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Author(s)	RAVI KUMAR H V
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Synthetic Studies on Highly Complex *N*-glycans and Glycopeptides

(高度に複雑なアスパラギン結合型糖鎖と糖ペプチドの合成研究)

A Thesis

Submitted for the Degree of

Doctor of Life Science

By

RAVI KUMAR H V

Transdisciplinary Life Science Course

Graduate School of Life Science

Hokkaido University

Japan, 2013

DECLARATION

I hereby declare that the matter embodied in this thesis entitled ***"Synthetic Studies on Highly Complex N-glycans and Glycopeptides"*** is the result of investigations carried out by me under the supervision of ***Prof. Shin-Ichiro Nishimura*** at the Laboratory of Advanced Chemical Biology, Transdisciplinary Life Science Course, Graduate School of Life Science, Hokkaido University, Japan and it has not been submitted elsewhere for the award of any other degree or diploma.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made whenever the work described has been based on the findings of the other investigators. Any omission that might have occurred by oversight or error of judgments is regretted.

RAVI KUMAR H V

CERTIFICATE

I hereby certify that the work described in this thesis entitled ***"Synthetic Studies on Highly Complex N-glycans and Glycopeptides"*** has been carried out by ***RAVI KUMAR H V***, under my supervision at the Laboratory of Advanced Chemical Biology, Transdisciplinary Life Science Course, Graduate School of Life Science, Hokkaido University, Japan.

Prof. Shin-Ichiro Nishimura

(Research Supervisor)

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Abbreviations

Ac	Acetyl
Ac_2O	Acetic Anhydride
ACN	Acetonitrile
Bn	Benzyl
$BF_3{\cdot}Et_2O$	Borantrifluoride diethyl ether complex
CAN	Ceric Ammonium nitrate
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
DBU	1,8-diazabicyclo[5,4,0]undec-7-ene
1,2-DCE	1,2-dichloroethane
DHB	Dihydroxybenzoicacid
DIEA	*N*,*N*-diisopropylethylamine
DMAP	*N*,*N*-dimethyl-4-aminopyridine
DMF	*N*,*N*-dimethylformamide
ESI	Electro spray ionization
Et	Ethyl
EtOAc	Ethylacetate
GalNAc	*N*-acetyl-D-galactosamine
GlcNAc	*N*-acetyl-D-glucosamine
HOBt	1-hydroxybenzotriazole
HSQC	1H-detected single quantum coherence spectrum
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
MALDI	Matrix assisted laser desorption ionization

Man	Mannose
MeOH	Methanol
MS	Molecular sieves
NaOMe	Sodium methoxide
NaN_3	Sodium azide
$NaNO_2$	Sodium nitrate
NaH	Sodium hydride
Neu5Ac	*N*-Acetylneuraminic acid
NGP	Neighboring group participation
NMR	Nuclear magnetic resonance
Py	Pyridine
SPh	Thiophenyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMSOTf	Trimethylsilyltrifluoromethanesulfonate
Tol	Toluene
UV	Ultra violet

Chapter 1

General Introduction

1-1. Role of Glycans

The glycosylation is one of the most common post-translational modifications. Encountered in around 50% of proteins are glycosylated and is understood to play a pivotal role in numerous vital processes, including protein folding, cell-cell recognition, immune surveillance, hormone activities, and inflammatory reactions.[1,2] Glycans in mammals are constructed with monsaccharides, glucose, galactose, mannose, xylose, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose, and negatively charged *N*-acetyl neuraminic acid, glucuronic acid and iduronic acids.

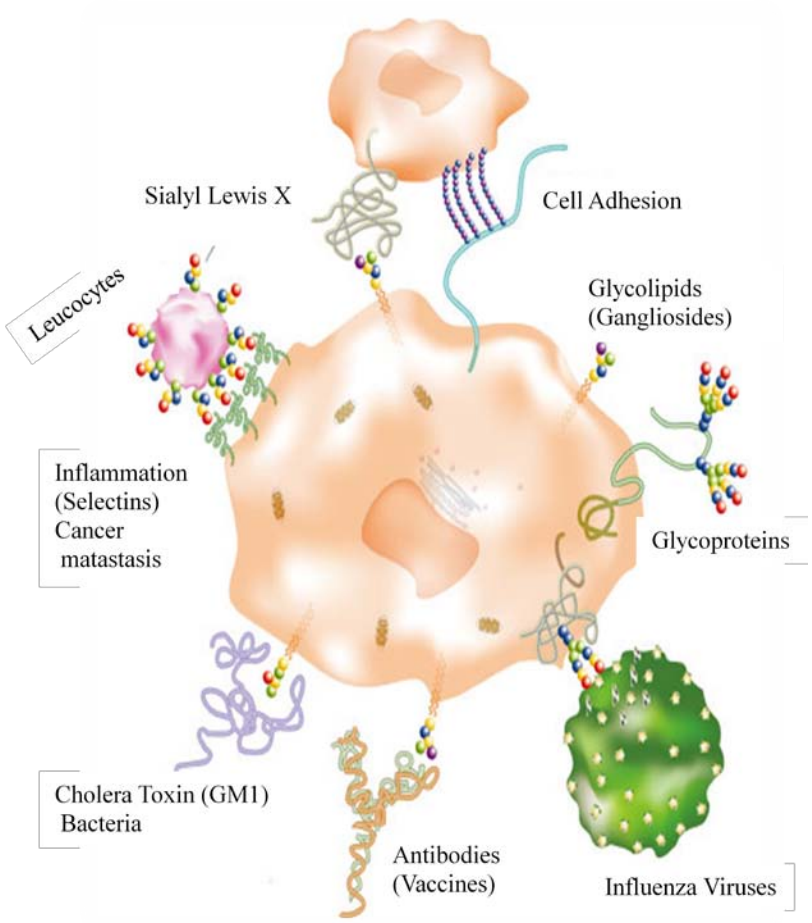

Fig 1-1-1. Role of carbohydrate chain in many biological functions.

Generally glycans are classified into two types: *N*-glycans and *O*-glycans. *N*-glycans are incorporated on Asn side chain, where Asn located in the Asn-X-Thr sequence (X: any amino acids except for proline). *N*-glycans are mainly classified into complex, hybrid, and high mannose type and known to have large variations by its branching structure.[3] On the other hand, in the case of *O*-glycans, small glycans are conjugated with the alchol moiety of Ser or Thr basically by GalNAc linkages.[4]

Recent studies on large-scale *N*-glycan analysis have revealed the importance of structural alteration of human serum/cellular *N*-glycans during disease progression. Many such glycans serve as potent biomarkers for the early diagnosis of disease and as new agents for therapeutic antibodies.[5] Amongest the many important mammalian proteins that are *N*-glycosylated, antibodies are of particular significance. Human IgG antibodies contain *N*-linked glycans and it is the key for modulating antibody-mediated responses.[6] Given that monoclonal antibodies (mAbs) are now widely accepted as the most important new class of therapeutic agents in development,[7] the importance of glycosylations becomes obvious.

Some cytokines utilized as therapeutic glycoproteins, such as erythropoietin,[8] granlocyte-colony stimulating factor,[9] α-interferon,[10] have reported to need glycans for their *in vivo* activity. Other hormones having no *N*-glycan, like insulin were also reported to show enhanced *in vivo* activity when a glycans was chemically introduced.[11]

In addition, as expression levels of highly branched *N*-glycans are enhanced distinctly during cancer cell proliferation and metastasis, it is clear that the construction of *N*-glycans compound libraries is of growing importance for the insight into the functions of cell surface glycoprotein. Up-regulation in the expression levels of hyper branched *N*-glycans were observed in prostate cancer patients serum (Fig 1-1-2).[12]

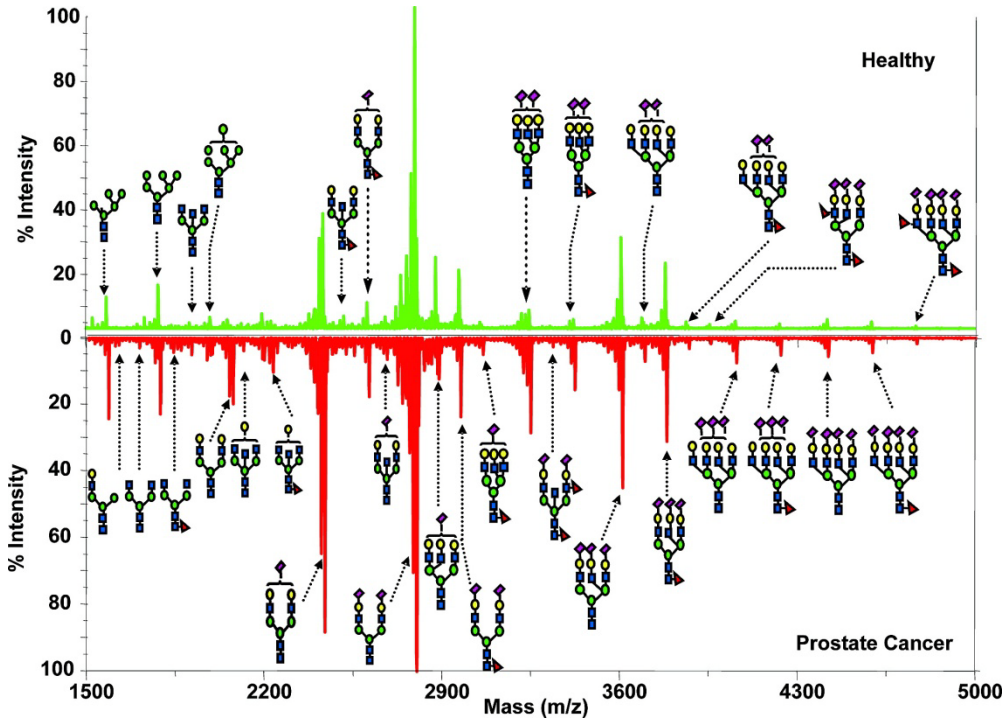

Fig 1-1-2. MALDI-TOF MS mirror spectra of permethylated *N*-glycans derived from human blood serum of healthy individual *vs.* a prostate cancer patient.

Thus, the glycans of glycoproteins or glycoconjugates play crucial roles in the maintenance and regulation of many biological functions. To understand the biological phenomenon as well as pharmaceutical applications, glycans and glycoconjugates are the real targets.

1-2. Glycans Synthesis

The biological properties of glycoproteins are influenced by the structure of their oligosaccharide as mentioned in section 1-1. However, the relationship between structure and function of *N*-glycans remains mostly unclear due to the structural complexity and heterogeneity in the dynamic posttranslational modification at the potential *N*-glycosylation sites.[13] The diversity of natural *N*-glycans is thus impairing the isolation of these compounds from glycoproteins. Therefore, the synthesis of *N*-glycans has been used to overcome the shortage of material for biological studies. Two general strategies are used for oligosaccharide production; enzymatic (including chemoenzymatic) synthesis and chemical synthesis.

In enzymatic and chemoenzymatic routes, saccharide intermediates are elaborated with enzymes, typically glycosyltransferases or glycosidases, to generate oligosaccharides.[14] Chemoenzymatic synthesis by its reliance on both chemical synthesis and enzymatic transformations. Enzymes can be used to affect glycosylation with absolute regio- and stereo-control. If the necessary enzyme is available, the desired bond can be formed, often with high efficiency. Availability of specific enzymes and price of the enzymes turns difficulty in the synthesis.

