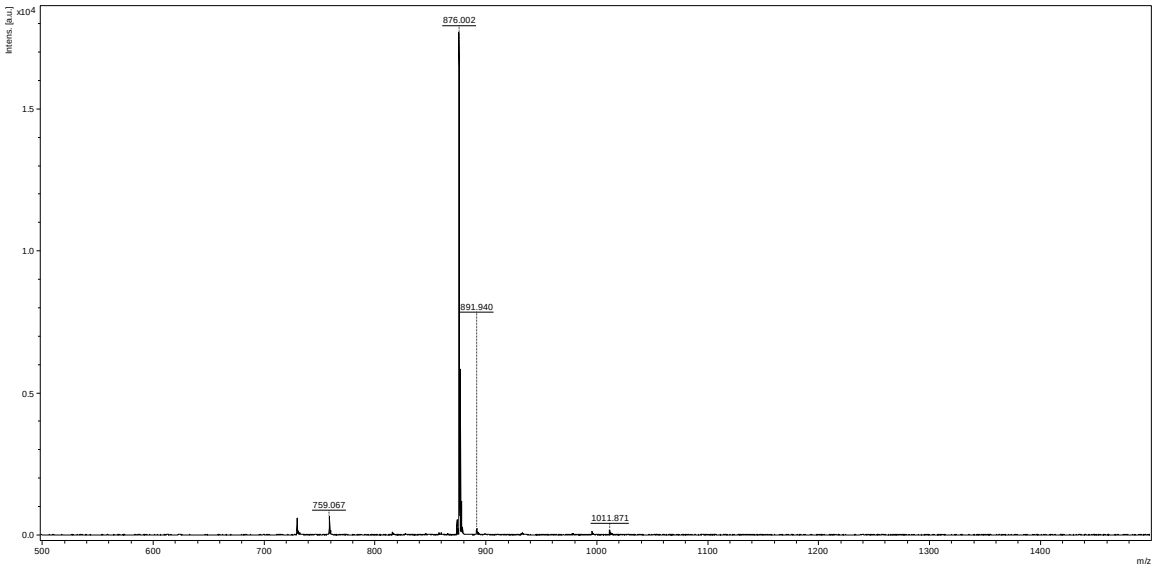




MALDI-MS: $[M + Na]^+$ $C_{26}H_{45}NNaO_{21}$ calcd for 730.2382, found 730.093.

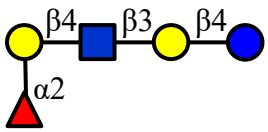
H0601

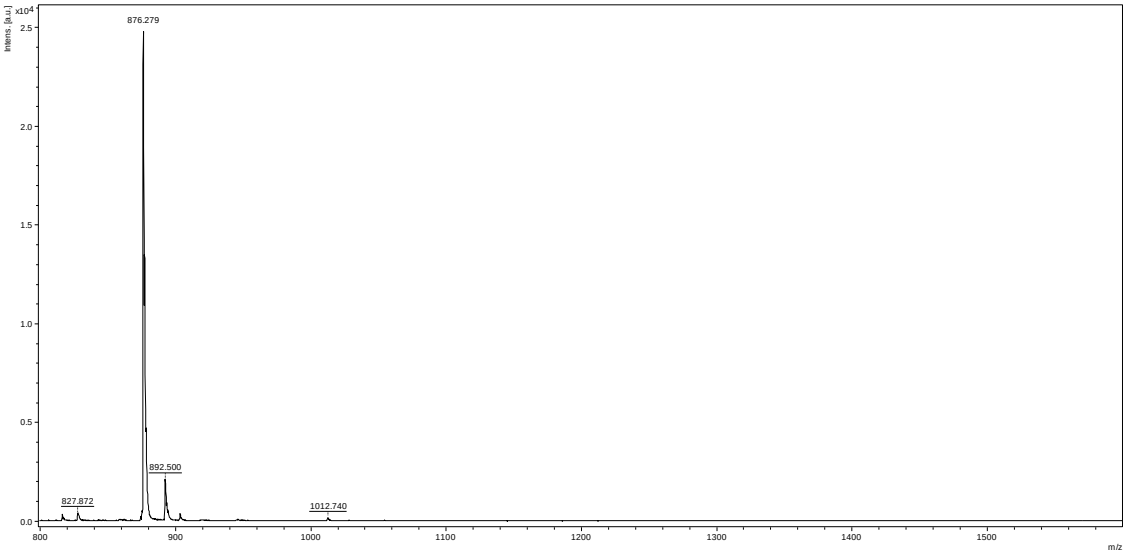




MALDI-MS: $[M + Na]^+$ $C_{32}H_{55}NNaO_{25}$ calcd for 876.2961, found 876.002.

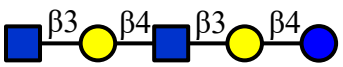
H0602

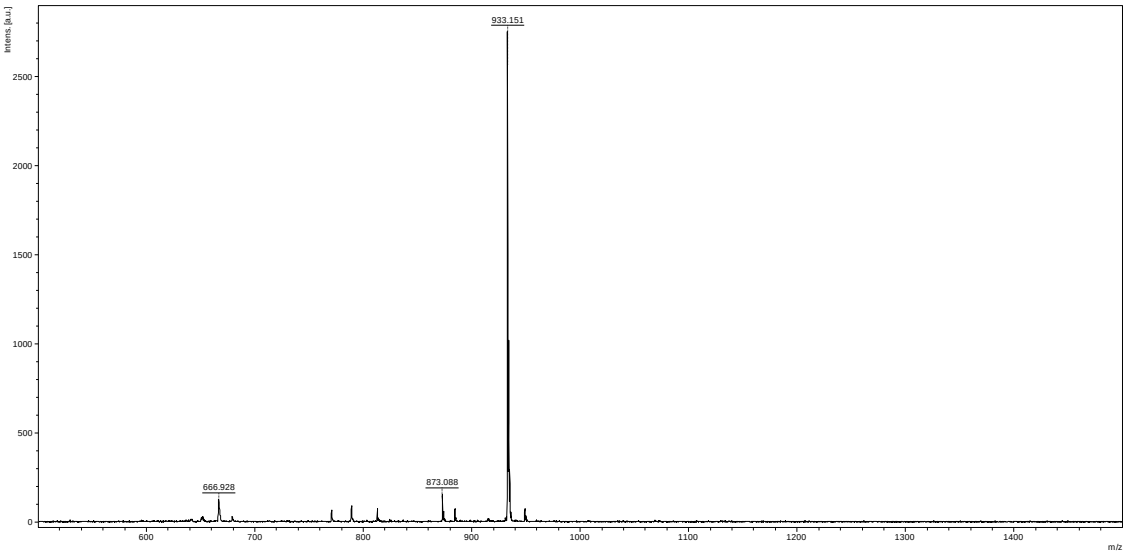




MALDI-MS: $[M + Na]^+$ $C_{32}H_{55}NNaO_{25}$ calcd for 876.2961, found 876.279.

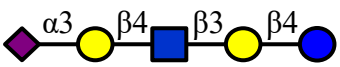
H0603

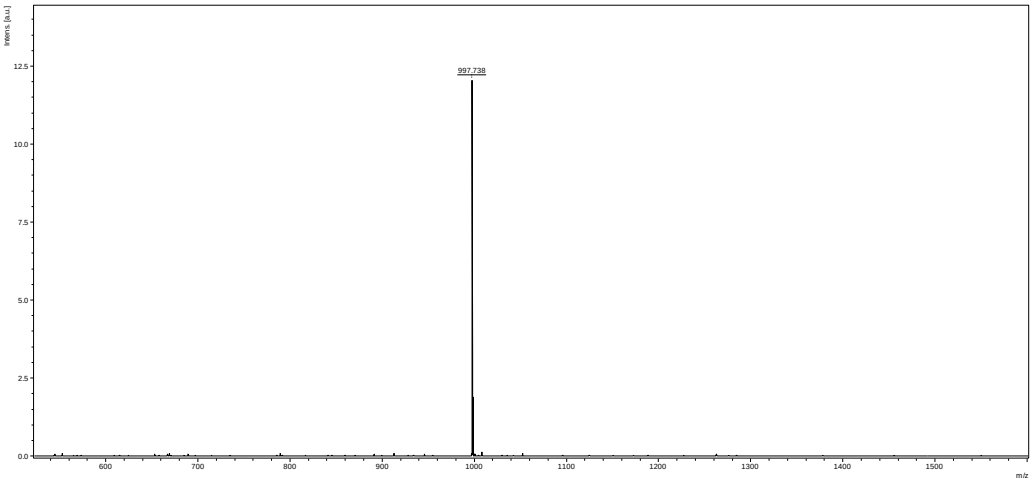




MALDI-MS: $[M + Na]^+$ $C_{34}H_{58}N_2NaO_{26}$ calcd for 933.3175, found 933.151.

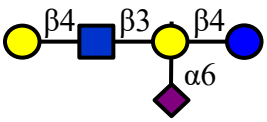
H0604

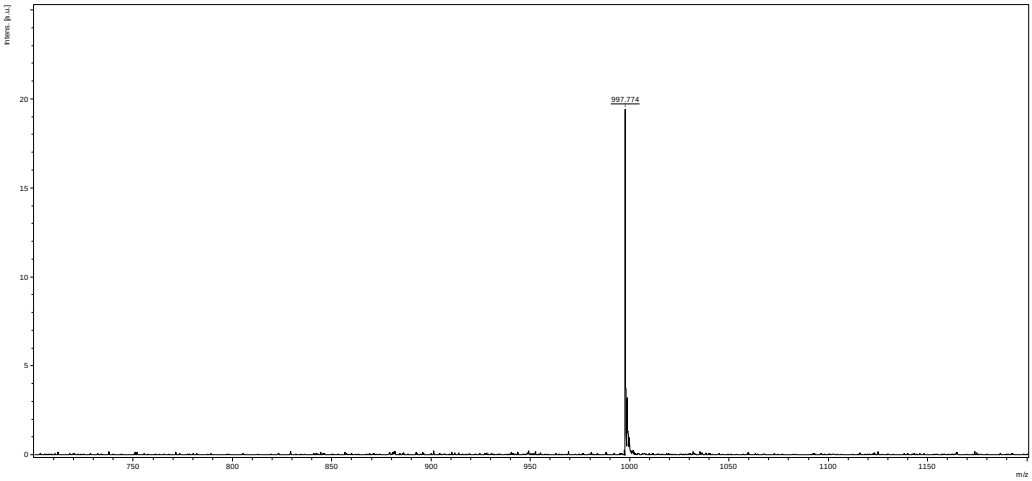




MALDI-MS: $[M - H]^-$ $C_{37}H_{61}N_2O_{29}$ calcd for 997.3360, found 997.738.

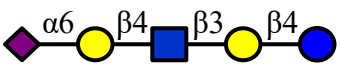
H0605

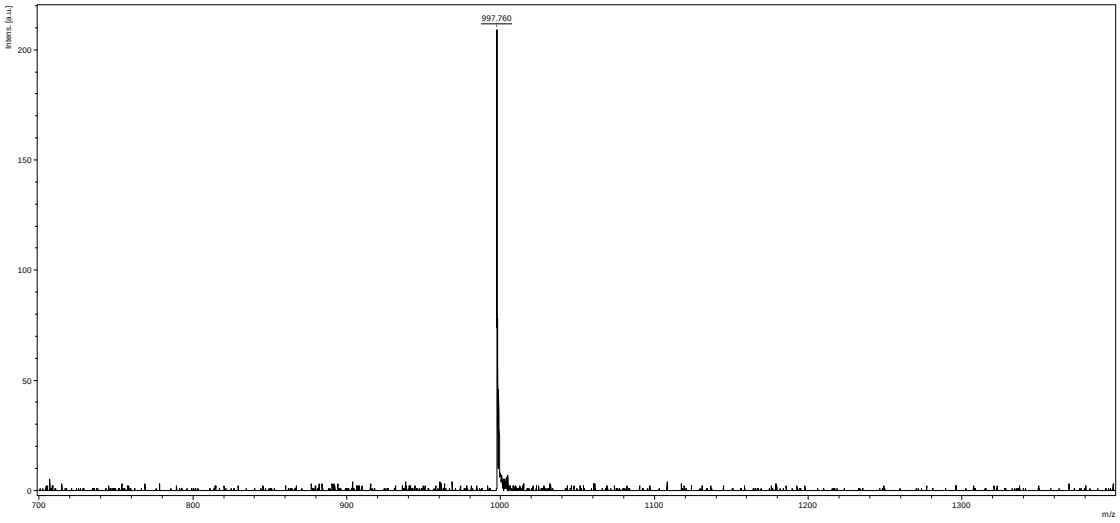




MALDI-MS: $[M - H]^-$ $C_{37}H_{61}N_2O_{29}$ calcd for 997.3360, found 997.774.

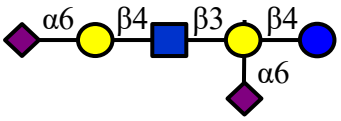
H0606

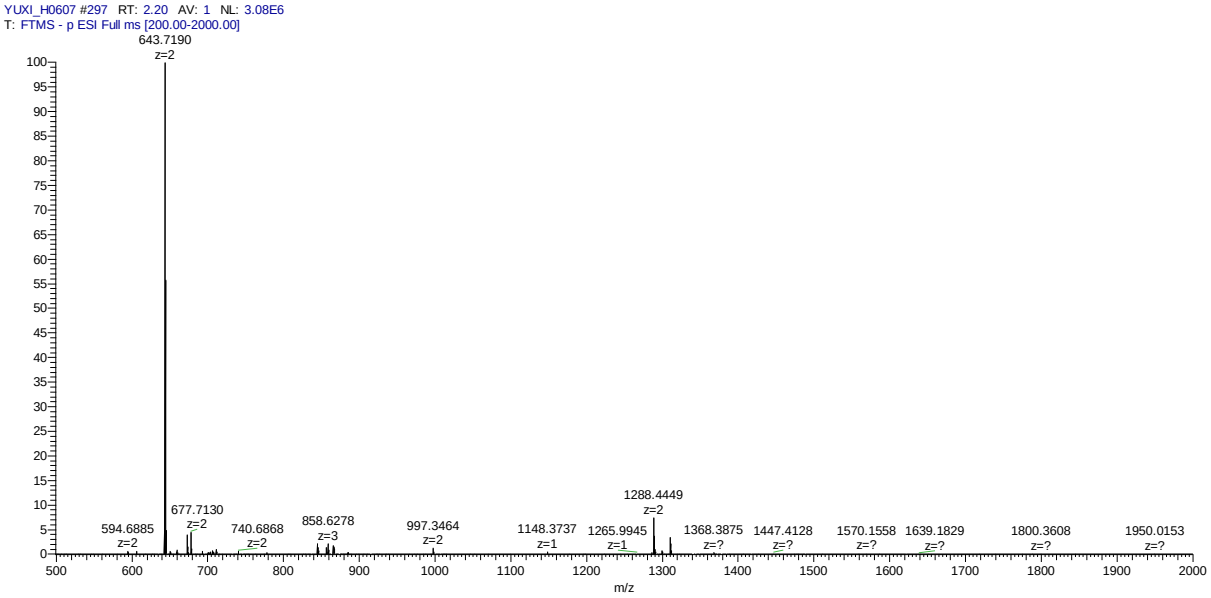




MALDI-MS: $[M - H]^-$ $C_{37}H_{61}N_2O_{29}$ calcd for 997.3360, found 997.760.