On the other hand chemical synthesis offers exceptional flexibility. Natural and non-natural saccharide building blocks can be assembled with natural and non-natural linkages. The chemical synthesis of oligosaccharides is formidable, it requires stereochemical and regiochemical control in glycosidic linkage formation. As chemical synthesis of glycans depends on the subtle balance of various factors[15]; protecting groups, anomeric leaving groups, activator systems, solvents and so on, even careful tunings of systems are needed, such as β-mannosylation[16] and α-sialylation[17] which had used to be recognized as difficult reactions. Stereo chemical control can be achieved by

employing stereospecific activation methods, using protecting groups that direct the orientation of the glycosidic bond through intermolecular (neighbouring group participation) or intramolecular (tethered aglycone delivery) participation, altering the steric environment around the anomeric position to bias the desired outcome, or exploiting the intrinsic stereoelectronic preferences for reaction at the anomeric position.

1-3. Synthesis of glycopeptides/glycoproteins.

Since natural and recombinant glycoproteins are typically produced as a mixture of heterogeneous glycoforms, synthesis of homogeneous glycoproteins carrying structurally defined oligosaccharides has become essential both for detailed structure function relationship studies and for developing glycoprotein-based therapeutics. To address the need, a variety of synthetic strategies have been explored for constructing large glycopeptides and glycoproteins. The introduction of new techniques, such as native chemical ligation and chemoselective ligation,[18] novel solid-phase synthesis,[19] and enzymatic oligosaccharide transfer[20] has significantly expanded, synthetic repertoire for constructing large homogeneous glycopeptides.

Particularly the endo-β-*N*-acetylglucosaminidase (ENGase) catalyzed oligosaccharide transfer for glycopeptide synthesis is attracted, because it allows the attachment of large oligosaccharides to a pre-assembled, unprotected GlcNAc-peptide/protein in a single step in a regio- and stereo- specific manner, thus providing a highly convergent approach. Both glycosyltransferases and glycosidases have been vigorously studied for synthetic purposes. In comparison with glycosyltransferase-catalyzed reaction that uses complex sugar nucleotide as the donor and usually has very stringent substrate specificity, glycosidase-catalyzed transglycosylation has several advantages, including the use of readily available donor substrates, the relaxed substrate specificity for acceptors, the easy access to the enzymes, and the potential for a single-step block oligosaccharide transfer (as in the case of endoglycosidases). Nevertheless, the use of glycosidases in synthesis is subject to two major limitations: the low transglycosylation yield and the product hydrolysis.

Significant progresses have been made in recent years to overcome these problems. A major breakthrough in the field is the invention of glycosynthases, a class

of novel glycosidase mutants that can promote glycosidic bond formation when a suitable activated glycosyl donor is provided, but do not hydrolyze the newly formed glycosidic linkage. Moreover, protein engineering, including directed evolution coupled with elegant screening methodology has led to the discovery of an expanding number of novel glycosynthases with enhanced transglycosylation activity and/or altered substrate specificity.

The novel glycosynthase, EndoM-N175A, that previously created has demonstrated a great potential for the synthesis of homogeneous glycoproteins carrying natural complex-type and high mannose-type *N*-glycans. Despite the lack of the product hydrolysis, however, the specific activity of the glycosynthase for transglycosylation was much lower than that of the wild type Endo-M. The enzymatic reactions catalyzed by the EndoM-N175A mutant are usually slow, requiring relatively large amounts of the mutant enzyme and/or extended incubation time. To improve the catalytic efficiency of the glycosynthase for practical use, a systematic mutagenesis was performed at the critical Asn-175 site of Endo-M, as well as site-directed mutations at other conserved Glu and Asp residues to probe whether those carboxylate residues are critical in the catalysis. The mutagenesis and subsequent enzymatic evaluation have led to the identification of an array of glycosynthase mutants that showed enhanced catalytic activity. In particular, the N175Q mutant was found to possess significantly enhanced transglycosylation activity for activated sugar oxazoline, whereas its hydrolysis activity for the product was diminished. Surprisingly, this mutant was also capable of efficiently transglycosylating *N*-glycan while having a significantly diminished product hydrolysis activity behaving as a typical “transglycosidase.”

The method was based on the assumption that the ENGase catalyzed reaction proceeds via a mechanism of the substrate assisted catalysis involving an oxazolinium ion intermediate, as demonstrated for some chitinases and *N*-acetyl-α-hexosaminidases. Although a detailed mechanism of ENGase-catalyzed transglycosylation is yet to be characterized, Fujita and co-workers recently reported that a disaccharide oxazoline of Manβ1,4GlcNAc could serve as a substrate for ENGase-catalyzed transglycosylation.[21] This observation suggested that the Endo-A- and Endo-M catalyzed transglycosylation might indeed proceed via an oxazolinium ion intermediate.

In conclusion, ENGase catalyzed transglycosylation produced varieties of glycoconjugates. But the main disadvantage is the chemical synthesis of the *N*-glycan core Manβ1,4GlcN disaccharide needs stereocontrolled β-glycosylation, followed by selective inversion of the *Glc* C-2 configuration and protecting group manipulations, makes the glycans synthesis most difficult and practical synthesis is impossible. Insertion of gal as well as Neu5Ac group at non reducing end also makes the glycans synthesis much difficult.

In this thesis, the author challenged to overcome this problem and tried to synthesize hyper branched *N*-glycopeptides by using novel synthetic route and the application of ENGase catalyzed transglycosylation. In chapter 2, I described the highly efficient synthesis of *N*-glycan core Manβ1,4GlcN disaccharide from natural abundant polysaccharide locust bean gum. Then di, tri and tetra antennary *N*-glycans synthesis were demonstrated using core Manβ1,4GlcN disaccharide in chapter 3. The synthesis of glycopeptides using di, and tetra antennary *N*-glycans by the application of ENGase catalyzed transglycosylation in chapter 4.

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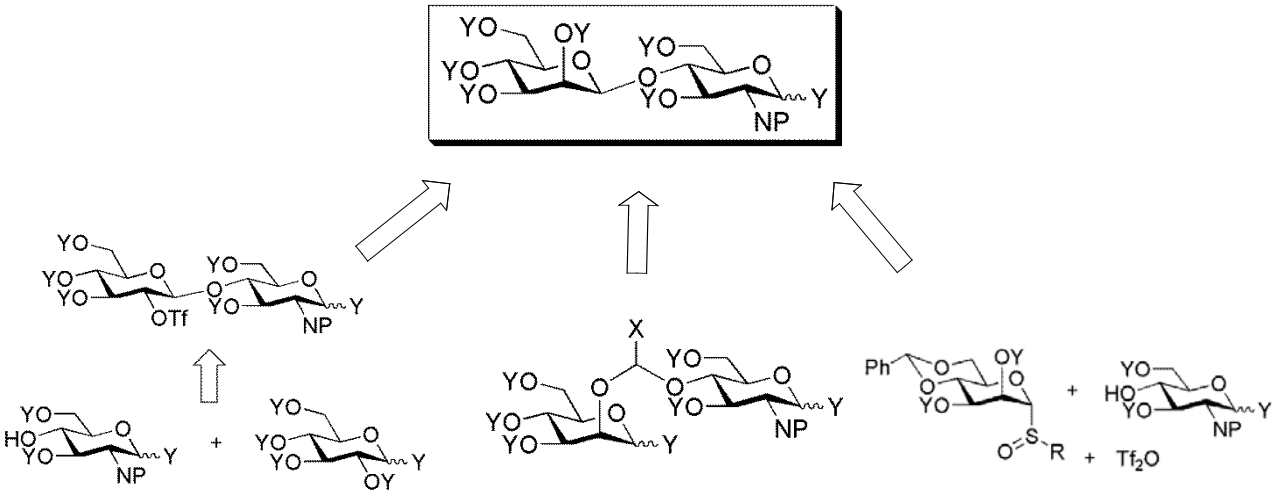
Chapter 2

Synthesis of a Core disaccharide as a Versatile Building Block for N-Glycans and Glycoconjugates

2-1. Introduction.

The *β*-*O*-mannopyranosidic bond, as present in the common core pentasaccharide of the *N*-linked glycoproteins,[1] in various mannans and glycosphingolipids[2] and in lipopolysaccharides,[3] is arguably the most difficult type of glycosidic linkage with which nature has challenged the synthetic chemist.[4] The formidable combination of steric and stereoelectronic factors that weigh against formation of the *β*-mannoside in classical glycosidation protocols has prompted the development of less direct routes, principally reduction of 2-ulososes,[5] inversion of *β*-glucosides,[6] radical anomeric inversion of α-mannosides,[7] direct *O*-alkylation of pyranoses,[8] and, most successfully, preattachment of the aglycon by means of a suitable tether to the *O*-2 position of mannosyl donors.[9] While the successful synthesis of oligosaccharides has been achieved through several of these methods,[10] a protocol for the direct coupling of aglycons to simple mannopyranosyl donors with high *β*-selectivity remains a very desirable goal.



S_N2 conversion from β-glucoside Intramolecular aglycone delevery(IAD) Crich's β-mannosylation

P, Y= Protecting groups

In the chemical synthesis of the core sugar chain structure, it is extremely difficult to form a bond of β-mannoglycoside (Manβ1,4-GlcNAc). The reason comes from the facts that a neighbouring group effect is not available since 2-OH group of mannose is linked at the axial position and the β-manno glycoside bond brought an electrically unstable structure against an anomer effect typically found in sugars. Few groups mentioned previously, discloses a chemical method for preparing a β-manno glycoside structure, which contains a complicated process and requires the time and cost of running. Other reasons why a bond of β-manno glycoside (Manβ1,4-GlcNAc) is difficult to be formed are that the acceptor of the glycosylation reaction is *N*-acetyl glucosamine of low solubility in the reaction medium and the reactivity of 4-OH group is low compared with the other OH groups (reactivities of OH groups; 1-OH>>6-OH>>2-OH>3OH>4-OH).

Hence, there is a need to develop a novel method to obtain a Manβ1,4-GlcNAc disaccharide for the practical synthesis of *N*-glycans.

2-2. Results and Discussions.

2-2-1. Synthesis of β1,4-mannobiose octaacetate **2**

Retrosynthetic analysis of hyperbranched *N*-glycans provide us β1,4-mannobiose octaacetate can be the key compound for the synthesis of all types of *N*-glycans. The corresponding retrosynthetic analyses are shown below.