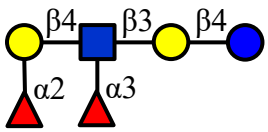
H0607

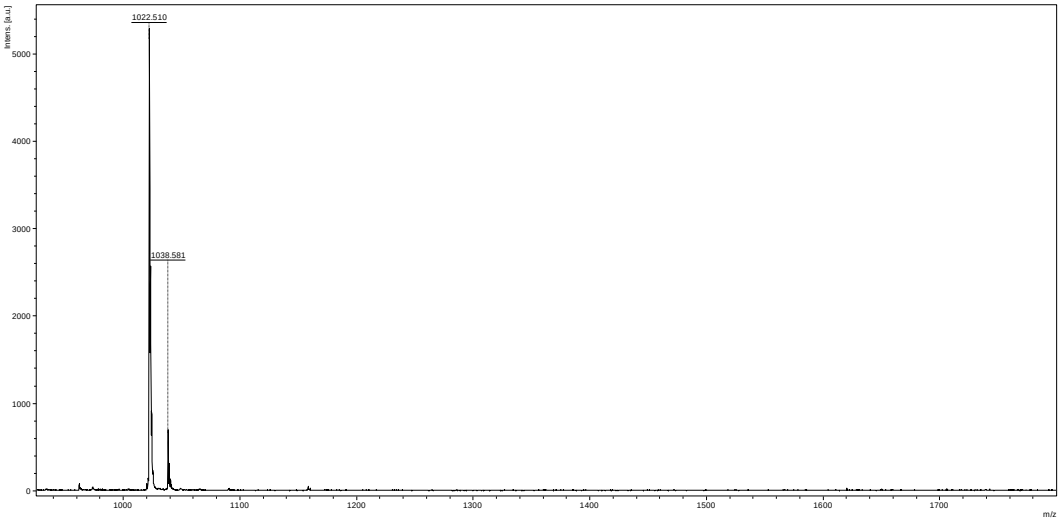




ESI-MS: $[M - 2H]2^-$ $C_{48}H_{77}N_3O_{37}$ calcd for 1287.4236/2, found 643.7190.

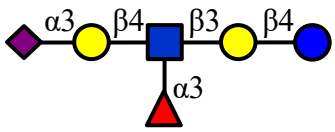
H0608

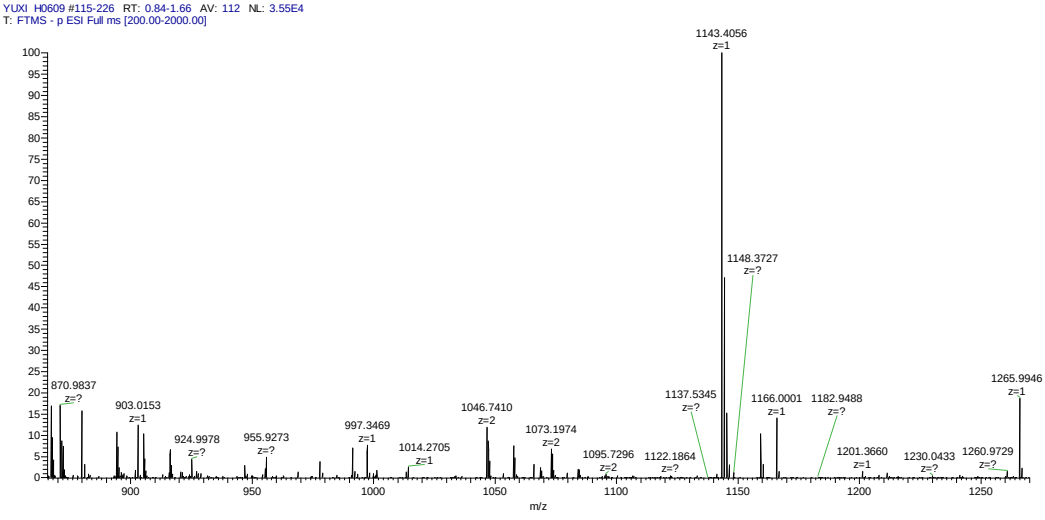




MALDI-MS: $[M + Na]^+$ $C_{38}H_{65}NNaO_{29}$ calcd for 1022.3540, found 1022.510.

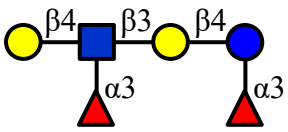
H0609

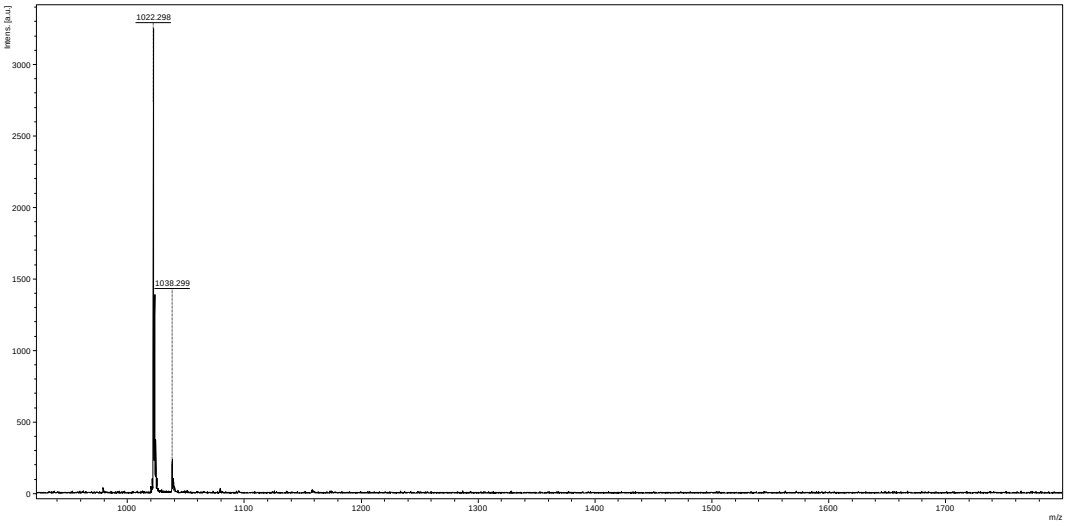




ESI-MS: $[M - H]^-$ $C_{43}H_{71}N_2O_{33}$ calcd for 1143.3939, found 1143.4056

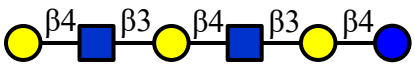
H0611

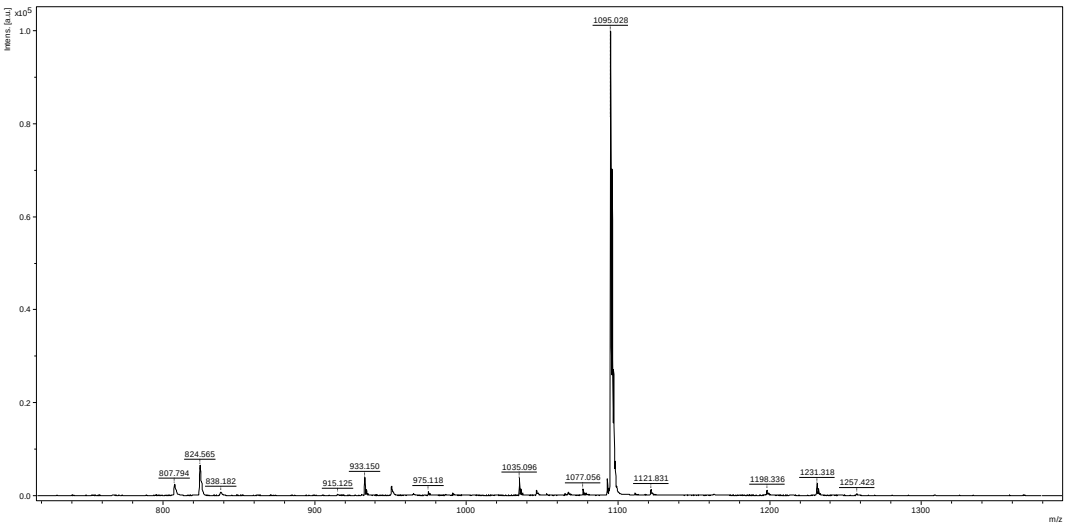




MALDI-MS: $[M + Na]^+$ $C_{38}H_{65}NNaO_{29}$ calcd for 1022.3540, found 1022.298.

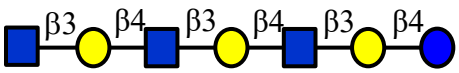
H0700

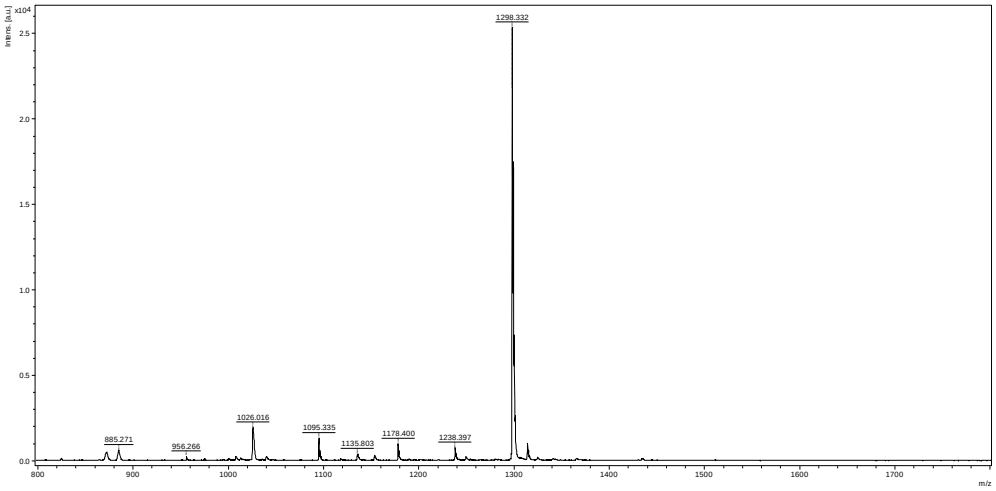




MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{31}$ calcd for 1095.3704, found 1095.028.

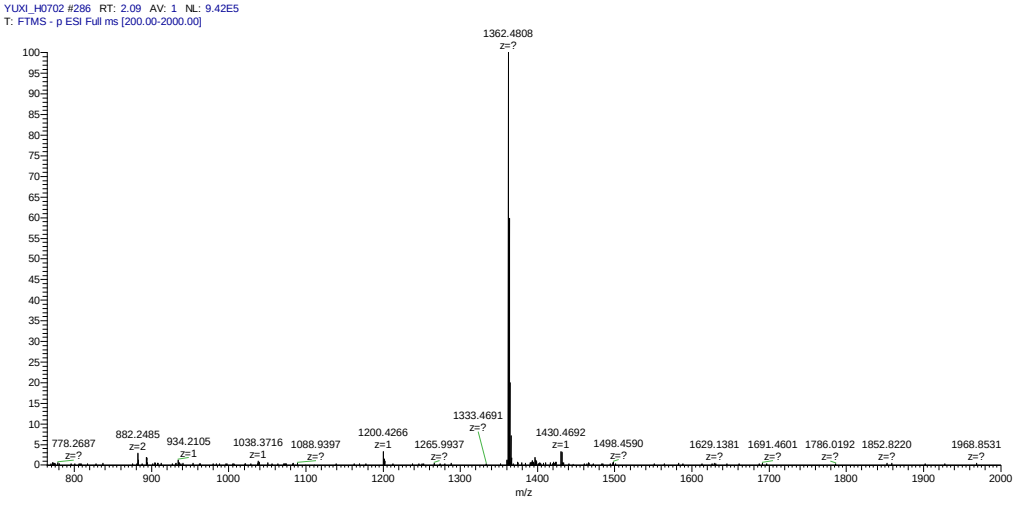
H0701





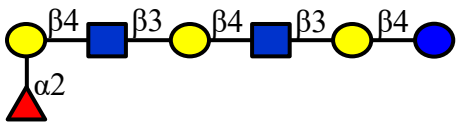
MALDI-MS: $[M + Na]^+$ $C_{48}H_{81}N_3NaO_{36}$ calcd for 1298.4497, found 1298.332.

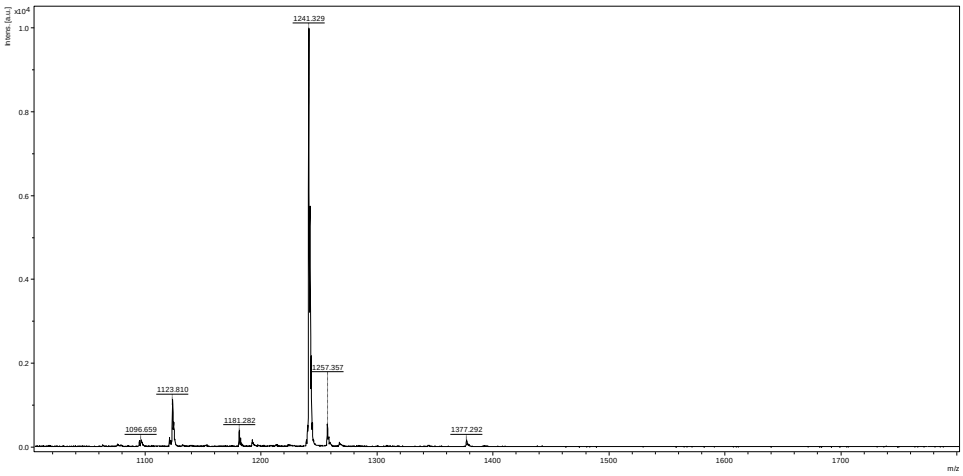
H0702



ESI-MS: $[M - H]^-$ $C_{51}H_{84}N_3O_{39}$ calcd for 1362.4682, found 1362.4808

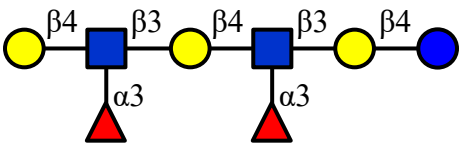
H0703

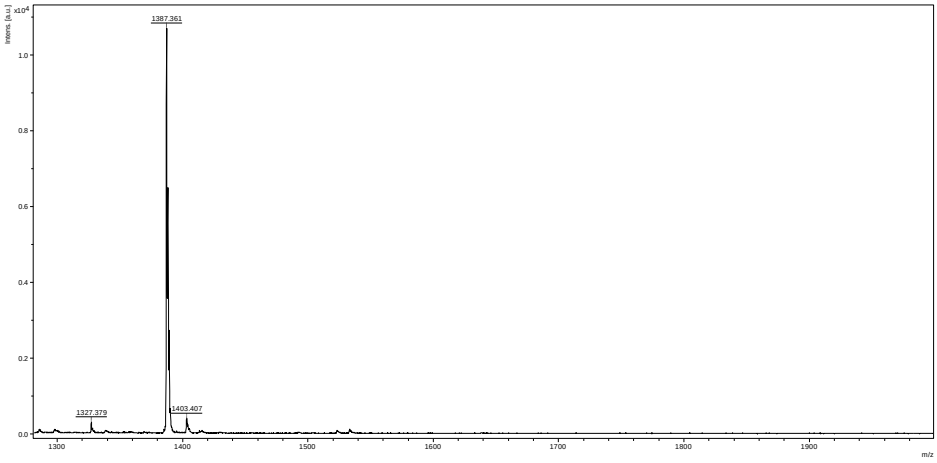




MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{35}$ calcd for 1241.4283, found 1241.329.