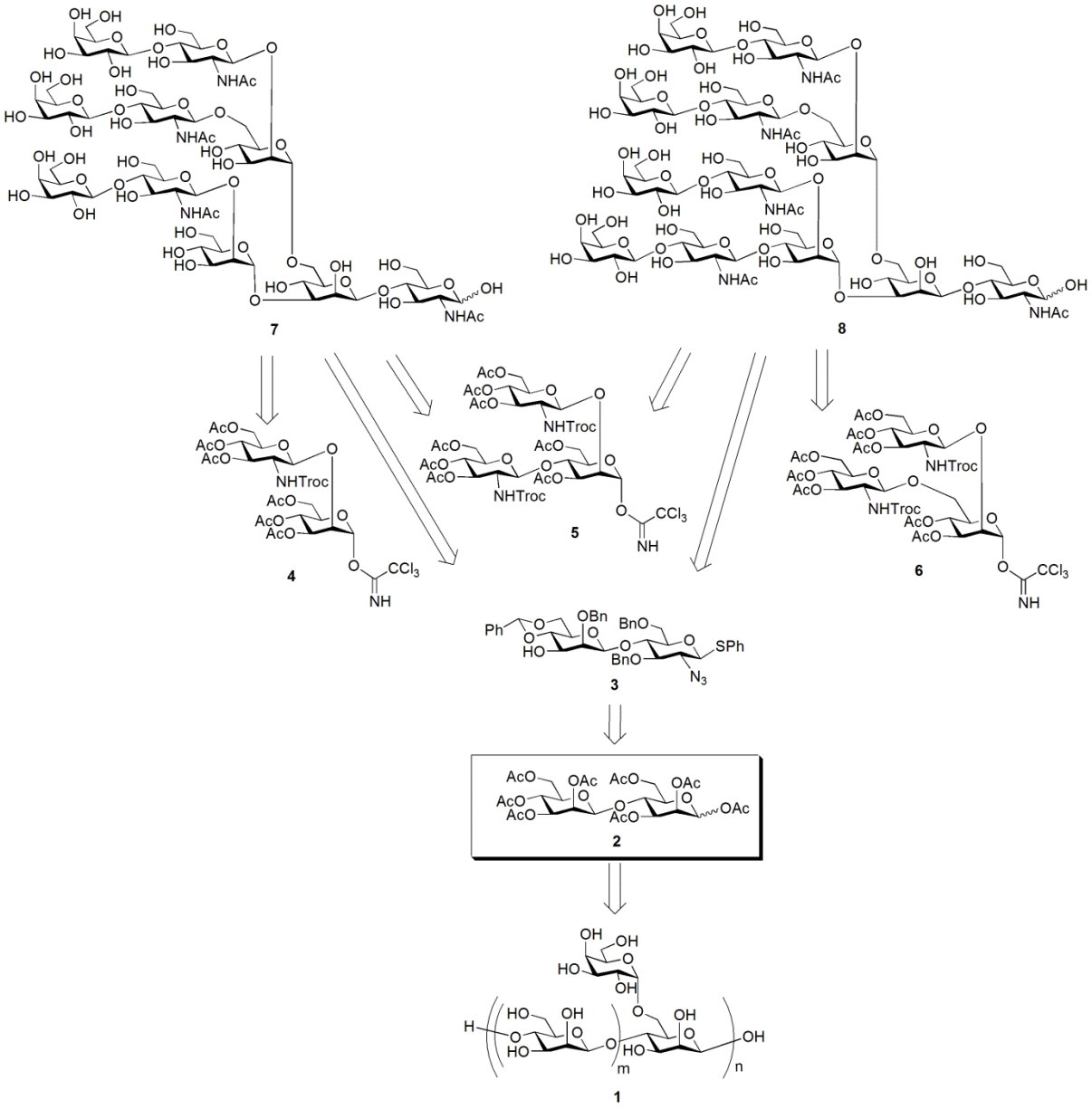
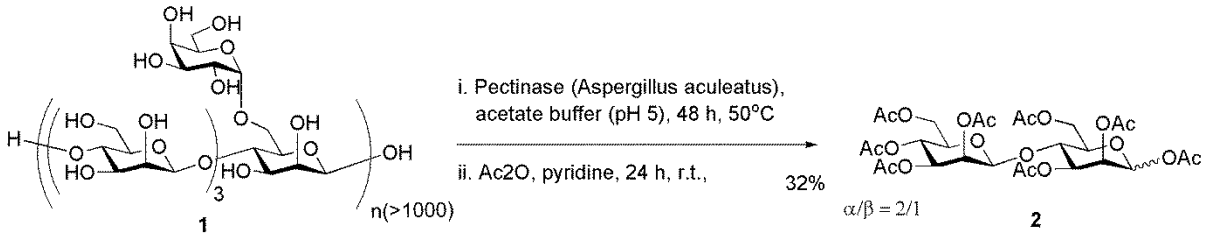


Fig. 2-2-1: Retrosynthesis of hyperbranched *N*-glycans.

Based on the difficulty in the synthesis of Manβ(1→4)GlcNAc, Our previous finding that the β1,4-mannobiose octaacetate[11] obtainable through the acid hydrolysis of guar gum can be converted into the compounds corresponding to the disaccharide unit, Manβ(1→4)GlcNAc, encouraged us to improve and optimize the procedure for the preparation of this important compound **2** from the most suited material among some abundant polysaccharides containing common formula of galactomannan shown in Scheme 2-2-1. In the present study, we investigated the degradation products of two abundant polysaccharides, guar gum and locust bean gum, under various hydrolytic conditions as listed in Table-2-2-1. Consequently, we discovered that the degradation of locust bean gum by treating with pectinase from *Aspergillus aculeatus* at 50ºC for 48 h in 50 mM acetate buffer (pH5.0) gives dominantly a disaccharide Manβ(1→4)Man and subsequent per-*O*-acetylation of the crude product facilitates the isolation of β1,4-mannobiose octaacetate **2** by silica gel chromatography in 32% overall yield (51 g) from crude locust bean gum (100 g) (Scheme 1).



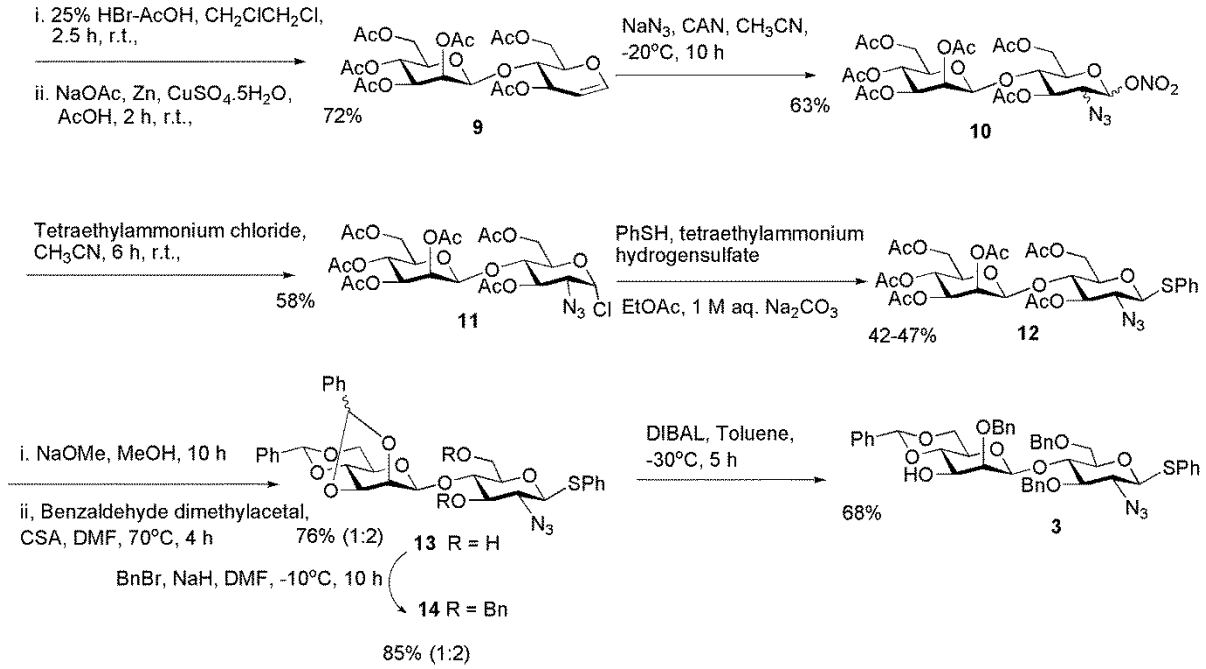
Scheme 2-2-1: Preparation of β1,4-mannobiose octaacetate 2.

Table 2-2-1: Preparation of β1,4-mannobiose octaacetate 2 from galactomannan.

No	Source	Hydrolysis Method	Reagent	Condition	Yield
1	Locust bean gum	Enzyme	Pectinase (Asperigillus aculeatus, 3,800 U/mL)	Acetate buffer, pH 5.0, 50 °C, 48 h	32%
2		Acid	0.5 N TFA	100 °C, 2 h	15%
3	Guar gum	Enzyme	Pectinase (Asperigillus aculeatus, 3,800 U/mL)	Acetate buffer, pH 5.0, 50 °C, 48 h	15%
4			Pectinase (Asperigillus aculeatus, 3,800 U/mL) + Mannanase (Cellvibrio japonicus, 5000 U/mL)	Acetate buffer, pH 5.0, 50 °C, 48 h	16%
5			α-galactosidase (Coffee beans, 9 U/mg) + Cellulase (Thermophiles, 2.92 U/mL)	Phosphate buffer, pH 6.5, 25 °C, 48 h and then 60 °C, 48 h	7%
6		Acid	0.5 N TFA	100 °C, 2 h	15%
7		Microwave	Water	150 W, 100 °C, 5 d	5%
8		Supercritical water	Water	180 °C, 10 mPa, 30 min	ND

2-2-2. Synthesis of key intermediate **3**

As anticipated, per-*O*-acetate **2** allowed for highly efficient synthesis of the key intermediate **3** by a general procedure as shown in Scheme 2-2-2. 2-Azido derivative **10** obtained readily by azidonitration of glycal **9** was treated by tetrabutylammonium chloride in acetonitrile to make the purification of the stereo-controlled gluco configured chloride **11** possible. Next, β-thioglycoside **12** derived by the nucleophilic substitution of **11** with thiophenol was subjected to de-*O*-acetylation, benzylidenation, and benzylation afforded regio-selectively protected disaccharide **13** as a mixture of stereo isomers at 2',3'-*O*-benzylidene group (exo : endo = 1 : 2). However, it was revealed that reductive ring opening reaction of compound **14** occurred at 2',3'-*O*-benzylidine ring[12] selectively in endo isomer in the presence of DIBAL and gave the desired disaccharide intermediate **3** having 3'-OH group in high yield.



Scheme 2-2-2: Synthesis of disaccharide intermediate **3**.

2-3. Conclusion.

We developed a novel synthetic strategy toward Phenyl -(2-*O*-benzyl-4,6-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside **3** by using the β1,4-mannobiose octaacetate **2** derived efficiently from abundant locust bean gum as a key starting material. We optimized the best condition to isolate β1,4-mannobiose from the abundant polysaccharide locust bean gum and then we developed the novel synthetic methodology for the synthesis of very important key compound **3**, which is very essential for the practical synthesis of *N*-glycans and glycopeptides.

2-4. Experimental Section.