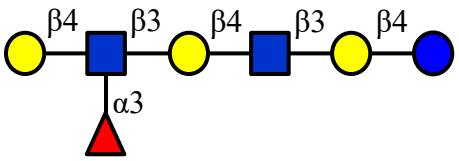
H0704

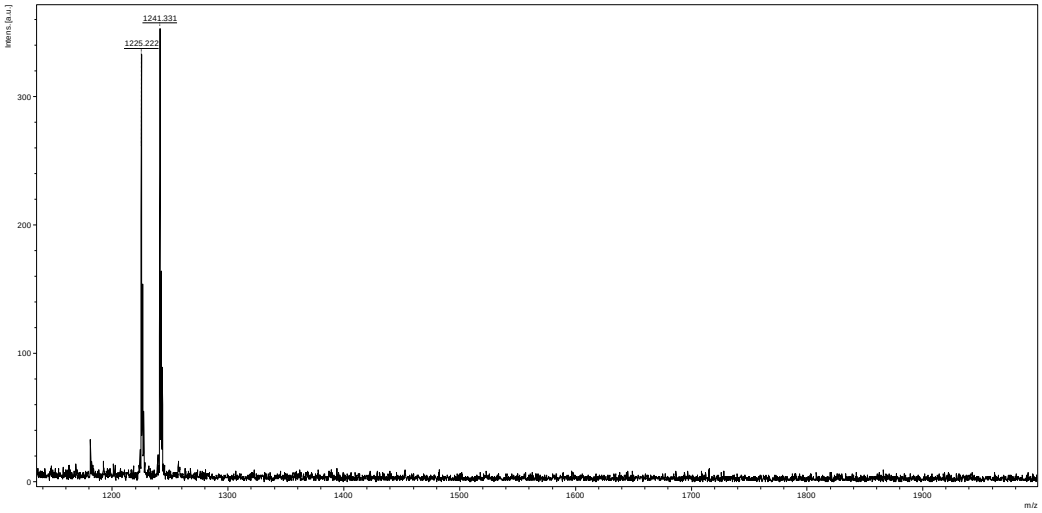




MALDI-MS: $[M + Na]^+$ $C_{52}H_{88}N_2NaO_{39}$ calcd for 1387.4862, found 1387.361.

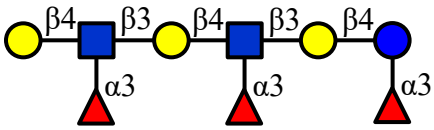
H0705

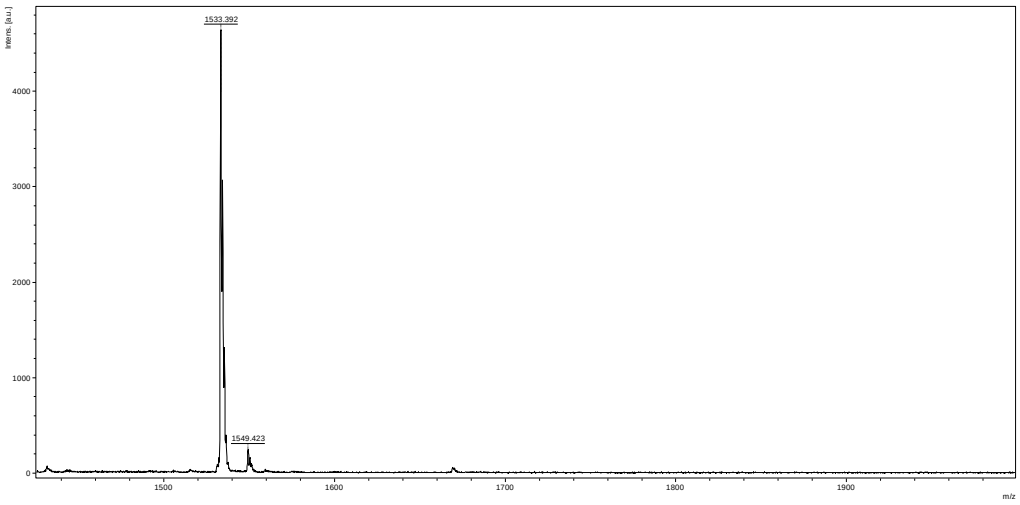




MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{35}$ calcd for 1241.4283, found 1241.331.

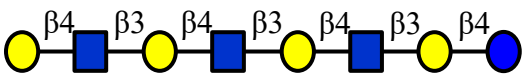
H0706

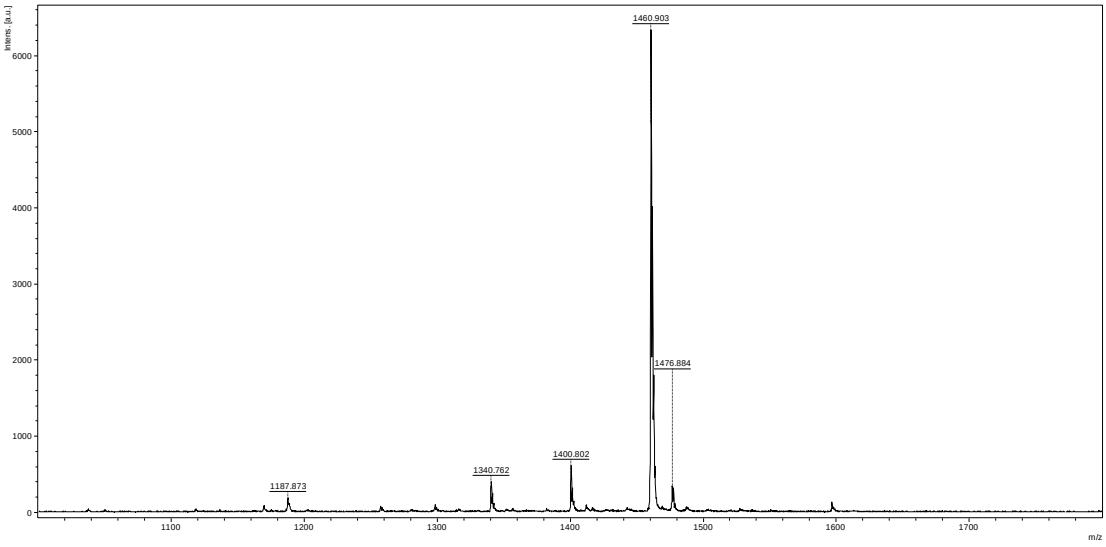




MALDI-MS: $[M + Na]^+$ $C_{58}H_{98}N_2NaO_{43}$ calcd for 1533.5441, found 1533.392.

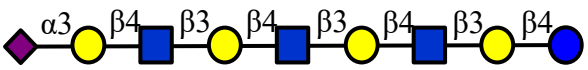
H0800

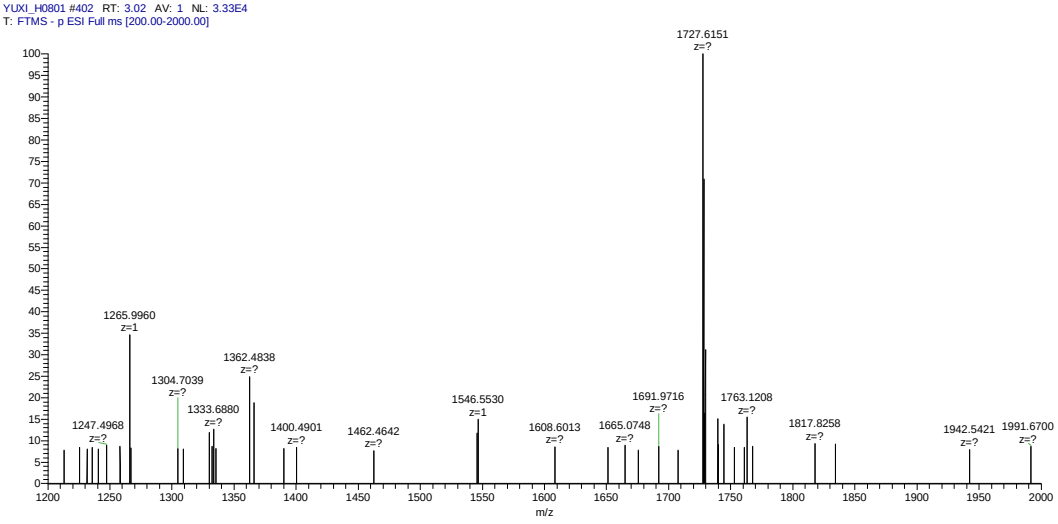




MALDI-MS: $[M + Na]^+$ $C_{54}H_{91}N_3NaO_{41}$ calcd for 1460.5026, found 1460.903.

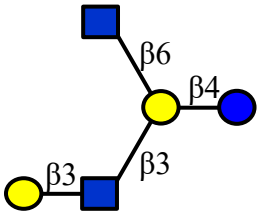
H0801

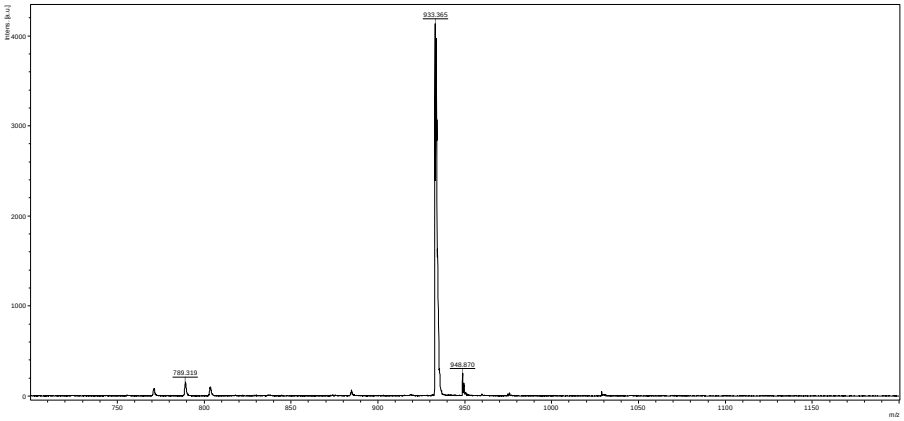




ESI-MS: $[M - H]^-$ $C_{65}H_{107}N_4O_{49}$ calcd for 1727.6004, found 727.6151

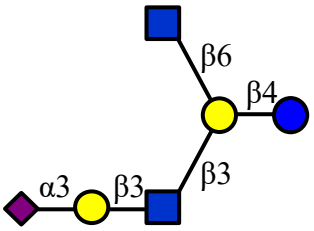
H0900

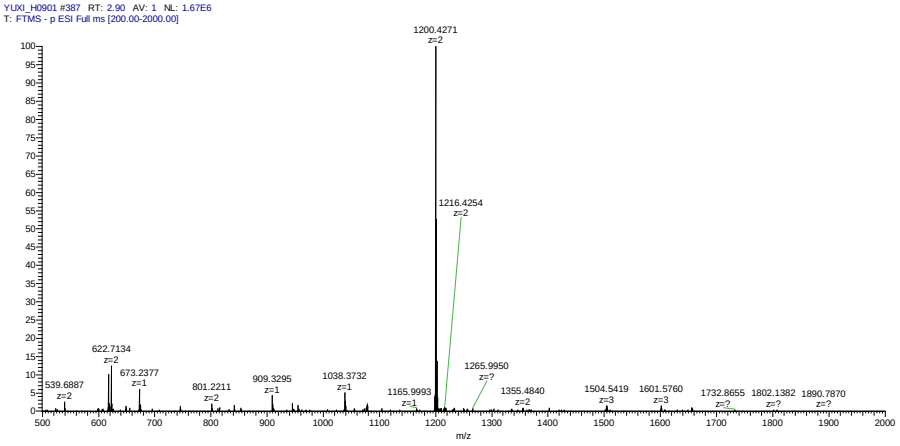




MALDI-MS: $[M + Na]^+$ $C_{34}H_{58}N_2NaO_{26}$ calcd for 933.3175, found 933.365.

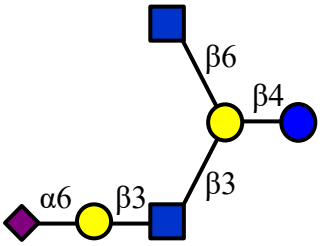
H0901

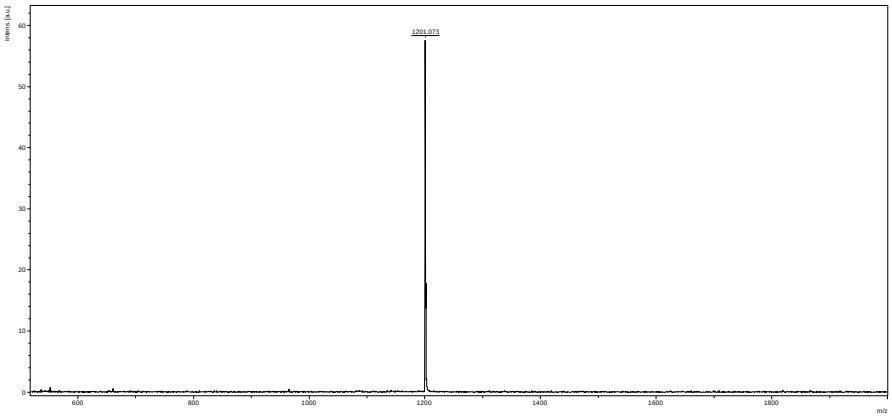




ESI-MS: $[M - H]^-$ $C_{45}H_{74}N_3O_{34}$ calcd for 1200.4154, found 1200.4271

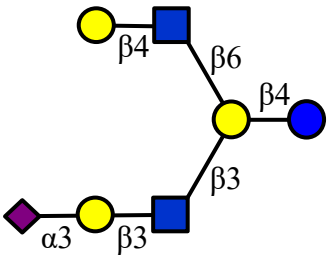
H0902

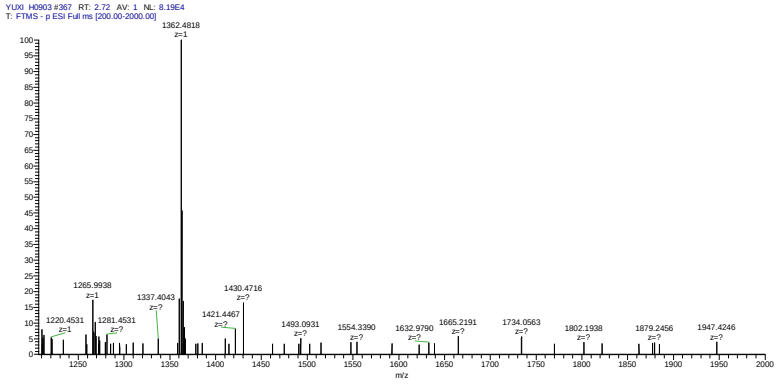




ESI-MS: $[M - H]^-$ $C_{45}H_{74}N_3O_{34}$ calcd for 1200.4154, found 1201.073

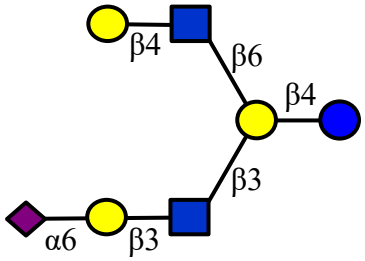
H0903

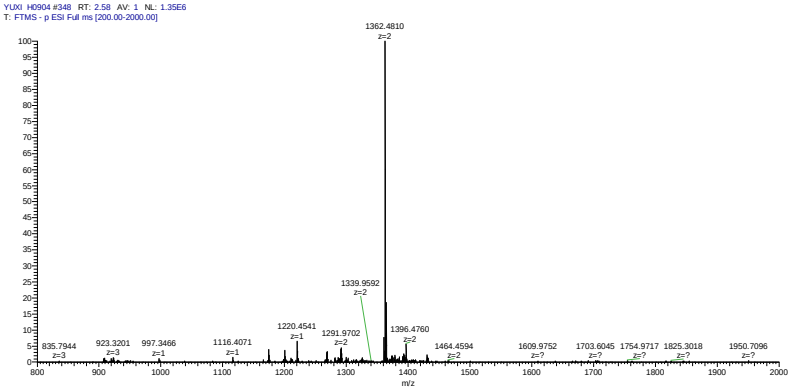




ESI-MS: $[M - H]^-$ $C_{51}H_{84}N_3O_{39}$ calcd for 1362.4682, found 1362.4818

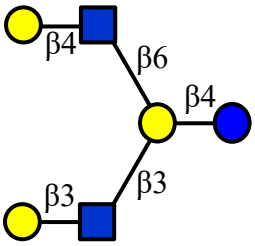
H0904

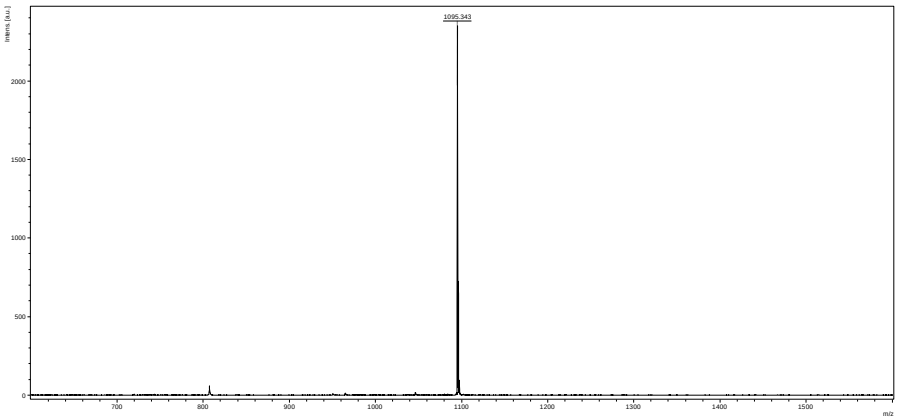




ESI-MS: $[M - H]^-$ $C_{51}H_{84}N_3O_{39}$ calcd for 1362.4682, found 1362.4810

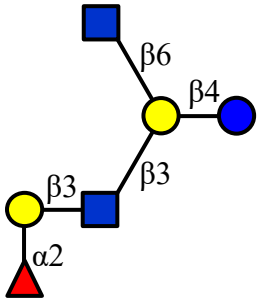
H0905

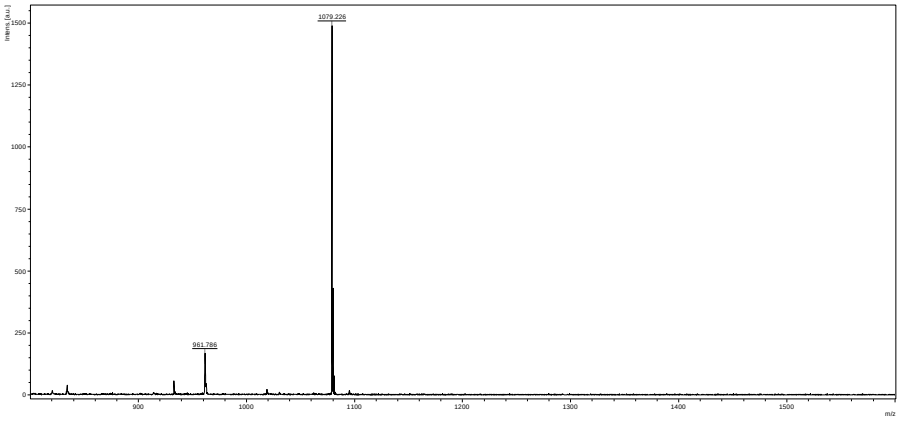




MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{31}$ calcd for 1095.3704, found 1095.343.

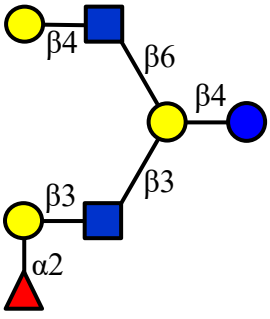
H0906

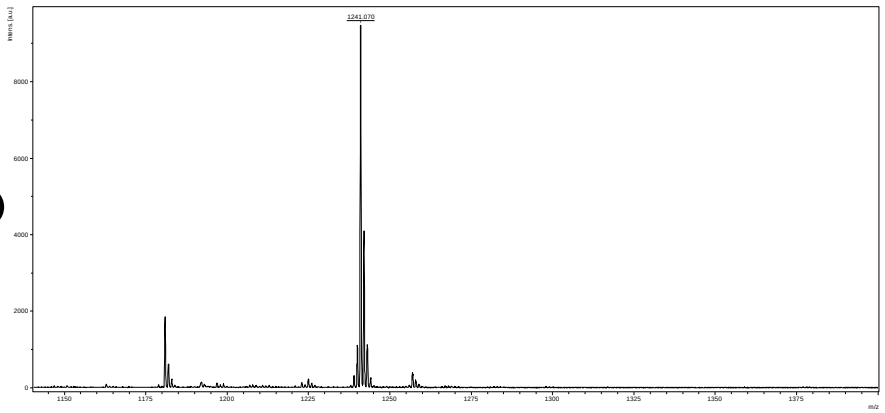




MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{30}$ calcd for 1079.3755, found 1079.226.

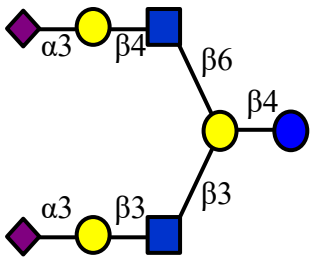
H0907

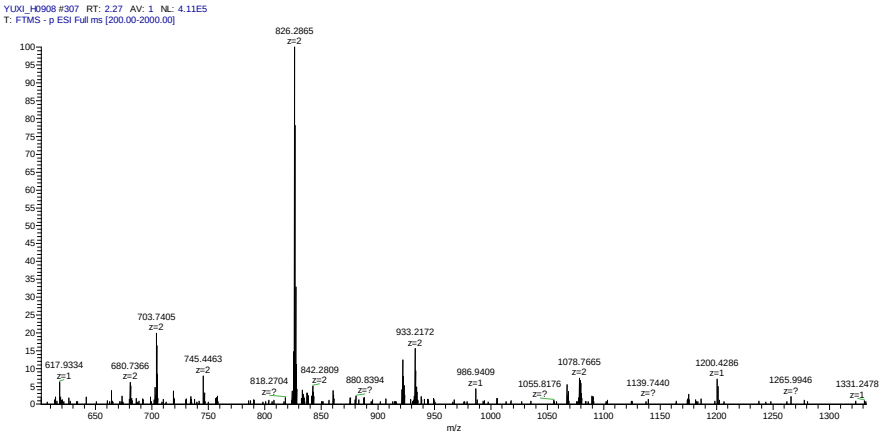




MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{35}$ calcd for 1241.428, found 1241.070.

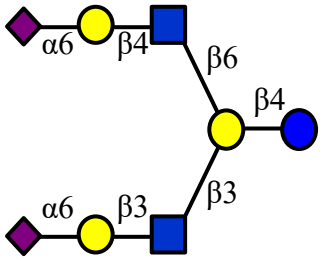
H0908

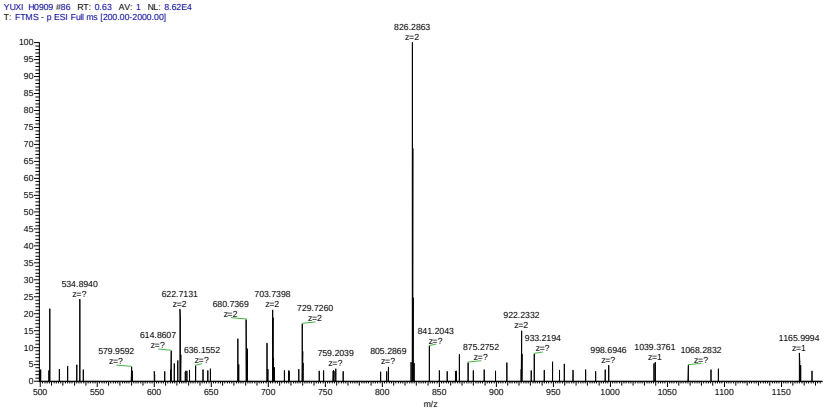




ESI-MS: $[M - 2H]^{2-}$ $C_{62}H_{100}N_4O_{47}$ calcd for 826.2784, found 826.2866.

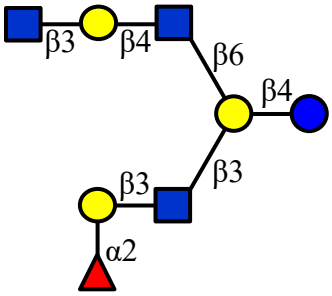
H0909

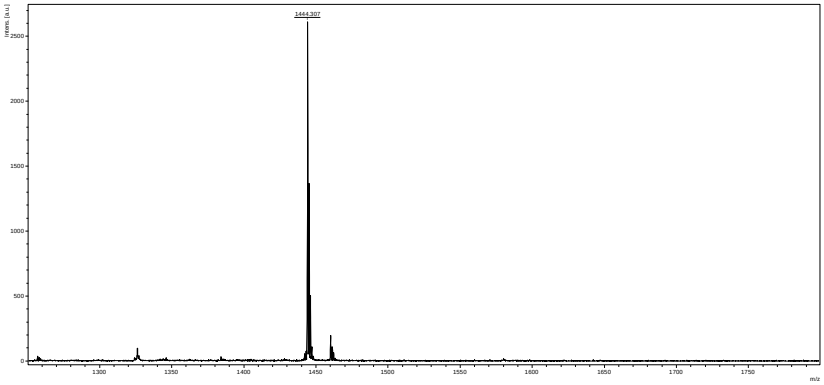




ESI-MS: $[M - 2H]^{2-}$ $C_{62}H_{100}N_4O_{47}$ calcd for 826.2784, found 826.2863.

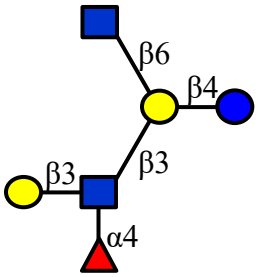
H0910

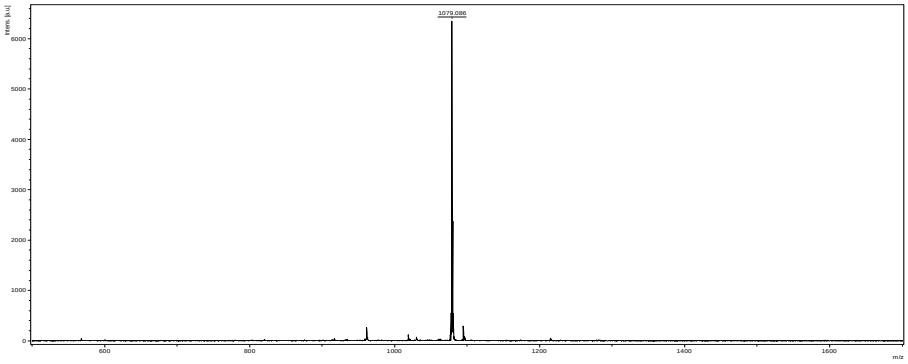




MALDI-MS: $[M + Na]^+$ $C_{54}H_{91}N_3NaO_{40}$ calcd for 1444.5077, found 1444.307.

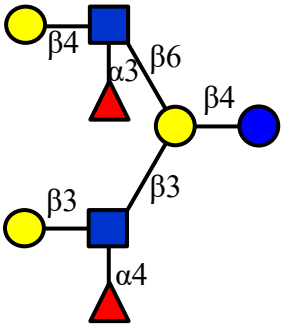
H0911

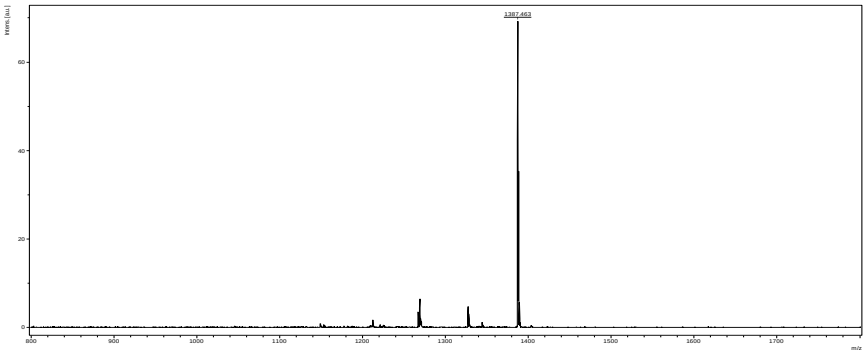




MALDI-MS: $[M + Na]^+$ $C_{40}H_{68}N_2NaO_{30}$ calcd for 1079.3755, found 1079.086.

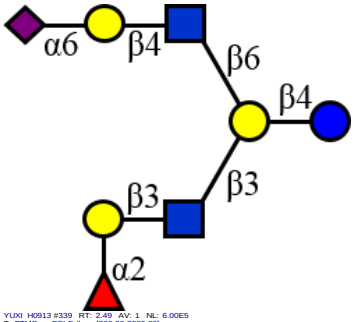
H0912

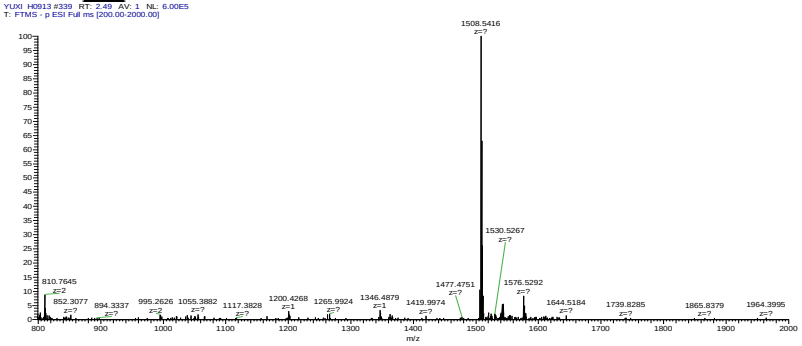




MALDI-MS: $[M + Na]^+$ $C_{52}H_{88}N_2NaO_{39}$ calcd for 1387.486, found 1387.478.