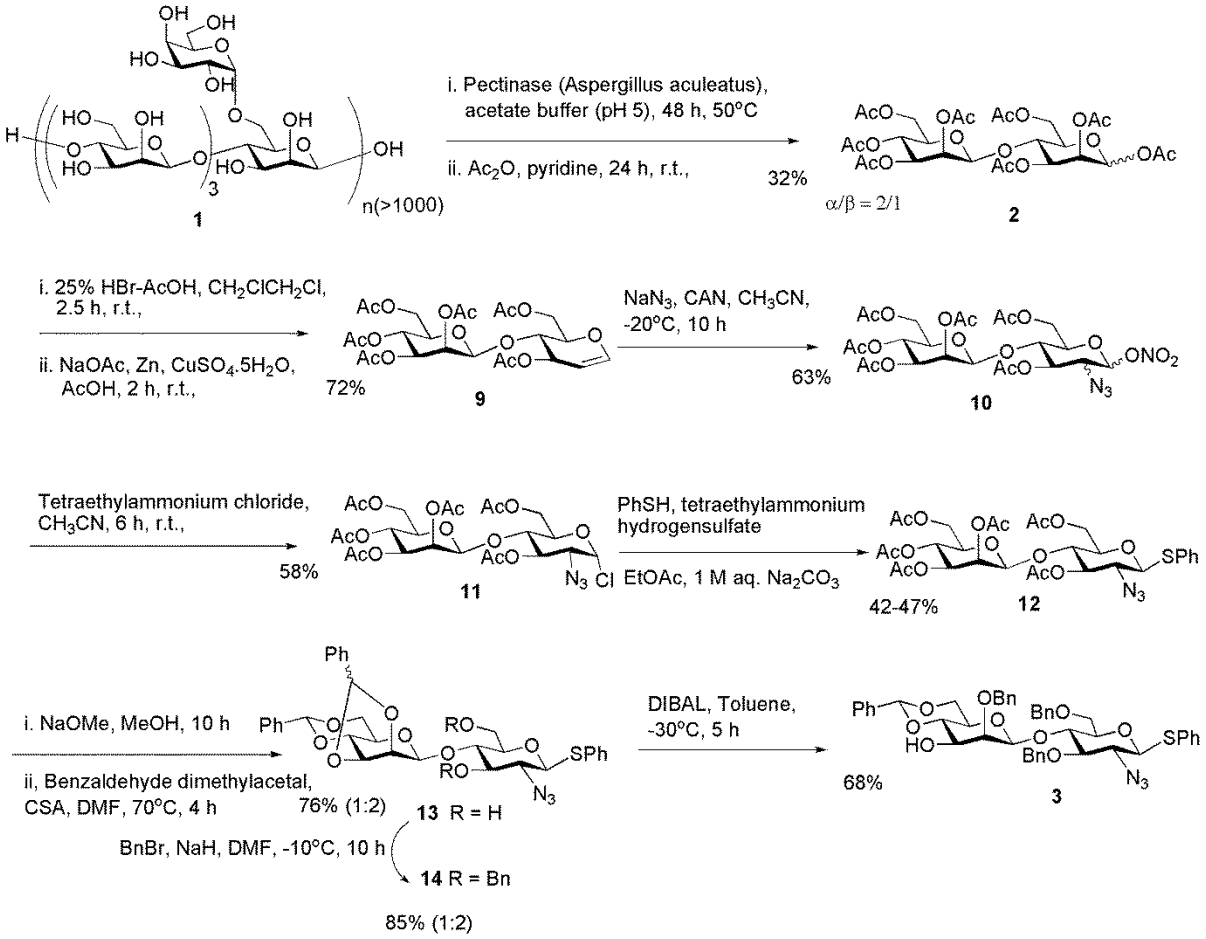
All reactions were carried out under a nitrogen atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Proton and carbon NMR was recorded with Varian UnityInova 500 MHz (Agilent Inc., USA; ^{1}H: 500 MHz, ^{13}C: 125 MHz). Chemical shifts are given in ppm and referenced to internal TMS (δ_H 0.00 in $CDCl_3$), $CHCl_3$ (δ_H 7.26 in $CDCl_3$) or $CDCl_3$ (δ_C 77.00). Assignments in ^{1}H NMR were made by first-order analysis of the spectra by using ACD/NMR processor software (Advanced Chemistry Development, inc.) and were verified by H-H COSY and HSQC experiments. 2D NMR of compounds were recorded at 300 K with a Bruker Avance 600 spectrometer at 600.03 MHz for proton frequency equipped with cryoprobe. For the complete assignments and structural determination, two dimentional homonuclear DQF-COSY, TOCSY with MLEV-17 and NOESY spectra were recorded in the indirect dimension using States-TPPI phase cycling. Additionally two dimentional heteronuclear ^{13}C edited HSQC and HSQC-TOCSY measurements were also recorded with echo-antiecho mode for sensitive enhancement. All NMR data were processed by NMRPipe software and analysed using the Sparky program. A high/low resolution electrospray ionization mass spectra (ESI-MS) were recorded by JMS-700TZ (JEOL, Japan) and Bruker ultraflex-I. TLC was performed on Merck pre-coated plates (20 cm × 20 cm; layer thickness, 0.25 mm; Silica Gel 60F_{254}); spots were visualized by spraying a solution of 90:5:5 (v/v/v) MeOH-*p*-anisaldehyde-concentrated sulfuric acid and heating at 250°C for ca. $^{1}/_{2}$ min, a solution of 95: 5 (v/v) MeOH-concentrated sulfuric acid and heating at 180°C for ca. $^{1}/_{2}$ min, and by UV light (256 or 365 nm) when applicable. Column chromatography was performed on Silica Gel N60 (spherical type, particle size 40−50 μm; Kanto Chemical Industry) with the solvent systems specified, and the ratio of solvent systems was given in v/v.

The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet,

dd = double doublet, t = triplet, m = multiplet, br = broad.

Materials: Locust bean gum (Asperigillus aculeatus) (G0753). Pectinase from Aspergillus aculeatus (P2611) and α-galactosidase (Coffee beans) (G8507) was purchased from Sigma-Aldrich Chemical Co.. Cellulase was purchased from Thermostable Enzyme Laboratory Co. Ltd (Lot: C1060302). B-mannanase (Cellvibrio japonicas) was purchased from Bicon (Japan) Ltd (Lot: 90901b). Solvents and other reagents were purchased from Sigma-Aldrich Chemical Co., Tokyo Chemical Industry (Tokyo, Japan), and Wako Pure Chemical Industries Ltd. (Tokyo, Japan) and used without further purification, unless otherwise noted.

Synthesis of Phenyl -(2-*O*-benzyl-4,6-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside (3).



2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-D -manno pyranose (2).

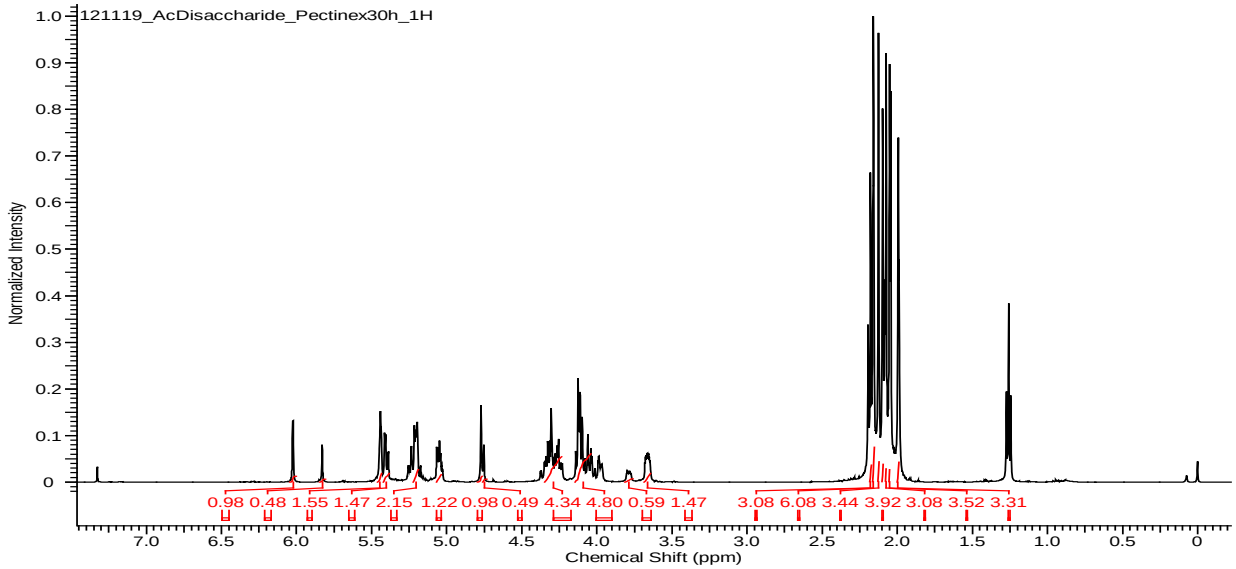
Locust bean gum (100 g) from sigma Aldrich (Lot # SLBC7065V) in 50 mM acetate buffer (2000 mL) was added 10 mL of pectinase from Aspergillus aculeatus and incubated at 50 °C for 48 h. Then the solution was refluxed for 10 min and poured into 99% ethanol (1000 mL) and filtered at r.t., the filtrate was concentrated in vacuo. coevaporated with toluene. Pyridine (1000 mL) and acetic anhydride (1000 mL) were added to resulting mixture at 0 ^{o}C. The solution was stirred at r.t., for 24 h. Then, the reaction mixture was poured into ice water and extracted with $CHCl_3$. Organic phase was washed with water, 1N HCl, sat. $NaHCO_3$, brine, dried (Na_2SO_4) and concentrated. The crude residue was purified by flash

column chromatography (hexane/EtOAc=2:1) to give **2** (51 g, 32%, α/β=2/1) as a white powder.

^{1}H NMR (500 MHz , $CDCl_3$, 25 ^{o}C, TMS) δ 6.03 (d, $J^{\alpha}_{1,2}$ = 2.0 Hz, H-1α), 5.81 (d, 1/3H, $J^{\beta}_{1,2}$ = 0.9 Hz, H-1β), 5.46-5.38 (m, 2H, H-2', H-2β and H-3α), 5.25-5.18 (m, 2 H, H-4', H-2α and H-3β), 5.07-5.00 (m, 1 H, H-3'), 4.75 (s, 2/3 H, H-1α'), 4.73 (s,1/3H, H-1β'), 4.38-4.22 (m, 3 H; H-6bβ, H-6aα, H-6bα, H-6a' and H-6aβ), 4.15-3.95 (m, 2+2/3H, H-5α, H-4β, H-4α and H-6'b), 3.77 (m, 1/3H, H-5 β), 3.64 (m, 1 H, H-5'), 1.99-2.19 (s, 24 H; 8x$COCH_3$).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 171.0, 170.4, 170.4, 170.3, 170.2, 170.2, 169.9, 169.9, 169.5, 169.4, 169.4, 169.3, 168.2, 168.1, 98.0, 98.0, 97.6, 97.6, 90.5, 90.4, 90.4, 73.6, 72.5, 72.2, 70.9, 70.6, 70.2, 68.8, 68.7, 68.7, 68.6, 68.3, 68.3, 68.3, 65.8, 62.5, 62.3, 60.3, 21.0, 20.8, 20.8, 20.7, 20.7, 20.7, 20.6, 20.6, 20.5.

HRMS (ESI): *m/z* calcd for $C_{28}H_{38}NaO_{19}$, $[M+Na]^+$ 701.19050, found 701.18935.



2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl-(1→4)-3,6-di-*O*-acetyl-D-glucal (9).

To a solution of **2** (20 g, 29.5 mmol) in dry 1,2-dichloroethane (74 mL, 0.4 M) was added 25% HBr in acetic acid (17.7 mL, 73.7 mmol) and stirred at r.t., for 2.5 h. Then, the reaction mixture was concentrated, co-evoparated with toluene and dissolved in EtOAc (10.0 mL). The resulting mixture was added to sodium acetate (24.2 g, 295 mmol), Zinc (19.3 g, 295 mmol), $CuSO_4.5H_2O$ (1.8 g, 7.4 mmol) in acetic acid (120 mL) and stirred at r.t., for 2 h. Then the reaction mixture was filtered through celite and washed with EtOAc and water. Separated the organic phase, organic phase was washed with H_2O, sat. $NaHCO_3$, brine, dried (Na_2SO_4) and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=60:40) to give **9** (11.9 g, 72%) as a white powder.