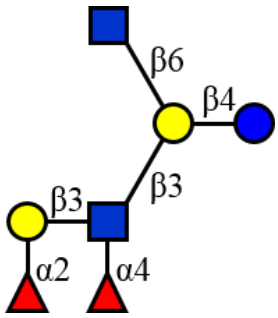
H0913

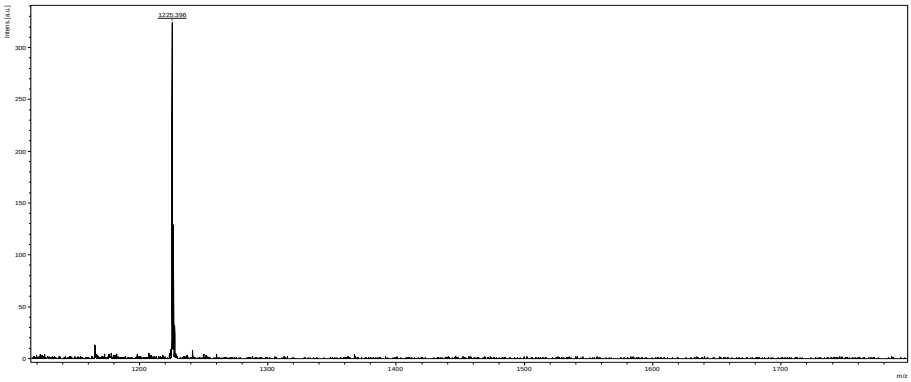




ESI-MS: $[M - H]^-$ $C_{57}H_{94}N_3O_{43}$ calcd for 1508.5261, found 1508.5416

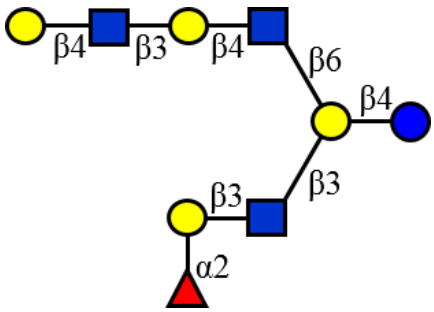
H0914

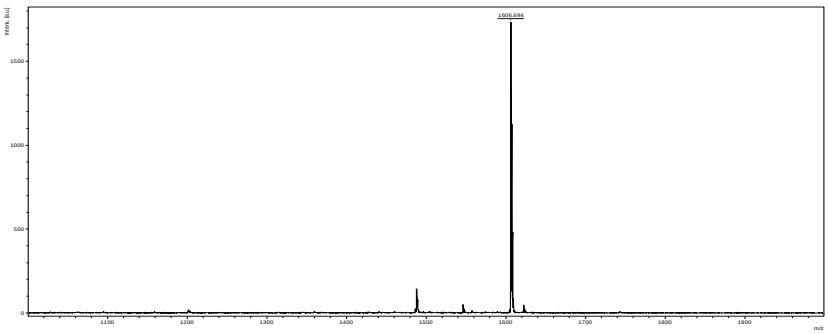




MALDI-MS: $[M + Na]^+$ $C_{46}H_{78}N_2NaO_{34}$ calcd for 1225.433, found 1225.396.

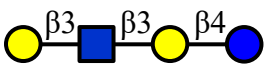
H0915

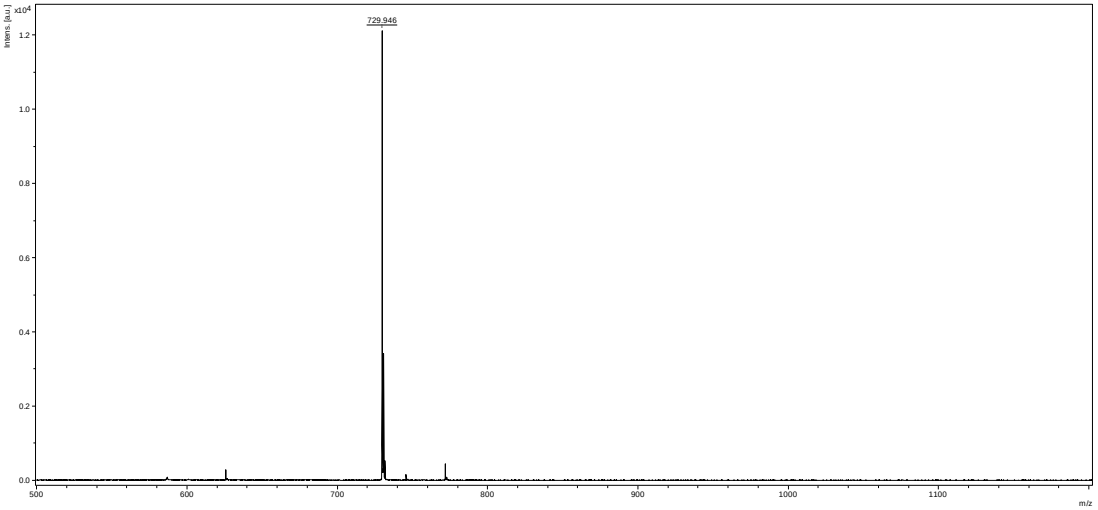




MALDI-MS: $[M + Na]^+$ $C_{60}H_{101}N_3NaO_{45}$ calcd for 1606.5605, found 1606.694.

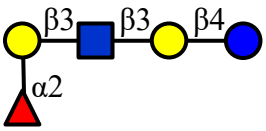
H1000

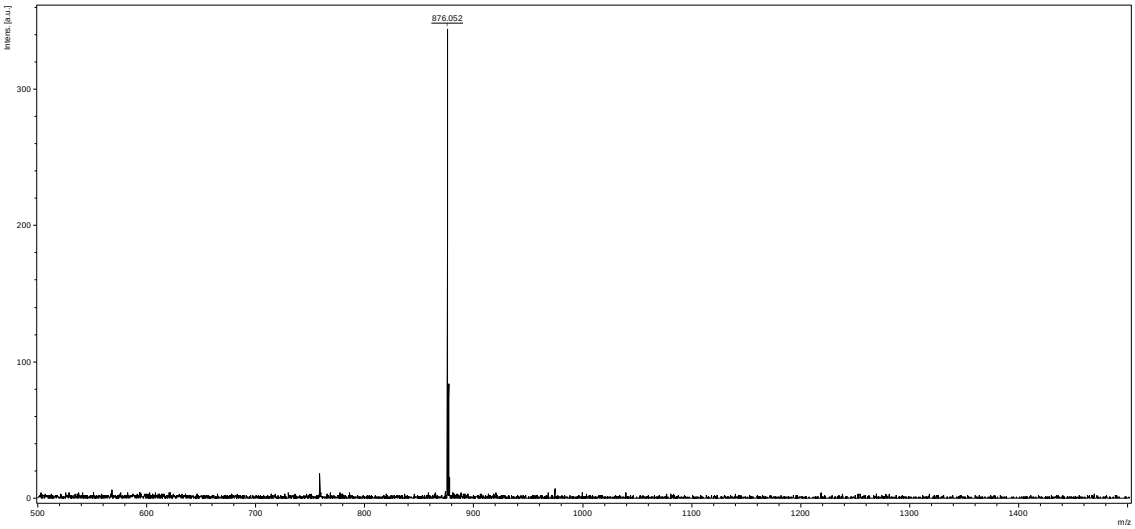




MALDI-MS: $[M + Na]^+$ $C_{26}H_{45}NNaO_{21}$ calcd for 730.2382, found 729.946.

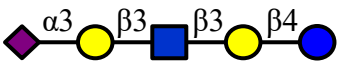
H1001

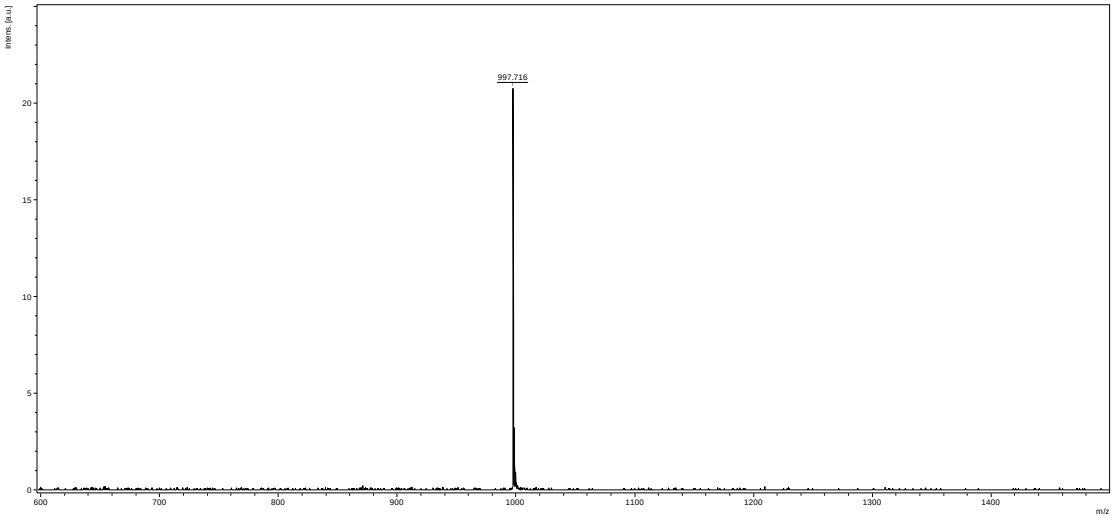




MALDI-MS: $[M + Na]^+$ $C_{32}H_{55}NNaO_{25}$ calcd for 876.2961, found 876.052.

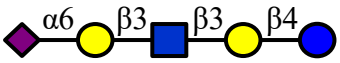
H1002

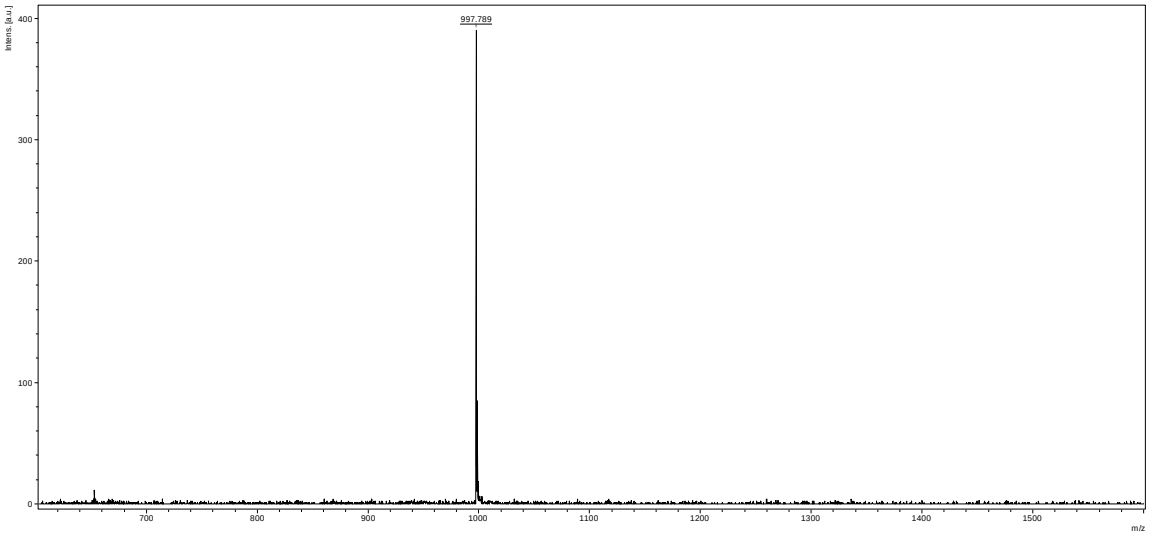




MALDI-MS: $[M - H]^-$ $C_{37}H_{61}N_2O_{29}$ calcd for 997.3360, found 997.716.

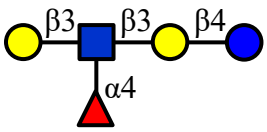
H1003

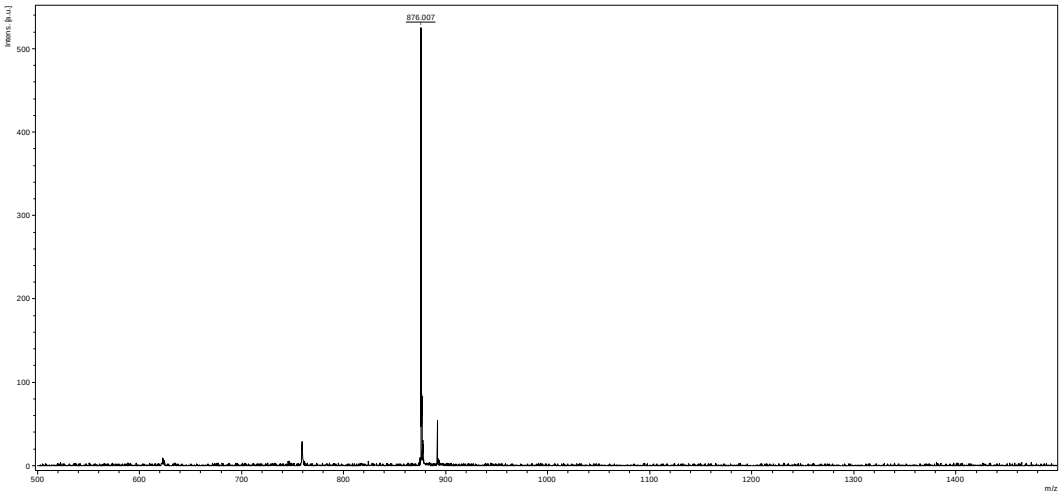




MALDI-MS: $[M - H]^-$ $C_{37}H_{61}N_2O_{29}$ calcd for 997.3360, found 997.789.

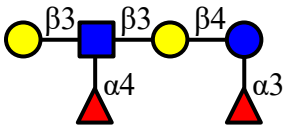
H1004

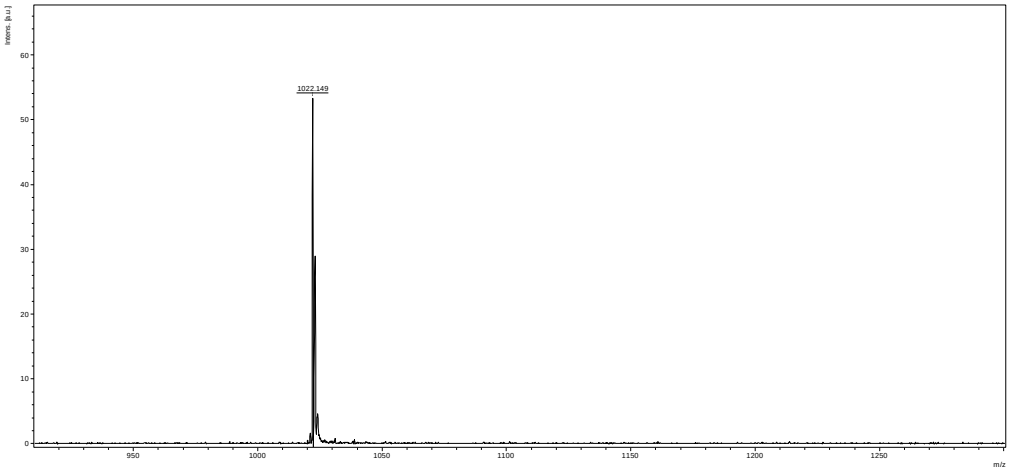




MALDI-MS: $[M + Na]^+$ $C_{32}H_{55}NNaO_{25}$ calcd for 876.2961, found 876.007.

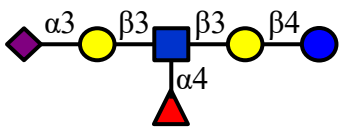
H1005

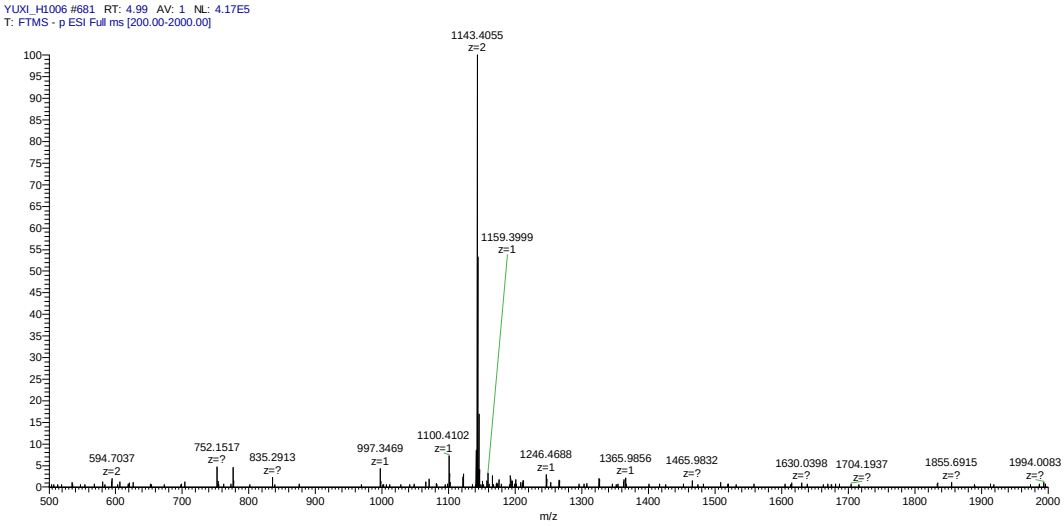




MALDI-MS: $[M + Na]^+$ $C_{38}H_{65}NNaO_{29}$ calcd for 1022.3540, found 1022.194.