^{1}H NMR (500 MHz , $CDCl_3$, 25 ^{o}C, TMS) δ 6.41 (d, J = 5.7 Hz, 1 H; H-1), 5.51 (dd, J = 3.1, 4.8 Hz, 1 H; H-3), 5.45 (d, J = 2.6 Hz, 1 H; H'-2), 5.22 (t, J = 10.0 Hz, 1 H; H'-4), 5.06 (dd, J = 3.4, 10.0 Hz, 1 H; H'-3), 4.88 (s, 1 H; H'-1), 4.79 (dd, J = 3.0, 6.1 Hz, 1 H; H-2), 4.42 (dd, J = 2.7, 12.1 Hz, 1 H; H-6b), 4.30 (dd, J = 5.7, 12.3 Hz, 1 H; H'-6b), 4.23 (dd, J = 5.3, 12.1 Hz, 1 H; H-6a), 4.19 - 4.09 (m, 2 H, 1H; H'-6a, 1Hx EA), 4.06 (dd, J = 6.1, 8.1 Hz, 1 H; H-4), 3.70 - 3.64 (m, 1 H; H'-5), 2.68, 2.12, 2.10, 2.09, 2.05, 2.00 (s each, 3 H each, 6x $COCH_3$).

^{13}C NMR (125 MHz, $CDCl_3$, 25°C, TMS) δ 168.0, 167.8, 167.7, 167.4, 167.3, 166.9, 143.0, 96.4, 95.3, 71.8, 71.4, 70.0, 68.2, 65.9, 63.3, 59.9, 59.2, 57.7, 18.5, 18.2, 18.1, 18.1, 18.1, 17.9.

HRMS (ESI): *m/z* calcd for $C_{24}H_{32}NaO_{15}$, $[M+Na]^+$ 583.16389, found 583.16322.

2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl-(1→4)-3,6-di-*O*-acetyl-

2-azido-2-deoxy-α-D-glucopyranosyl chloride (11).

To a solution of **9** (11.5 g, 20.5 mmol) in dry acetonitrile (205 mL) was added cerium ammonium nitrate (41.1 g, 74.9 mmol), sodium azide (2.0 g, 30.8 mmol) at -20 ^{o}C and stirred at -20 ^{o}C for 12 h. Then, the reaction mixture was poured into ice cold water and extracted with $CHCl_3$. Organic phase was washed with H_2O, brine, dried (Na_2SO_4) and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=60:40) to gave **10** (9 g, 66%) as a white powder. To obtained compound **10** (8.7 g, 13.1 mmol) in dry acetonitrile (175 mL) was added tetraethylammonium chloride (10.9g, 65.5 mmol) and stirred at r.t., for 6 h. Then, the reaction mixture was concentrated and dissolved in EtOAc. Organic phase was washed with H_2O, brine, dried (Na_2SO_4) and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=64:36) to give **11** (4.8 g, 58%) as a white powder.

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 6.08 (d, J = 3.7 Hz, 1 H; H-1), 5.53 (t, J = 9.8 Hz, 1 H; H-3), 5.40 (d, J = 3.1 Hz, 1 H; H'-2), 5.21 (t, J = 9.9 Hz, 1 H; H'-4), 5.05 (dd, J = 3.4, 9.9 Hz, 1 H; H'-3), 4.73 (s, 1 H; H'-1), 4.42-4.32 (m, 2 H; H-6b, H'-6b), 4.32-4.26 (m, 2 H; H-5, H-6a), 4.15-4.12 (m, 1 H; H'-6a), 3.86 (t, J = 9.6 Hz, 1 H; H-4), 3.72 (dd, J = 3.8, 10.4 Hz, 1 H; H-2), 3.64 (ddd, J = 2.6, 5.2, 9.8 Hz, 1 H; H'-5), 2.19, 2.17, 2.13, 2.10, 2.05, 1.99 (s each, 3 H each, 6x$COCH_3$).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS) δ 170.4, 170.3, 170.1, 169.8, 169.5, 169.4, 97.2, 91.9, 91.8, 73.8, 73.6, 72.7, 72.4, 71.5, 70.4, 69.2, 68.1, 65.6, 62.5, 62.3, 20.7, 20.6, 20.6.

HRMS (ESI): *m/z* calcd for $C_{24}H_{32}ClN_3NaO_{15}$, $[M+Na]^+$ 660.14196, found 660.1409.

Phenyl-(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1→4)-3,6-di-*O*-

acetyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside (12).

To a solution of **11** (3.5g, 5.5 mmol) in ethylacetate (35 mL) was added tetrabutylammonium hydrogensulfate (1.9g, 5.5 mmol), thiophenol (1.7 mL, 16.5 mmol) and 1 M aqueous sodium carbonate (35 mL) and stirred at r.t., for 3 h. Then, separated the layers, organic phase were washed with H_2O, sat. $NaHCO_3$, brine, dried (Na_2SO_4) and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=65:35) to give **12** (1.8 g, 47%) as a white powder.

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.53 (t, J = 6.5 Hz, 2 H; Ar-H), 7.34-7.29 (m, 3 H; Ar-H), 5.34 (d, J = 2.9 Hz, 1 H; H'-2), 5.17 (t, J = 9.9 Hz, 1 H; H'-4), 5.05 (t, J = 9.4 Hz, 1 H; H-3), 4.98 (dd, J = 3.2, 10.0 Hz, 1 H; H'-3), 4.61 (s, 1 H; H'-1), 4.44 (d, J = 10.3 Hz, 1 H; H-1: H-6b), 4.38 (dd, J = 2.1, 12.1 Hz, 1 H; H'-6b), 4.32 (dd, J = 5.3, 12.4 Hz, 1 H; H-6a), 4.18 (dd, J = 4.6, 12.2 Hz, 1 H; H'-6b), 3.68-3.62 (m, 1 H; H-4), 3.59 (dddd, J = 2.5, 4.8, 7.0, 9.3 Hz, 2 H; H-5, H'-5), 3.29 (t, J = 10.0 Hz, 1 H; H-2), 2.13, 2.11, 2.09, 2.02, 2.01, 1.95 (s each, 3 H each, 6xCH_3CO).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 170.3, 170.2, 169.8, 169.6, 169.5, 133.9, 130.6, 129.0, 128.7, 97.3, 97.2, 85.8, 76.5, 74.2, 73.3, 72.5, 70.7, 68.1, 68.1, 65.8, 62.9, 62.3, 62.2, 20.8, 20.7, 20.6, 20.6, 20.5.

HRMS (ESI): *m/z* calcd for $C_{30}H_{37}N_3NaO_{15}S$, $[M+Na]^+$ 734.18431, found 734.18347.

Phenyl -(2,3:4,6-di-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-2-azido-2-deoxy-1-thio-β-D-glucopyranoside (13).

To a solution of **12** (700 mg, 0.98 mmol) in dry methanol (2 mL, 0.5 M) was added sodium methoxide (27 mg, 0.49 mmol) and stirred at r.t., for 12 h. Then the solution was neutralized by Dowex 50W-X8 [H^+] resin. After the filtration, the reaction mixture was concentrated in

vacuo. and dried for 3 h. Dry *N,N*-dimethylformamide (10 mL, 0.1 M), benzaldehyde dimethylacetal (0.4 mL, 3.0 mmol) and (±)-10-camphorsulfonic acid (69 mg, 0.3 mmol) were added under nitrogen and stirred at 70 ^{o}C under reduced pressure for 3 h. Aq. $NaHCO_3$ was slowly added at r.t., and extracted with EtOAc. The organic phase was dried (Na_2SO_4) and concentrated. The crude residue was purified by flash column chromatography to give diastereomeric mixture (hexane/EtOAc=72:28) to give **13a** and **13b** as mixture in 1:2 (475 mg, 76%) as a white powder.

Phenyl -(2,3:4,6-di-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-2-azido-2-deoxy-1-thio-β-D-glucopyranoside 13a (Exo):

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.54 (dd, J = 3.0, 6.5 Hz, 2 H; H-Ar), 7.50 (dd, J = 2.2, 7.2 Hz, 2 H; H-Ar), 7.46-7.41 (m, 2 H; H-Ar), 7.39-7.32 (m, 9 H; H-Ar), 6.29 (s, 1 H; H-CPh), 5.61 (s, 1 H; H-CPh), 5.00 (d, J = 2.4 Hz, 1 H; H'-1), 4.57 (dd, J = 6.2, 7.7 Hz, 1 H; H'-6b), 4.49 (d, J = 10.0 Hz, 1 H; H-1), 4.43-4.37 (m, 2 H; H'-2, H'-4), 3.96-3.94 (m, 1 H; H-6b), 3.93-3.88 (m, 1 H; H-6a), 3.84 (t, J = 10.2 Hz, 1 H; H'-3), 3.77-3.71 (m, 2 H; H-3, H'-6a), 3.66 (t, J = 9.3 Hz, 1 H; H-4), 3.54 (dt, J = 5.2, 10.0 Hz, 1 H; H'-5), 3.45 (td, J = 2.6, 9.3 Hz, 1 H; H-5), 3.28 (dd, J = 9.5, 10.0 Hz, 1 H; H-2), 2.03 (s, 2 H; -OH).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 137.8, 136.7, 133.4, 131.1, 129.4, 129.3, 129.2, 128.6, 128.5, 128.3, 126.2, 104.6, 104.6, 101.9, 100.4, 100.4, 86.0, 80.6, 78.2, 76.6, 75.4, 73.8, 68.7, 65.5, 64.7, 61.3, 60.4.

HRMS (ESI): *m/z* calcd for $C_{32}H_{33}N_3NaO_9S$, $[M+Na]^+$ 658.18352, found 658.18226.

Phenyl -(2,3:4,6-di-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-2-azido-2-

deoxy-1-thio-β-D-glucopyranoside 13b (Endo):

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.56-7.50 (m, 4 H; H-Ar), 7.48 (dd, *J* = 2.2, 7.3 Hz, 2 H; H-Ar), 7.41-7.37 (m, 3 H; H-Ar), 7.36-7.32 (m, 6 H; H-Ar), 5.97 (s, 1 H), 5.54 (s, 1 H), 5.04 (d, *J* = 2.7 Hz, 1 H; H'-1), 4.48-4.43 (m, 2 H; H-1, H'-6b), 4.42-4.36 (m, 2 H; H'-2, H'-4), 3.89 (d, *J* = 8.9 Hz, 1 H; H-6b), 3.80 (t, *J* = 10.3 Hz, 1 H; H'-3), 3.73-3.67 (m, 2 H; H-3, H'-6a), 3.64-3.55 (m, 3 H; H-4, H'-5, H-6a), 3.31 (td, *J* = 2.6, 9.4 Hz, 1 H; H-5), 3.26 (dd, *J* = 9.4, 10.0 Hz, 1 H; H-2), 2.04 (s, 2 H; -OH).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 136.7, 136.2, 133.5, 131.0, 129.6, 129.2, 129.1, 128.6, 128.4, 128.3, 126.7, 126.2, 105.2, 105.1, 101.6, 99.7, 99.7, 85.8, 80.0, 78.6, 78.3, 76.0, 75.4, 74.9, 68.8, 65.6, 64.8, 60.9.