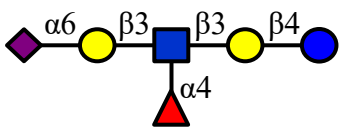
H1006

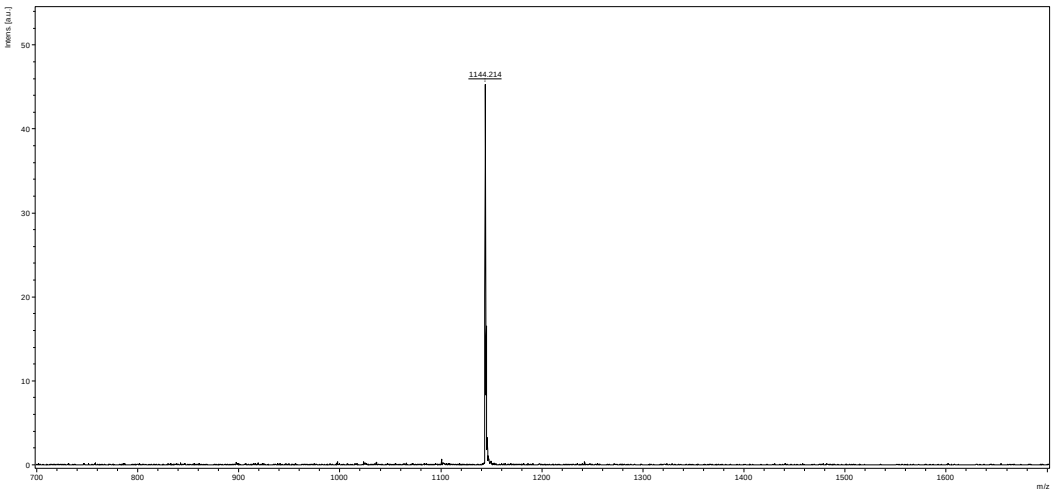




ESI-MS: $[M - H]^-$ $C_{43}H_{71}N_2O_{33}$ calcd for 1143.3939, found 1143.4055

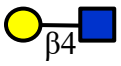
H1007

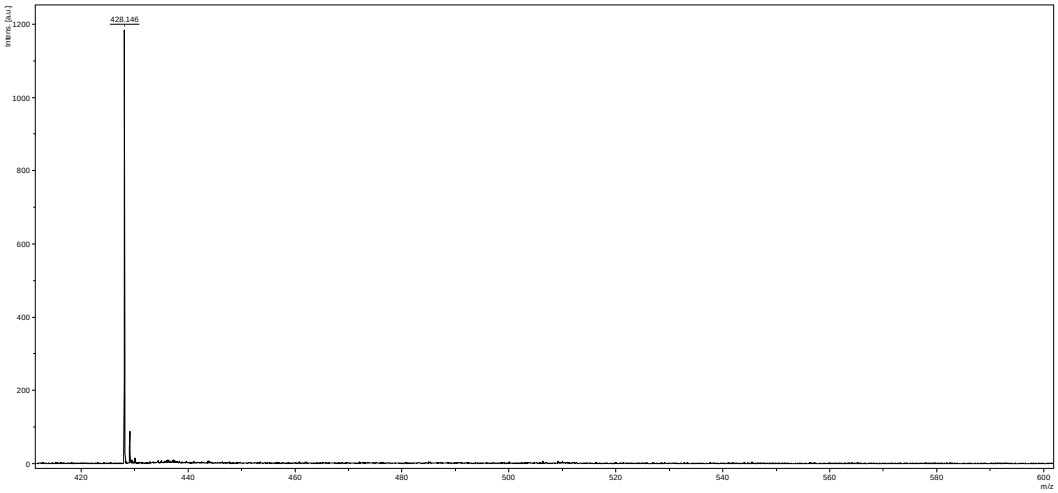




MALDI-MS: $[M - H]^-$ $C_{43}H_{71}N_2O_{33}$ calcd for 1143.3939, found 1144.214

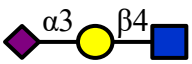
H1100

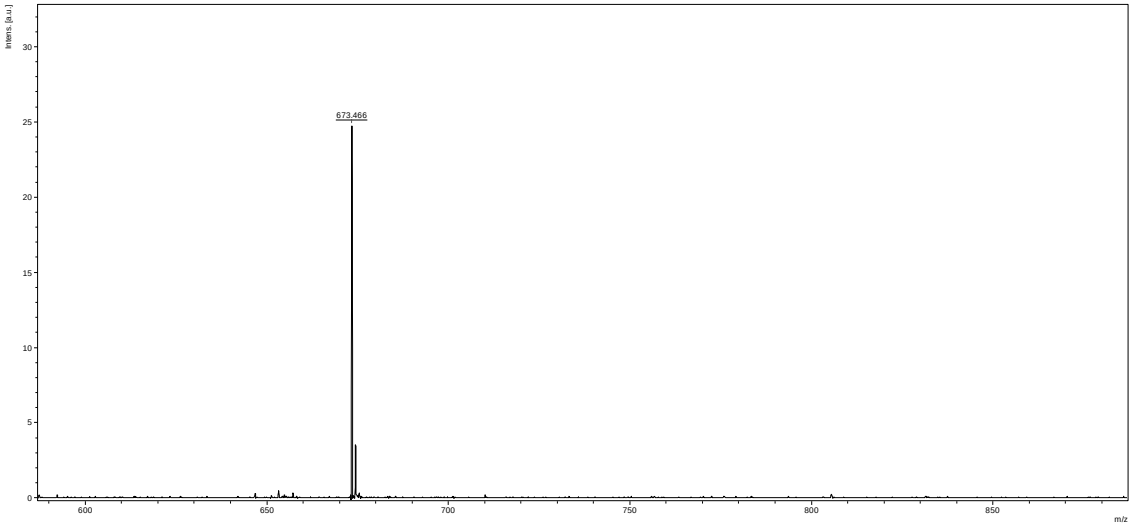




MALDI-MS: $[M + 2Na - H]^+$ $C_{14}H_{24}NNa_2O_{11}$ calcd for 428.1145, found 428.146.

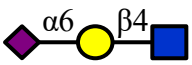
H1101

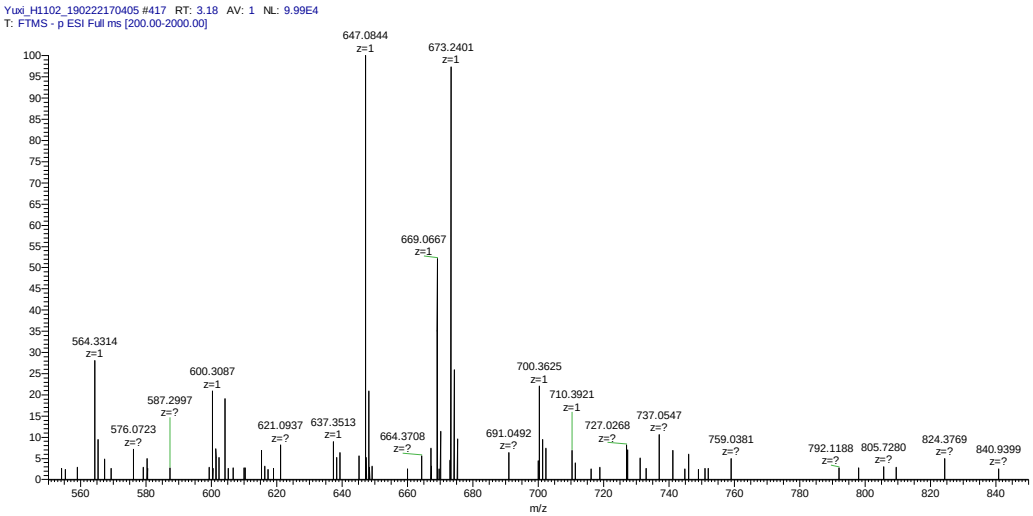




MALDI-MS: $[M - H]^-$ $C_{25}H_{42}N_2O_{19}$ calcd for 674.2382, found 673.466

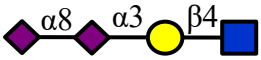
H1102

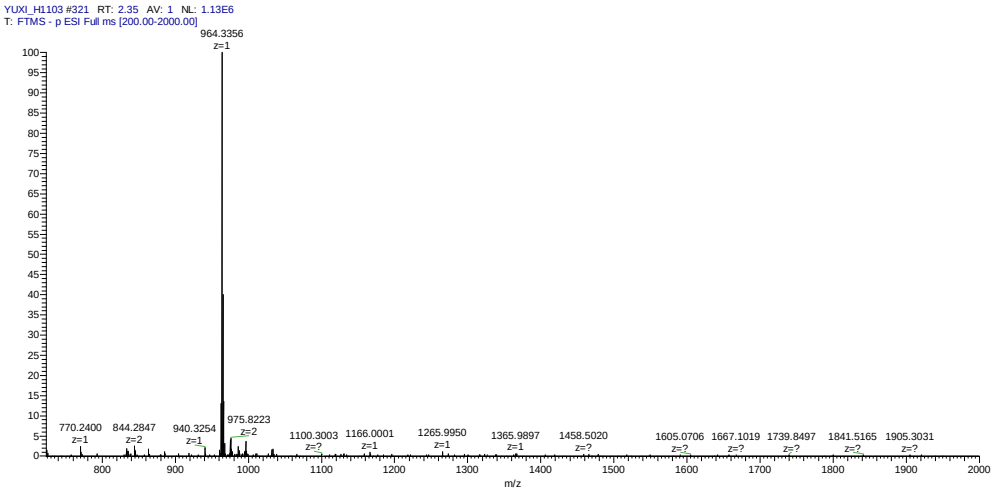




ESI-MS: $[M - H]^-$ $C_{25}H_{42}N_2O_{19}$ calcd for 674.2382, found 673.2401

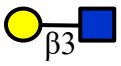
H1103

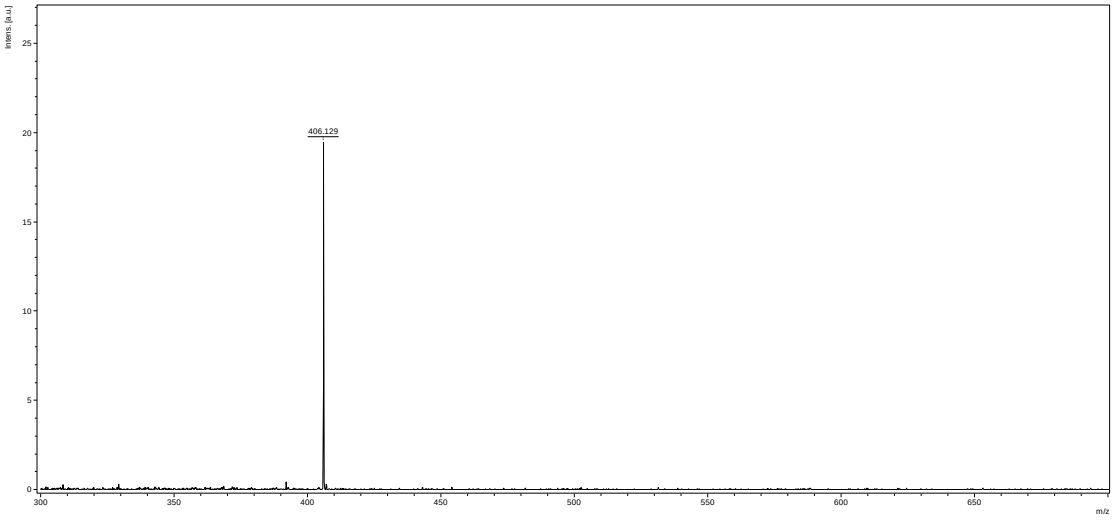




ESI-MS: $[M - H]^-$ $C_{36}H_{58}N_3O_{27}$ calcd for 964.3258, found 964.3356

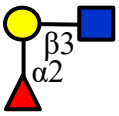
H1200

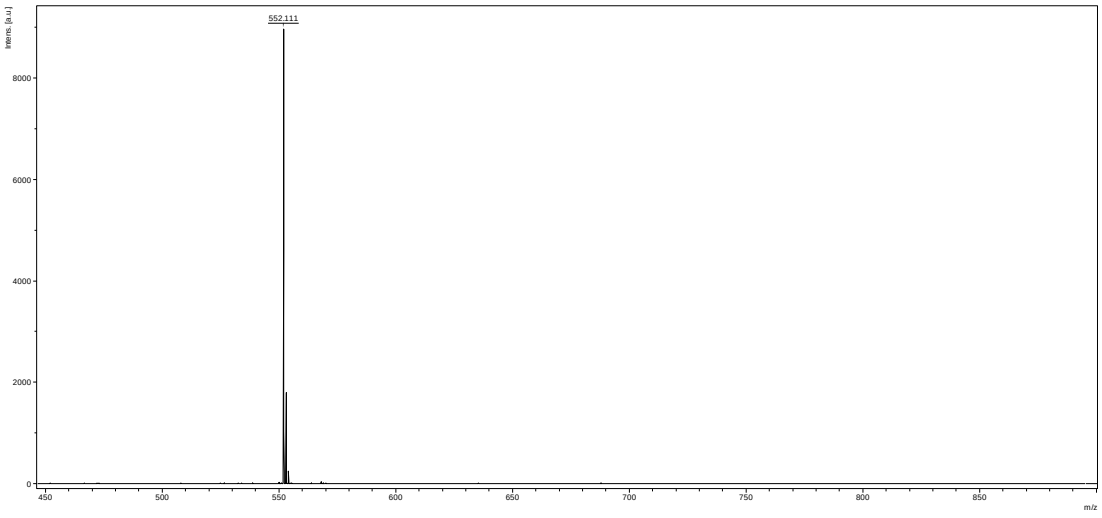




MALDI-MS: $[M + Na]^+$ $C_{14}H_{24}NNaO_{11}$ calcd for 406.1325, found 406.129.

H1201





MALDI-MS: $[M + Na]^+$ $C_{20}H_{35}NNaO_{15}$ calcd for 552.1904, found 552.111.