HRMS (ESI): *m/z* calcd for $C_{32}H_{33}N_3NaO_9S$, $[M+Na]^+$ 658.18352, found 658.18330.

Phenyl -(2,3:4,6-di-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside (14).

To a solution of mixture **6a** and **6b** (475 mg, 1.0 mmol) in dry *N*,*N*-dimethyl formamide (7.5 mL, 0.1 M) was added 50% sodium hydride (108 mg, 2.2 mmol), cooled to -15 ^{o}C and stirred for 15 min. Then added benzyl bromide (267 µL, 2.2 mmol) and stirred for 10 h. Allowed to r.t., aq. $NaHCO_3$ was slowly added and extracted with EtOAc. The organic phase was washed with water, brine, dried (Na_2SO_4) and concentrated. The crude residue was purified by flash column chromatography to give diastereomeric mixture (hexane/EtOAc=85:15) to give **7a** and **7b** as mixture in 1:2 (519g, 85%) as a white powder.

Phenyl -(2,3:4,6-di-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside 14a (Exo):

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.58 (d, J = 7.2 Hz, 2 H; H-Ar), 7.49-7.46 (m, 2 H; H-Ar), 7.45-7.40 (m, 4 H; H-Ar), 7.39-7.33 (m, 12 H; H-Ar), 7.32-7.26 (m, 5 H; H-Ar), 6.26 (s, 1 H; H-CPh), 5.46 (s, 1 H; H-CPh), 5.07 (d, J = 10.8 Hz, 1 H; H_2CPh), 4.91 (d, J = 1.8 Hz, 1 H; H'-1), 4.82 (d, J = 10.8 Hz, 1 H; H_2CPh), 4.59 (d, J = 11.7 Hz, 1 H; H_2CPh), 4.41 (dd, J = 5.4, 7.8 Hz, 1 H; H_2CPh), 4.37 (s, 1 H; H'-3), 4.35 (s, 1 H; H-1), 4.06-3.95 (m, 3 H; H-4, H'-2, H'-6b), 3.83 (dd, J = 8.4, 9.6 Hz, 1 H; H'-4), 3.79-3.70 (m, 2 H; H-6ab), 3.56-3.44 (m, 3 H; H-3, H-5, H'-6a), 3.35 (t, J = 9.7 Hz, 1 H, H-2), 3.14 (dt, J = 5.1, 9.9 Hz, 1 H; H'-5).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 138.4, 138.3, 137.8, 137.0, 134.0, 130.7, 129.2, 129.2, 129.0, 128.5, 128.4, 128.3, 128.2, 128.0, 127.8, 127.8, 126.4, 126.2, 103.9, 103.9, 101.8, 99.9, 99.8, 85.9, 83.2, 78.9, 75.3, 74.2, 73.5, 68.6, 68.2, 65.3, 64.6.

HRMS (ESI): *m/z* calcd for $C_{46}H_{45}N_3NaO_9S$, $[M+Na]^+$ 838.27742, found 838.27809.

Phenyl -(2,3:4,6-di-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside 14b (Endo):

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.61-7.57 (m, 2 H; H-Ar), 7.52 (dd, J = 1.9, 7.3 Hz, 2 H; H-Ar), 7.44 (dd, J = 2.1, 7.5 Hz, 2 H; H-Ar), 7.41-7.37 (m, 2 H; H-Ar), 7.35-7.31 (m, 9 H; H-Ar), 7.31-7.27 (m, 5 H; H-Ar), 7.27-7.24 (m, 3 H; H-Ar), 6.01 (s, 1 H; H-CPh), 5.27 (s, 1 H; H-CPh), 5.04 (d, J = 10.5 Hz, 1 H; H_2CPh), 4.97 (d, J = 2.4 Hz, 1 H; H'-1), 4.77 (d, J = 10.5 Hz, 1 H; H_2CPh), 4.72 (d, J = 12.0 Hz, 1 H; H_2CPh), 4.50 (d, J = 12.0 Hz, 1 H; H_2CPh), 4.35 (d, J = 10.2 Hz, 1 H; H-1), 4.27 (dd, J = 6.0, 7.5 Hz, 1 H; H'-3), 4.12 (dd, J = 2.4, 5.4 Hz, 1 H; H'-2), 4.09-4.04 (m, 2 H; H-4, H'-6b), 3.85-3.74 (m, 2 H; H-6ab), 3.68 (dd, J = 7.5, 9.9 Hz, 1 H; H'-4), 3.53 (t, J = 9.3 Hz, 1 H; H-3), 3.45 (d, J = 9.6 Hz, 1 H; H-5), 3.36 (t, J = 9.9 Hz, 1 H; H-2), 3.32-3.25 (m, 1 H; H'-6a), 3.14 (dt, J = 5.2, 10.0 Hz, 1 H; H'-5).

^{13}C-NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 138.1, 137.7, 137.4, 137.1, 134.1, 130.6, 129.3, 129.1, 128.6, 128.4, 128.2, 128.1, 127.9, 127.6, 126.4, 126.2, 105.0, 104.9, 101.6, 101.5, 98.4, 98.3, 85.9, 83.0, 79.9, 79.0, 76.5, 76.3, 76.0, 75.4, 73.7, 73.7, 73.7, 68.5, 68.2, 65.4, 64.4.

HRMS (ESI): *m/z* calcd for $C_{46}H_{45}N_3NaO_9S$, $[M+Na]^+$ 838.27742, found 838.27840.

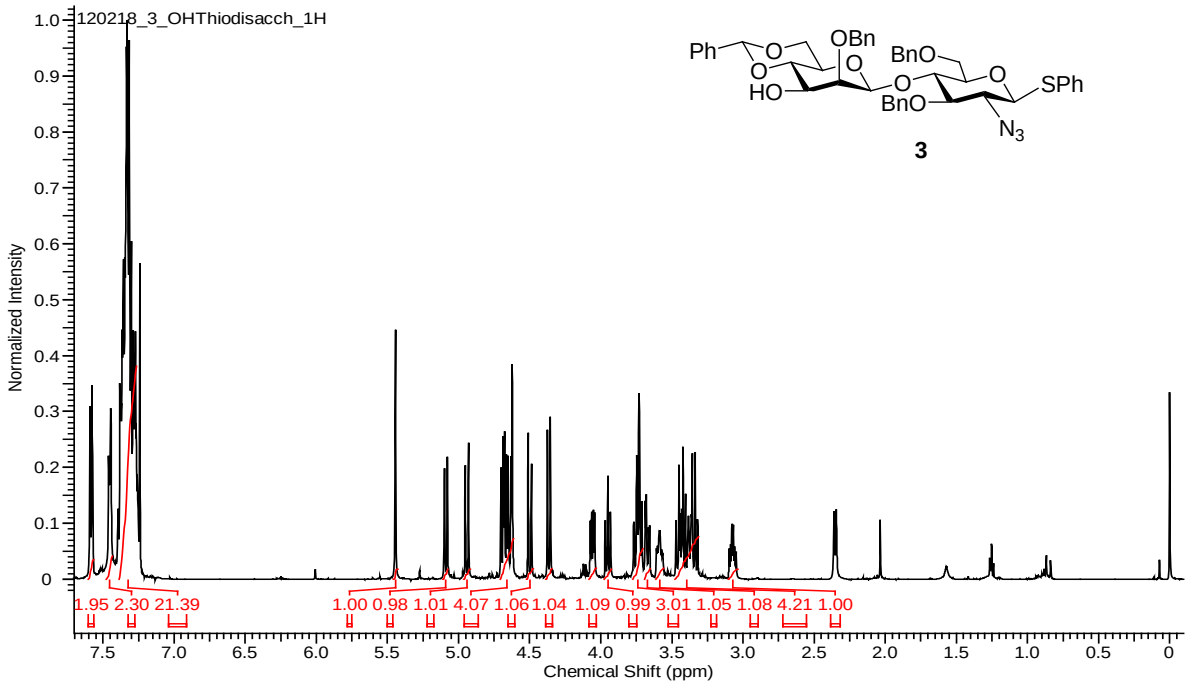
Phenyl -(2-*O*-benzyl-4,6-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside (3)

To a solution of **14b** (150 mg, 0.18 mmol) in toluene (920 µL, 0.2 M) was added 1 M DIBAL (540 µL, 0.54 mmol) in toluene and stirred at -30 ^oC for 2 h. Then, added 1 M DIBAL (360 µL, 0.36 mmol) in toluene and stirred at -30 ^oC for another 3 h. After, the reaction mixture was quenched with 10% aq. KOH at 0 ^oC and extracted with diethyl ether, dried (Na_2SO_4) and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=80:20) to give **3** (102mg, 68%) as a white powder.

^{1}H-NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.60-7.56 (m, 2 H; H-Ar), 7.45 (dd, *J* = 2.0, 7.3 Hz, 2 H; H-Ar), 7.39-7.26 (m, 21 H; H-Ar), 5.44 (s, 1 H; H-CPh), 5.09 (d, *J* = 9.9 Hz, 1 H; H_2CPh), 4.94 (d, *J* = 11.5 Hz, 1 H; H_2CPh), 4.71-4.63 (m, 2 H; H_2CPh), 4.62 (s, 1 H; H'-1), 4.50 (d, *J* = 11.8 Hz, 1 H; H_2CPh), 4.36 (d, *J* = 9.9 Hz, 1 H; H-1), 4.06 (dd, *J* = 4.8, 10.4 Hz, 1 H; H'-6b), 3.95 (t, *J* = 9.3 Hz, 1 H; H-4), 3.78-3.70 (m, 3 H; H'-2, H'-4, H-6b), 3.70-3.65 (m, 1 H; H-6a), 3.61-3.55 (m, 1 H; H'-3), 3.48-3.31 (m, 4 H; H-2, H-3, H-5, H'-6a), 3.07 (dt, *J* = 5.0, 9.6 Hz, 1 H; H'-5), 2.35 (d, *J* = 8.4 Hz, 1 H; OH).

^{13}C-NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 138.1, 138.0, 137.6, 137.2, 134.1, 130.5, 129.1, 129.1, 128.6, 128.5, 128.3, 128.2, 128.2, 128.1, 128.0, 128.0, 127.7, 126.3, 102.0, 101.6, 85.8, 83.4, 79.2, 79.1, 78.9, 78.9, 75.9, 75.5, 73.7, 73.7, 71.0, 68.4, 67.0, 64.2.

HRMS (ESI): *m/z* calcd for $C_{46}H_{47}N_3NaO_9S$, $[M+Na]^+$ 840.29307, found 840.2930.



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Chapter 3

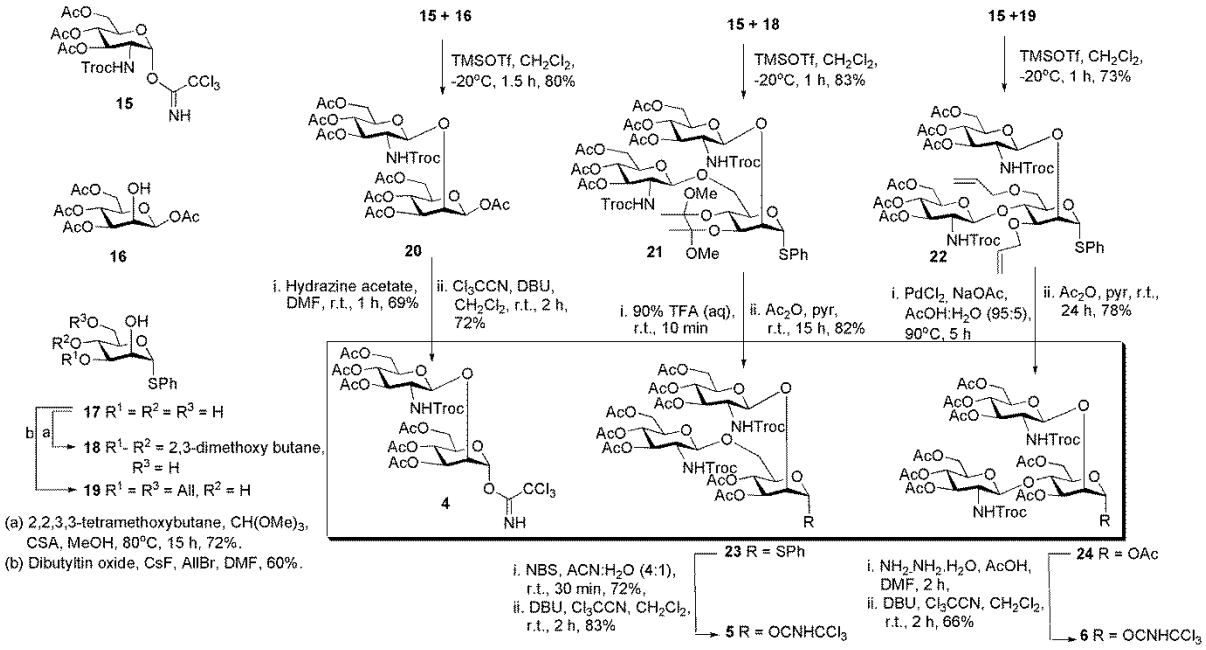
Synthesis of Multiantennary Complex Type N-Glycans by Use of Modular Building Blocks

3-1. Introduction.

The rapidly growing demand for recombinant therapeutic glycoproteins[1] has improved the analytical tools[2–4] capable of determining the vast number of structures of *N*-glycans present in biological material. At the same time glycobiologists have revealed more details about the interplay of protein *N*-glycosylation[5] with cellular functions. Due to the microheterogeneity of *N*-glycoproteins only a few of these oligosaccharides can be isolated from natural sources[6,7] in more than analytical amounts and thus the biological roles of *N*-glycans remain difficult to elucidate.[8] These circumstances have stimulated the chemical synthesis of *N*-glycans as a method to provide sufficient quantities for biological testing by using classical approaches[9] or miniaturized methods.[10] Seminal work utilizing chemically synthesized multiantennary fragments of *N*-glycans has revealed a multivalency effect for the asialoglycoprotein receptor, which also shows preferences for certain types of branched *N*-glycans.[11] In order to keep up with the growing number of identified *N*-glycans[2] and the demand for *N*-glycans on glycoarrays[12] we have developed a versatile set of building blocks that allow the synthesis of the most frequent core structures of complex type *N*-glycans. Based on the pioneering work of Ogawa[13], Paulsen[14], Carlo Unverzagt[15] and Danishefsky[16] we have established a robust and general method for the double regio- and stereo selective glycosylation of differently functionalized core disaccharide equipped with a benzylidene protected β-mannose as a key component. After optimizing this approach for biantennary *N*-glycans the building blocks were modified for the convergent synthesis of multiantennary *N*-glycans.

3-2. Results and Discussions.

3-2-1: Synthesis of disaccharide/trisaccharide donors **4**, **5** and **6**



Scheme 3-2-1: Synthesis of glycosyl donors **4**, **5** and **6**

To construct glycosyl donors **4**, **5**, and **6**, monosaccharide derivatives 15-19[17-19]were prepared by the use of free mannose and glucosamine hydrochloride. Then with normal glycosylation reactions with proper acceptor and donors gives respective intermediates followed by the protective group manipulation gives the donors **4**, **5** and **6** in high yields. Glycosylation with donor **15** and acceptor **16** afford disaccharide intermediate **20** in 80% followed hemiacetal formation and chloroacetonitrile treatment in the presence of DBU afford disaccharide donor **4** with 72% in two steps.

Excess donor **15** was glycosylated with acceptor **18** to afford trisaccharide **21** in 83%. Deprotection of methoxy group followed by the acetylation of **21** gives thiotrisaccharide **23** in 82%. Thiophenol deprotection of **23** using NBS followed by the treatment of trichloroacetonitrile in the presence of DBU afford trisaccharide donor **5** in high yield.

Trisaccharide **22** was obtained by the glycosilation with excess donor **15** and acceptor **19**. Deallylation followed by acetylation afford trisaccharide **24**, during deallylation in the presence of Pd cleaves thiophenol at the anomeric position, it means thiophenol protection

was sensitive towards palladium catalysed reactions. Hemiacetal formation followed by the treatment of trichloroacetonitrile with DBU to compound **24** afford trisaccharide donor **6** with good yield.

The synthesized trisaccharide donors and intermediates were analyzed and characterized by using 2D NMR (DQF COSY, TOCSY, NOESY, HMBC and HSQC) spectroscopy and all signals were assigned.

Table 3-2-1: ^{1}H-NMR chemical shift.

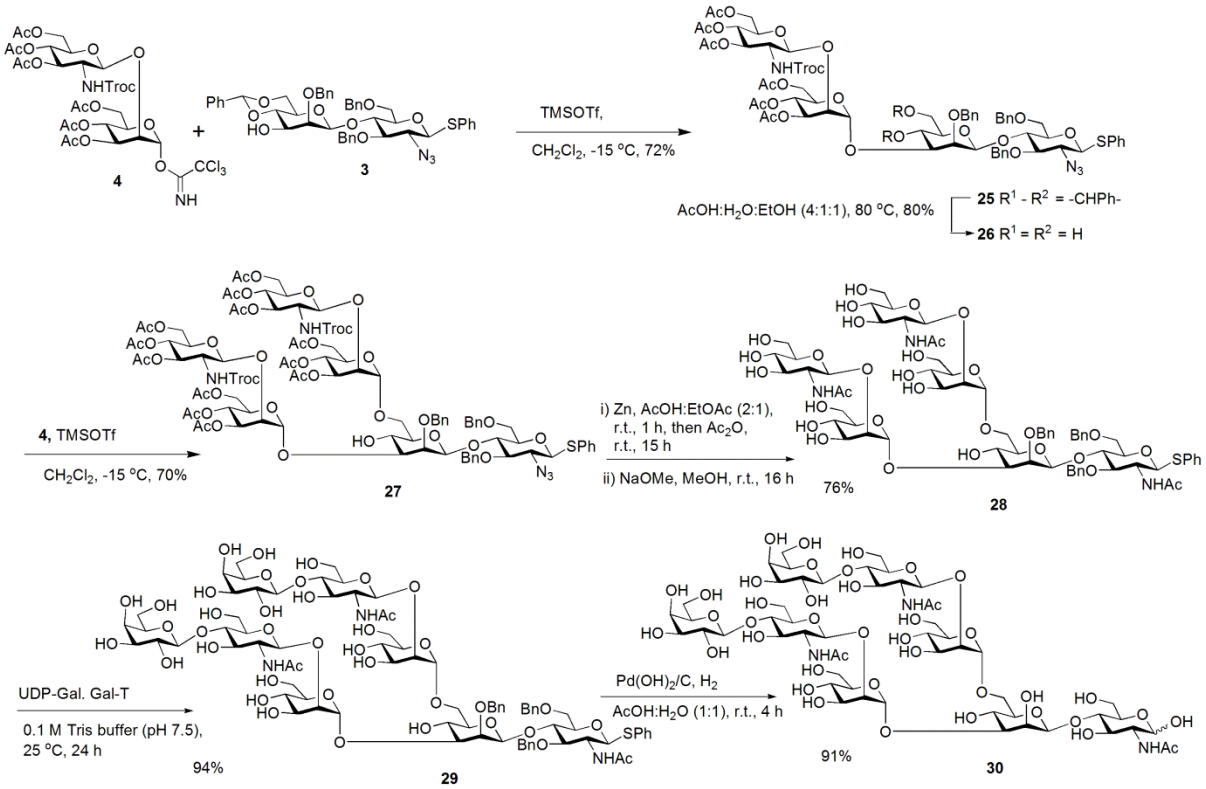
Residues		5	22	24α	24β	6
Man I	H1	6.25	5.41	5.67	5.96	6.13
	H2	4.42	4.29	4.31	4.16	4.36
	H3	5.21	3.81	4.83	5.08	5.13
	H4	5.48	4.07	3.95	3.99	4.03
	H5	3.91	4.21	3.64	3.91	4.03
	H6a	3.22	3.59	4.24	4.28	4.28
	H6b	4.16	3.75	4.15		4.37
	Ph-CH		7.30-7.50			
NTrocGlc II	H1	5.27	5.02	4.52	4.53	4.60
	H2	3.22	3.44	3.88	3.77	3.77
	H3	5.71	5.53	5.17	5.17	5.20
	H4	5.01	5.07	5.08	5.08	5.05
	H5	3.76	3.73	3.67	3.67	3.72
	H6a	4.05	4.19	4.09	4.09	4.05
	H6b	4.30	4.32			4.27
	NTroc-CH_2	4.73	4.55	4.73	4.73	4.59
	NTroc-CH_2	4.84		4.79	4.79	4.81
NTrocGlc III	H1	4.33	4.88	4.66	4.66	4.69
	H2	3.96	3.74	3.69	3.69	3.68
	H3	5.15	5.18	5.24	5.24	5.24
	H4	5.07	5.11	5.06	5.06	5.06
	H5	3.67	3.71	3.71	3.71	3.68
	H6a	4.12	4.14	4.39	4.39	4.07
	H6b	4.27	4.25			4.32
	NTroc-CH_2	4.74	4.63	4.61	4.61	4.71
	NTroc-CH_2	4.83	4.85	4.70	4.70	4.75
	CO-CH_3	2.02-2.10	1.95-2.11	2.04-2.18	2.04-2.18	1.94-2.16
	3OAllyl CH_2		4.17-4.23			
	6OAllyl CH_2		3.97-4.09			
	Allyl CH		5.93-5.99			
	Allyl CH_2		5.36-5.21			

Table 3-2-2: ^{13}C-NMR chemical shift

Residues		5	22	24α	24β	6
Man I	C1	94.6	85.8	91.3	91.2	95.6
	C2	71.7	75.7	74.2	74.3	73.8
	C3	69.7	77.4	72.5	70.4	70.6
	C4	65.2	76.2	73.3	73.5	73.7
	C5	71.9	71.6	73.7	71.5	71.8

3-2-2: Synthesis of bi-antennary *N*-glycan **30**

After synthesizing the disaccharide core structure **3** as an acceptor and donors **4**, **5** and **6,** we switched to construct hyper branched glycans structures.



Scheme 3-2-2: Preparation of bi-antennary *N*-glycan.