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4 ENZYMATIC SYNTHESIS OF O-ANTIGEN FROM P. AERUGINOSA SEROTYPE O11

4.1 Introduction

Lipopolysaccharides (LPS) are large molecules consisting of a lipid and a polysaccharide which contains O-antigen, outer core and inner core. They are found in the outer membrane of Gram-negative bacteria. These polysaccharides serve as an important molecular which mediate interaction between host cell and surrounding bacterium. Bacterial polysaccharides are extremely diverse, and their structural variation results in different serotypes or serogroups. For example, over 180 O-PS structures and 60 CPS structures have been identified for *Escherichia coli*. These high diversified LPS protect bacteria from immune clearance and act as the major virulence factor. [99] LPS are outstanding vaccine candidate for preventing bacterial infection.[100-102] There are several bacterial PS based vaccine which are commercially available. For example, Pfizer's PSs conjugate vaccine (PCV) Prevnar contains CPSs from 7 serotypes of *S. pneumoniae* conjugated with a protein, and its newer version Prevnar 13 contains protein-conjugated CPSs from 13 serotypes. These two vaccines represent Pfizer's top selling drugs, with an annual revenue of over 6 billion USD. Another example is Merk's 23-valent Pneumococcal PSs vaccine (PPSV), which contains solely PSs from 23 serotype of *S. pneumoniae* strains. [103-104] Pseudomonas aeruginosa is one representative of gram negative, rod-shape bacterium which is widely distributed in the environment. It is an opportunistic pathogen which can cause disease in patient with burn, cut wound or cystic fibrosis in the hospital. Treatment for Infections of P. aeruginosa is lacking efficient antibiotic due to its resistance to many medicines. Currently Doctor uses a combination antibiotic treatment that contains cefepime, aztreonam, ciprofloxacin, levofloxacin, aminoglycosides, ticarcillin and

ceftazidime with considerations about how sever of the infection in the patient. The hospitalized patients are the major infected people due to their long present in hospital and weakness of immune system of body. Infections of *P. aeruginosa* causing a total of 51000 healthcare infections in the USA annually.[105-108]

Lipopolysaccharides (LPS) as a major component of the outer membrane of *P. aeruginosa* is composed of variable length of polysaccharides (O-antigen), fat acid chains (lipid A) and conservative inner and outer core oligosaccharides. [109] Lipid A usually contains 6 fat acid chains or more which are the most challenging part in vitro synthesis because of its highly hydrophobicity and complexity. Core oligosaccharides are attached to the lipid A. they are comprised of 3-deoxy-D-mannooctulosonic acid (KDO), modified KDO with phosphate, D-glucose, D-mannose, D-galactose. More than 20 heterogenous serotype of O-antigens of *P. aeruginosa* are the most variable component which leads to different immunological reactivity.

LPS-Conjugated vaccines are the most widely characterized and studied research field because of its accessibility and efficient immunogenicity. *P. aeruginosa* strain was classified into 20 major serotypes, O1 to O20, by The International Antigenic Typing Scheme (IATS)[110]

Table 4.1 O-Antigen repeat units of the IATS reference strains

Serotype	*O-antigen repeat unit*
O1	4-D-GalNAc-β4-D-GlcNAc3NAcA-D-3-D-FucANc-3-QuiNAc
O2	4- β-D-ManNAc3NAmA-4-L-GulNAc3NAcA- β3-D-FucNAc
O3	2-L-Rha3OAc-6-D-GlcNAc-4-L-FucNAc-β3-D-QuiNAc4NSHb
O4	2-L-Rha-3-L-FucNAc-3-L-FucNAc-3-D-QuiNAc
O5	4-β-D-ManNAc3NAmA-β4-D-ManNAc3NAcA- β3-D-FucNAc
O6	3-L-Rha-4-D-GalNAc3OAcAN-4-D-GalNFoA-3-D-QuiNAc
O7	4-Pse4OAc5NRHb7NFo--β2,4-D-Xyl-3-D-FucNAc
O8	4-Pse4OAc5NAc7NFo--β2,4-D-Xyl-3-D-FucNAc
O9	4-Pse4OAc5NAc7NRHb--2,4-D-FucNAc-β3-D-QuiNAc
O10	3-L-Rha2OAc-4-L-GalNAcA-3-D-QuiNAc
O11	2-β-D-Glc3-L-FucNAc-3-D-FucNAc
O12	8-8eLeg5NAc7NAc-2,3-L-FucNAm-3-D-QuiNAc
O13	2-L-Rha-3-L-Rha-4-D-GalMac3PAcA-β3-D-QuiNAc
O14	2-D-Glc-3-L-Rha-3-L-Rha-4-D-GalNAc3A-β3-D-QuiNAc

O15	2- β-D-Ribf-4-D-GalNAc
O16	4-β-D-ManNAc3NAmA-β4-D-ManNAc3NAcA- β3-D-FucNAc
O17	4- β-D-Ribf-4-D-GalNAc
O18	4-L-GulNAc3NAmA-β4-D-ManNAc3NAcA- β3-D-FucNAc
O19	3-L-Rha-4-L-GalNAcA-3-D-QuiNAc
O20	4-L-GulNAc3NAmA-β4-D-ManNAc3NAcA- β3-D-FucNAc4OAc

O-Antigen from *P. aeruginosa* serotype O11 is ideal starting target for biosynthesis because of its simplicity of oligosaccharide structure. (**Figure 4.1**) Successful synthesis O-antigen can be a fundamental stone to develop vaccine to protect human from this specific type of *P. aeruginosa*. Currently the polysaccharides are isolated from bacteria culture as heterogeneous mixtures. Access to the pure and homogenous forms of LPS is tremendous benefit to such research field. In this study we will address this problem by employing and developing step wise chemoenzymatic approaches to get uniform pure O-Antigen.

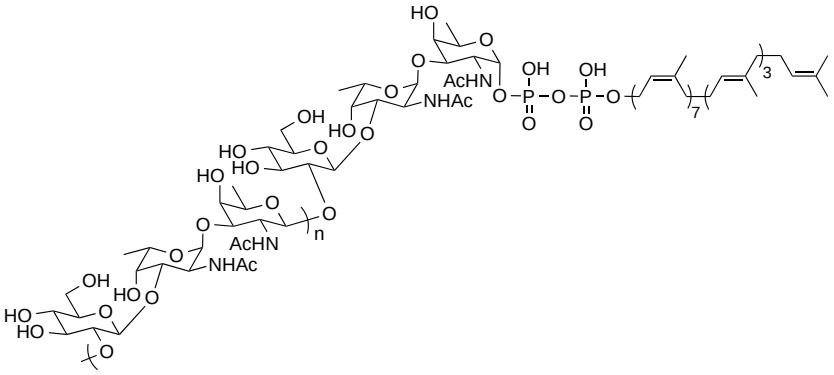


Figure 4.1 P. aeruginosa serotype O11 O-Antigen

4.2 Results and Discussion

4.2.1 Enzymatic preparation of UDP-L-FucNAc and UDP-ManNAcA

O-Antigen is synthesized on the inner membrane of cytoplasmic surface. Biosynthesis of proposal target requires these rare corresponding active form of sugar (UDP-Sugar). Our lab has

developed a highly efficient one-pot multienzyme (OPME) system for hundred milligram scale production of sugar nucleotide. We successfully utilized this theory into biosynthesis of UDP-L-FucNAc and UDP-ManNAcA by taking advantage of sugar nucleotide salvage pathway to avoid time-consuming, tedious purification step. [111]

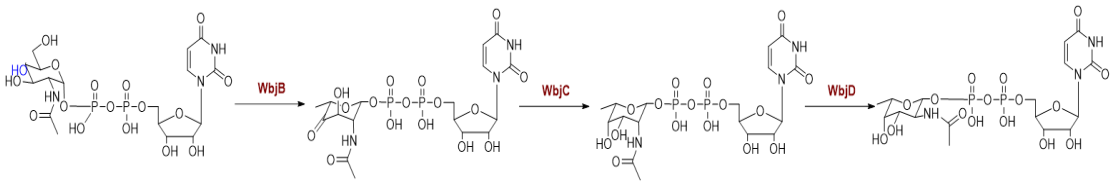


Figure 4.2 Biosynthesis of UDP-L-FucNAc

WbjB and WbjC were cultured at 37°C for overnight. After that inductions were activated by adding a low concentration of IPTG (0.2mM) at low temperature 16 °C. Ni-NTA affinity chromatography were employed to purify both enzymes with 6His tag to 90% with high yield. The estimate yield of both pure WbjB and WbjC from 1L culture is around 10 mg.

UDP-L-FucNAc was enzymatically synthesized by converting substrate UDP-GlcNAc which is more common sugar nucleotide. In our lab we have already addressed problem about large scale enzymatic synthesis of common UDP-sugar such as UDP-GlcNAc, UDP-GalNAc, UDP-GlcA, UDP-GalA, GDP-Fuc and CMP-Sia.[112] UDP-GlcNAc was sequentially epimerized by WbjB, WbjC and WbjD. Due to highly instability of UDP-L-FucNAc, the precursor UDP-PneNAc was enzymatically synthesized with NADPH as a reductant in hundred milligram scale. WbjD and Corresponding Glycotransferase WbjE will be added together to get L-FucNAc transferred without purification of unstable UDP-L-FucNAc.

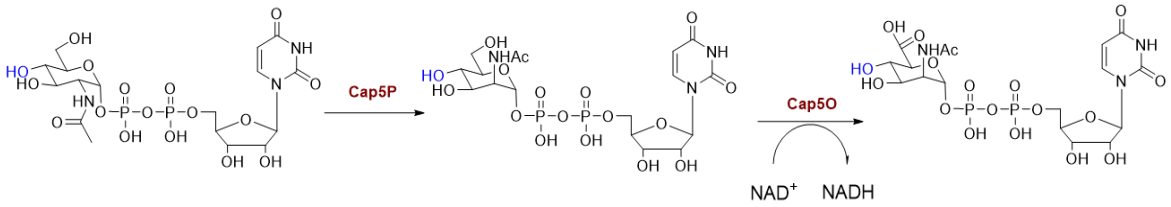


Figure 4.3 Biosynthesis of UDP-ManNAcA

UDP-ManNAcA was stepwise enzymatical synthesized by Cap5P, an epimerase to flip up NHAc group on C2 position, and Cap5O, oxidize C6 with NAD+. Cap5P and Cap5O were purified following the abovementioned method. [113] Given the stability of UDP-ManNAcA, hundred milligram scale production was achieved in the presence of Mn^{2+} and NAD+. The Total Yield of UDP-ManNAcA atfter P2 purification can reach to 80%.

4.2.2 Enzymatic Extension of DFucNAc-PP-C11-phenol

Facile chemoenzymatic synthesis of O-repeat unit-PP-Lipid is the starting point to access the production of *P. aeruginosa* serotype O11 vaccine. In order to biosynthesize this compound first monosaccharide-PP-lipid, corresponding sugar nucleotide and Glycotransferase are three requirements. In this manuscript we propose a substrate, monosaccharide-PP-C11-phenol, which can be recognized by first glycotransferase WbjE.

Natural undecaprenol C55 would be the best lipid part candidate which is involved into the bacterial polysaccharide synthesis pathway. However due to the low water solubility of lipid and extremely difficulty in synthesis. We can start with truncated C11 chain which shows relatively high activity towards several steps of glycosylation. In fact, first monosaccharide with two phosphate and lipid are the minimum requirement for the formation of first glycosidic bond on first sugar.

In the biosynthesis of LPS from *P. aeruginosa*, we first transfer UDP-L-PneNAc prepared by WbjB, WbjC to DFucNAc-PP-C11-phenol with configuration α1-3. One pot mutil-enzyme strategy (WbjD and WbjE) was applied to first glycosylation step due to instability of UDP-L-FucNAc. Chemical coupling a phenol group to lipid part enable us to overcome the lack of UV or florescent detection. This advantage will greatly facilitate us when we purify the

bacterial Polysaccharide by HPLC. The next glycosylation step is transferring Glucose to LFucANc by WbjA with β1-3 linkage.

Each step will be monitored by HPLC and Mass spectrum in negative mode, after 80% conversion rate, next step will start. C18 was used for separation based on hydrophobicity of lipid and phenol monitored by 280 nm UV detector under a gradient condition (solution A: water, solution B: acetonitrile; flow rate: 1ml/min B%: 10%-40% within 30min).

In the future Wzy, a bacterial PS polymerase, and Wzz, a polymerase regulator, both will work on the polymerization of O repeat unit of RU-PP-lipid which may shows better immunogenicity towards *P. aeruginosa serotype* O11.

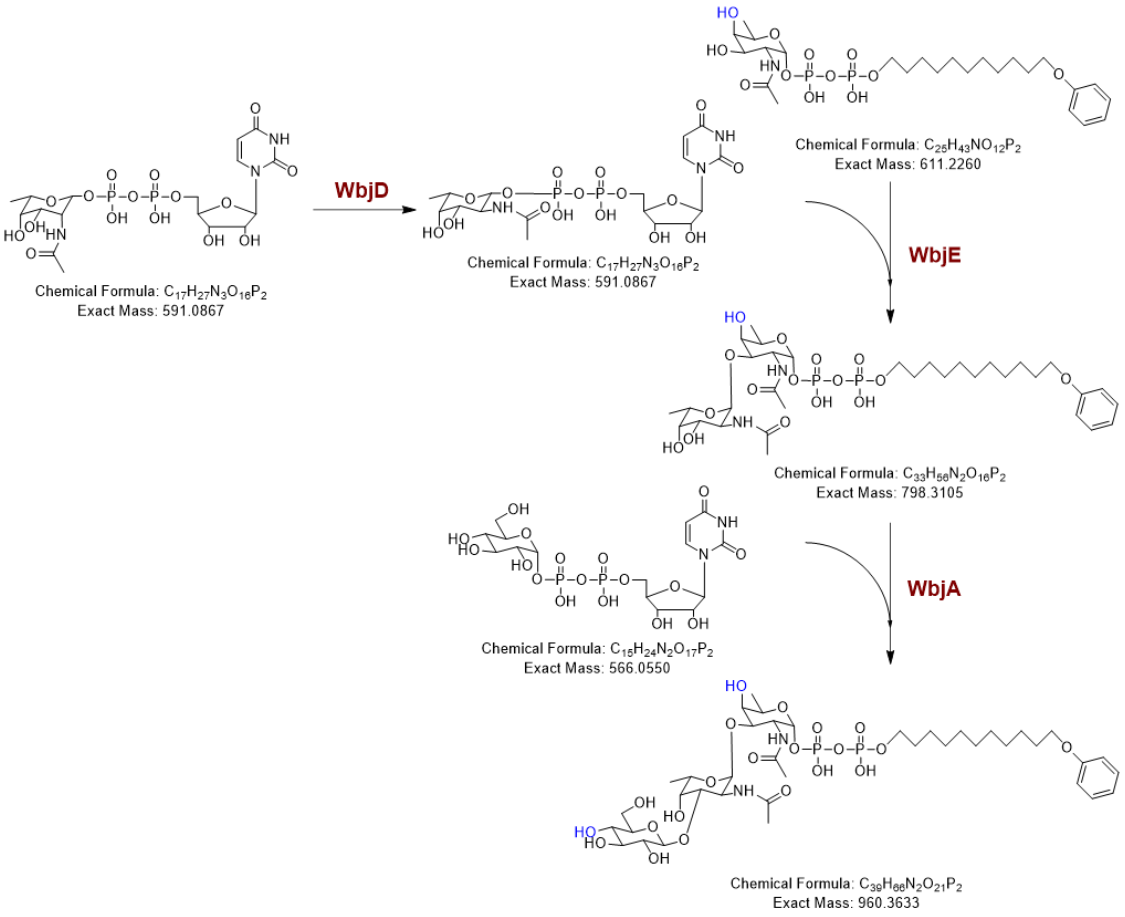


Figure 4.4 Biosynthesis of O-Antigen from P. aeruginosa serotype O11

WbjD and WbJE and WbjA were induced at low temperature 16 °C and low concentration of IPTG (0.2mM) to get entirely soluble form of enzymes.

As shown in **Figure 27**, B is before the IPTG induce, A is after IPTG induce, FT is flow through after Ni-NTA affinity column. All these enzymes with 6 his tags were purified as shown in the SDS gel.

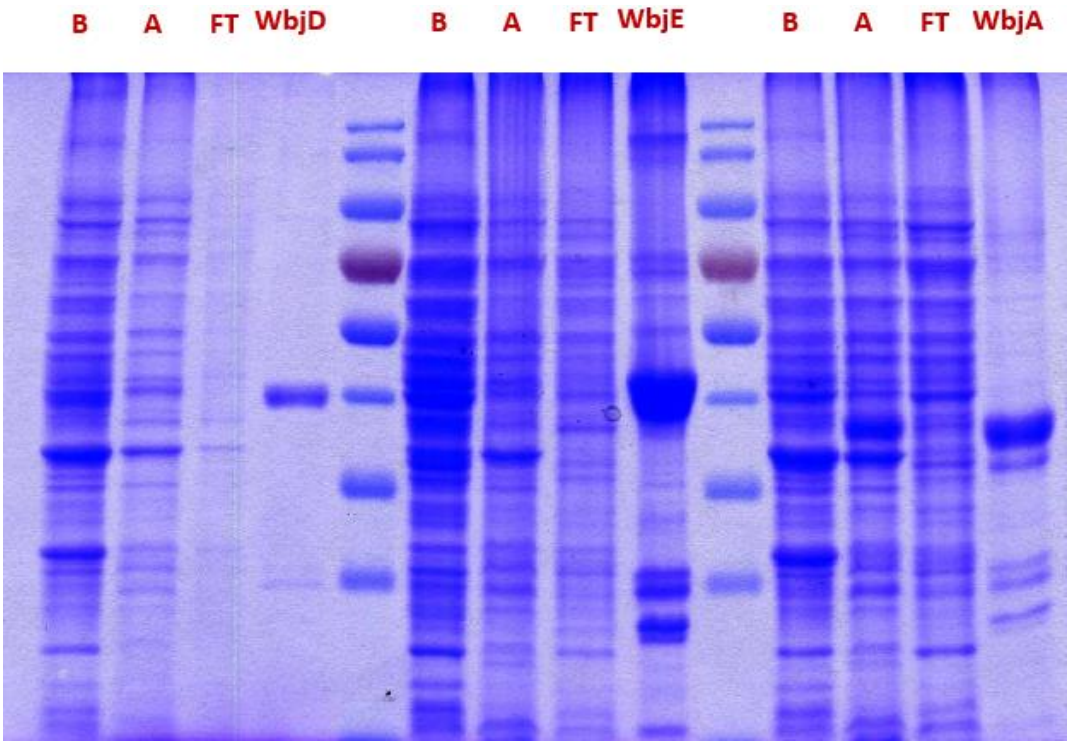


Figure 5 SDS-PAGE analysis of purified WbjD, WbjE and WbjA

4.3 Conclusion

Investigation into biosynthesis of O-antigen from *P. aeruginosa* is an excellent direction for synthesis of such macromolecule comparing to chemical method. Enzymatic synthesis method shows big advantages of less step, highly specificity toward substrate, easy purification and high yield. In the last decade many publications of the O-antigen cluster sequence and detailed information about LPS structures enable researcher to advance the real biosynthesis of

different type of O-antigen. Here we report that O-antigen DGlcβ3LFucNAcα3DFucNAc-PP-C11-Phenol was synthesized via WbjE and WbjA. This ongoing project will be further finished by synthesizing enough amount of final compound to get NMR result. The biosynthetic O-Antigen from *P. aeruginosa* serotype O11 could be valuable structure to be studied as potential candidate for an anti *P. aeruginosa* vaccine. However single repeat unit may not enough to activate strong immunogenicity. Longer repeat unit of O-antigen is needed for eliciting antibodies. [114]

4.4 Experimental Section

4.4.1 Materials

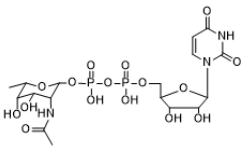
All Chemical reagents were purchased from sigma unless otherwise noted. Ni-NTA agarose was purchased from Qiagen. UDP-GlcNAc was prepared by utilizing salvage pathway via two enzymes (NahK and AGX1). [115] WbjB, WbjC, WbjD, Cap5P, Cap5O, WbjE and WbjA expression E. Coli strain were obtain by collaborating with Dr. Goldberg Joanna's lab from Emory University.

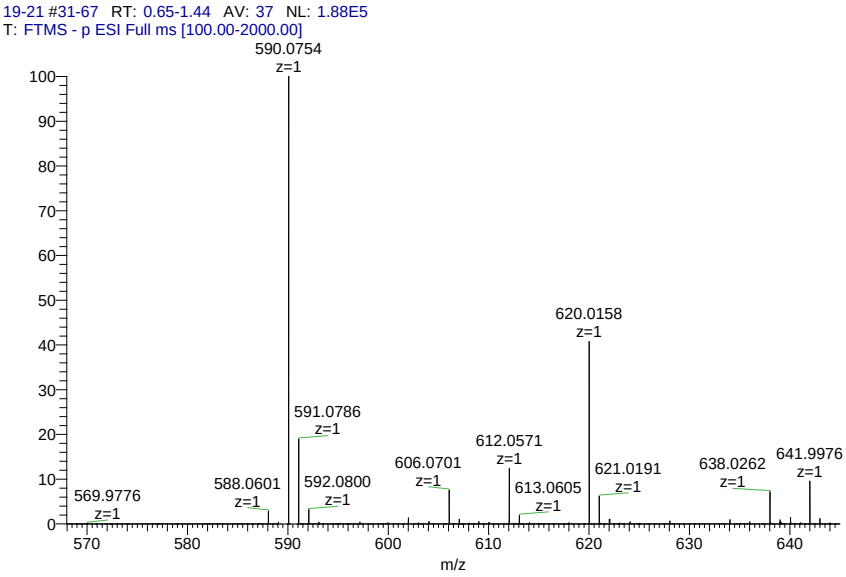
4.4.2 Expression, and purification of Enzymes

E. coli cells harboring the recombinant plasmids were grown in high salt LB medium at 37°C with 20 rpm shaking until OD_{600} reached 0.6-0.8, followed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2mM. The cells were harvested by centrifugation at 4000 rpm after 20h induction at 16°C and stored at -20°C until used. A Ni-NTA resin (Qiagen) column was utilized to purify desire the proteins by following the manufacture instructions. The purified proteins were desalted by PD-10 column (GE life science, USA) against 50mM Tris-HCl PH 8.0 and 10% glycerol. Purified enzymes were kept at -20°C until used.

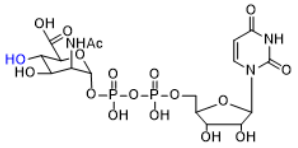
4.4.3 One pot multi-enzyme synthesis of UDP-PneNAc, UDP-ManNAcA and trisaccharide-PP-C11-Phenol

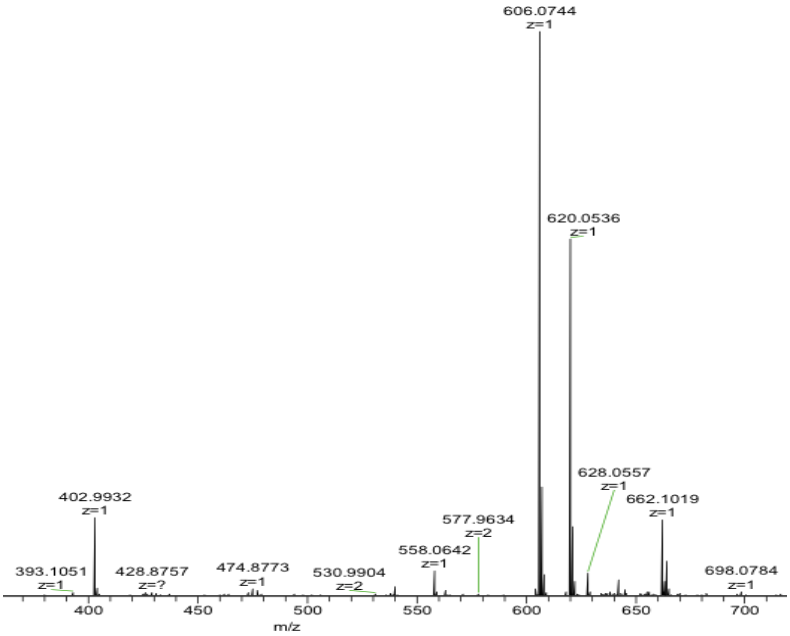
Production of large scale of UDP- PneNAc and UDP-ManNAcA were performed in a 10 ml reaction system containing 50mM PH8.0 Tris HCl buffer, 20mM UDP-GlcNAc, 20mM $MnCl_2$, 40mM NADPH for UDP-PneNAc, 20mM NAD+ for UDP-ManNAcA and corresponding enzymes. The reaction systems were incubated at 37°C water bath for overnight. The generation of UDP-PneNAc and UDP-ManNAcA were monitored by TLC (Isopropanol: NH_4OH: H_2O=7:3:2) and Capillary electrophoresis (20mM sodium tetraborate, 254nm, 20min and 75um diameter of capillary) and confirmed by MALDI-MS. After converting rate reach to 80%, the reactions were quenched by boiling for 10 min, centrifuged to remove precipitants and concentrated for separation. After that the concentrated reaction system were loading to a gel filtration column (Bio-Gel P2, Bio-Rad), fractions containing UDP-PneNAc or UDP-ManNAcA were pooled and lyophilized. Glc-β3-LFucANc-α3-DFucNAc-PP-C11-Phenol was sequentially enzymatic synthesized by adding WbjE and WbjA. UDP-L-FucNAc was transferred to DFucNAc in a 2ml reaction system containing 50mM PH8.0 Tris HCl buffer, 20mM UDP-PneNAc, 20mM $MnCl_2$, 20mM substrate and WbjD and WbjE. UDP-Glc was transferred to LFucNAc in a 2ml reaction system containing 50mM PH8.0 Tris HCl buffer, 20mM UDP-Glc, 20mM $MnCl_2$, 20mM substrate and WbjA. the reaction mixture was quenched by boiling for 10 min, centrifuged to remove precipitant and concentrated for loading to the P2 gel filtration.



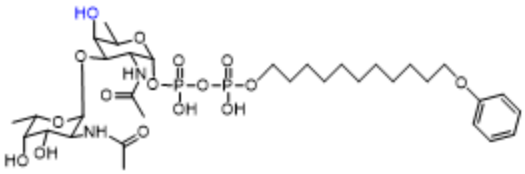


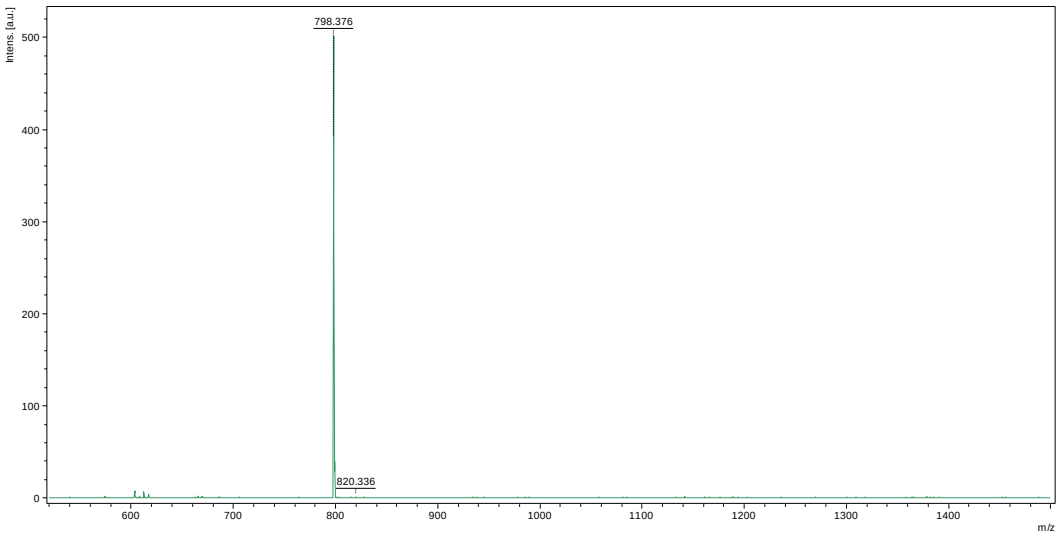
ESI-MS: $[M - H]^-$ $C_{17}H_{26}N_3O_{16}P_2$ calcd for 590.08, found 590.0754.



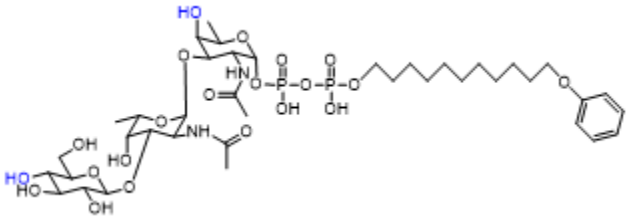


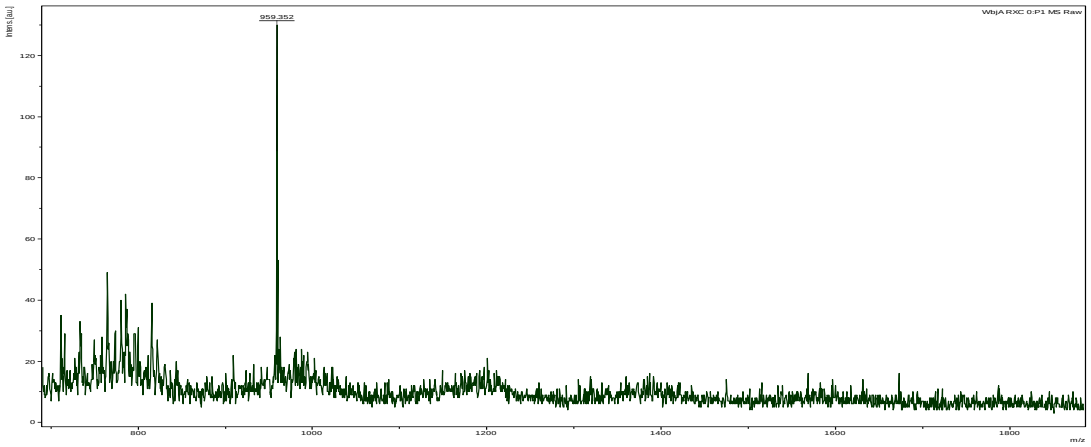
ESI-MS: $[M - H]^-$ $C_{17}H_{24}N_3O_{18}P_2$ calcd for 620.05, found 620.0536.





MALDI-MS: $[M - H]^-$ $C_{33}H_{55}N_2O_{16}P_2$ calcd for 797.31, found 798.376.





MALDI-MS: $[M - H]^-$ $C_{39}H_{65}N_2O_{21}P_2$ calcd for 959.3663, found 959.352.

4.4.4 HPLC method for purification of O-antigen

Gel filtration was first used for brief purification and desalting of O-antigen. But the final compound is still not pure enough to run NMR. Therefore disaccharide-PP-C11-Phenol reaction mixture was separated by high performance liquid chromatography (HPLC) and Detected by UV detector at 280 nm. Solvent A: 30mM potassium phosphate, PH 6.0; 5mM tetrabutylammoniumhydrogen sulfate, 2% acetonitrile and solvent B: acetonitrile was used as mobile phase. Xbridge peptide BEH C18 250*4mm (waters) was used as stationary phase. Sample was injected into HPLC and eluted by a gradient of solvent B (20%-70%).

4.5 Reference

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APPENDICES

Publications

Research publications. (*: Co-first author)

1. Zhang, J.; Chen, C.; Gadi, M. R.; Gibbons, C.; **Guo, Y.**; Cao, X.; Edmunds, G.; Wang, S.; Liu, D.; Yu, J.; Wen, L.; Wang, P. G., Machine-Driven Enzymatic Oligosaccharide Synthesis by Using a Peptide Synthesizer. *Angew Chem Int Ed Engl* **2018,** *57* (51), 16638-16642.
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