To synthesize bi-antennary *N*-glycan, firstly I did glycosylation reaction with acceptor **3** and donor **4** to afford tetrasaccharide **25**. The benzylidine group was deprotected to obtain the tetrasaccharide acceptor **26** was glycosylated using same donor **4** to afford hexasaccharide **27**. 2D NMR spectroscopy analysis provided complete information of sterio- and regio selectivity of compound **27** (Figure 3-2-1). The NTroc and azide group of **27** were converted into acetamide with Zn in acetic acid followed by the treatment with acetic anhydride, followed by the deacetylation gives free hydroxyl group containg sugar **28**. Galactosylation of non-reducing end with UDP-Gal and β1,4-galactosyltransferase goes smoothly even in the presence of benzyl and thiophenol group at reducing end afforded octasaccharide **29**. After complete conversion, the product **29** was purified by using HPLC with column: Inertsil ODS-3 (250×20 mm), Eluent A: H_2O, Eluent B: CH_3CN, eluent (A/B = 97/3) was employed from 0 min, then the ratio of eluent B was increased linearly from 3% to 60% over 50 min with a flow rate of 5.0 ml/min. Palladium catalyzed dehydrogenation of **29** afforded free bi-

antennary sugar **30** in high yield. Due to the presence of thio group the replacement of fresh palladium is needed to complete the hydrogenation reaction. Synthesized compounds were completely assigned by using 2 D NMR spectroscopy and tabulated (Table 3-2-3 and 3-2-4).

Figure 3-2-1: HSQC spectrum of bi-antennary *N*-glycan intermediate **27**.

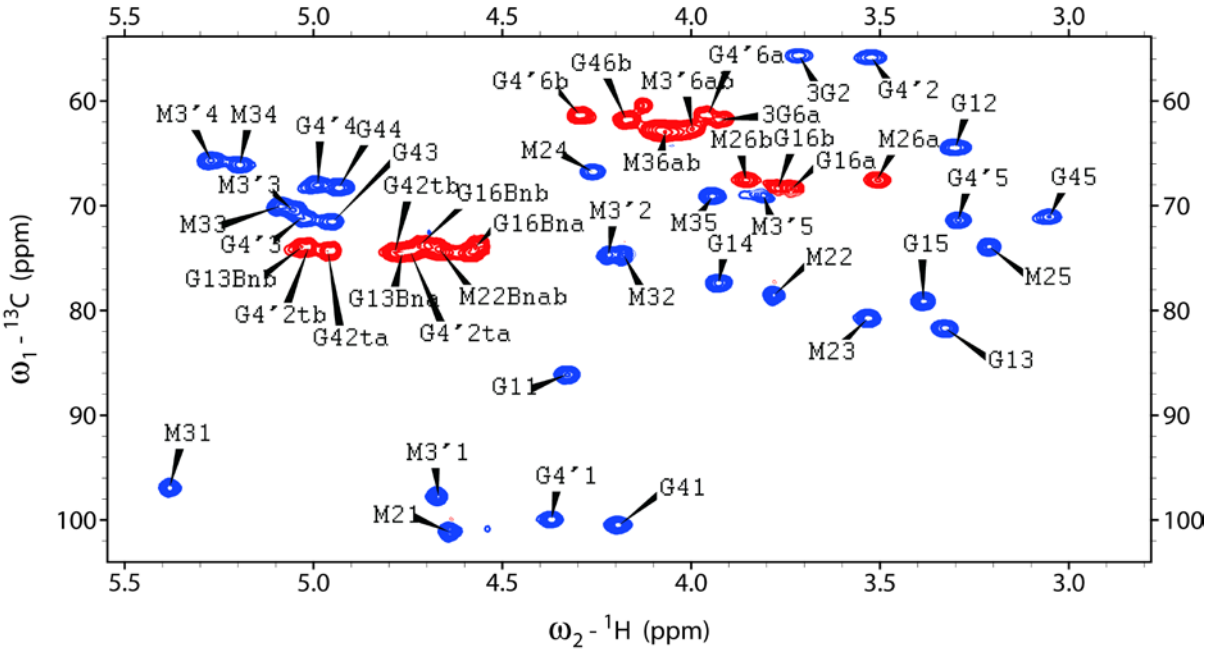
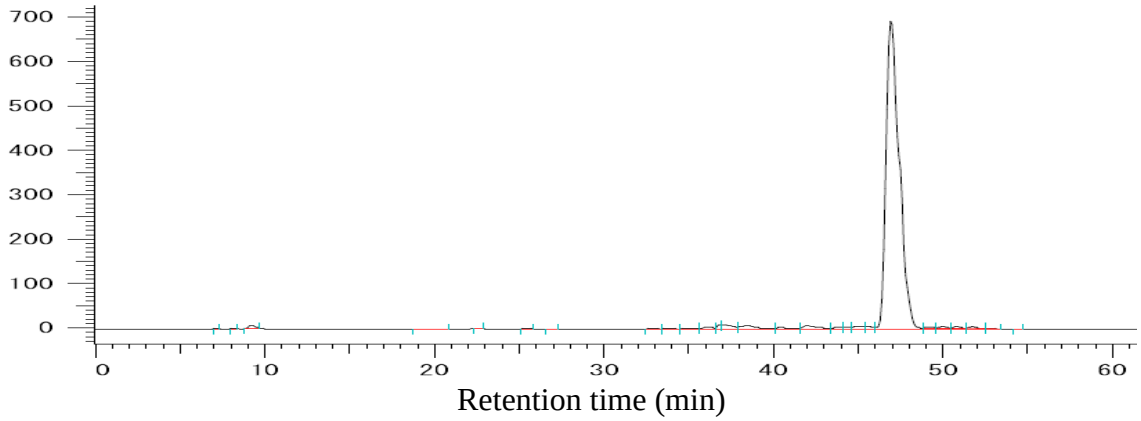


Figure 3-2-2: RP HPLC of (A) Hexasaccharide **28** and (B) Octasaccharide **29**.

Column: Inertsil ODS-3 (250×20 mm), Eluent A: H_2O, Eluent B: CH_3CN, eluent (A/B = 97/3) was employed from 0 min, then the ratio of eluent B was increased linearly from 3% to 60% over 50 min with a flow rate of 5.0 ml/min.

A.



B.

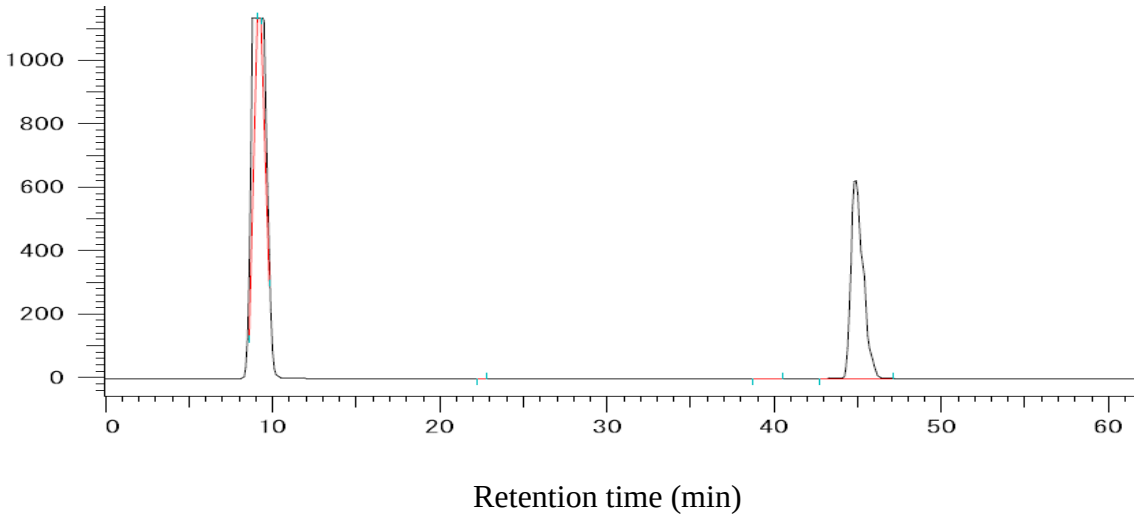


Table 3-2-3: ^{1}H-NMR chemical shift.

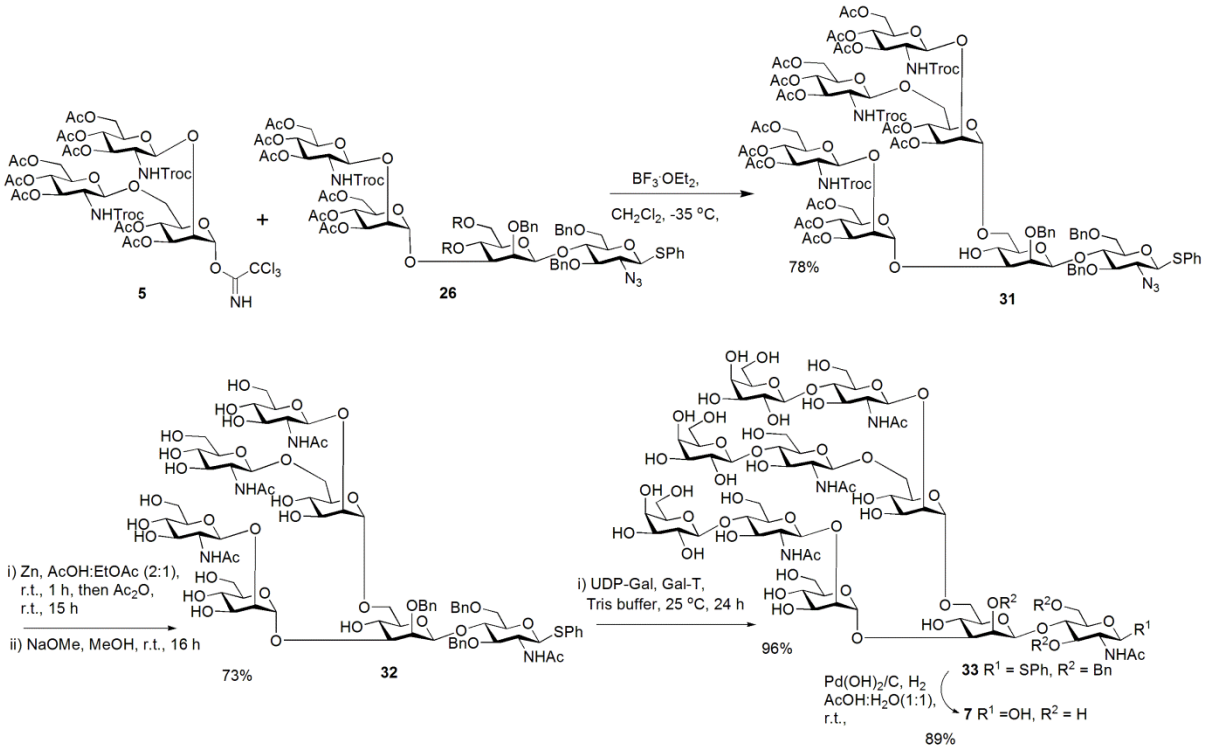
Residues		27	28	29	30
GlcNAc I	H1	4.33	4.66	4.64	5.13
	H2	3.3	3.64	3.69	3.81
	H3	3.32	3.44	3.42	3.64

Residues		27	28	29	30
GlcNAc IV	H1	4.19	4.46	4.47	4.51
	H2	3.71	3.63	3.65	3.66
	H3	4.95	3.48	3.49	3.64

Table 3-2-4: ^{13}C-NMR chemical shift

Residues		27	28	29	30		Residues		27	28	29	30
GlcNAcI	C1	86.1	86.2	86.2	90.5		GlcNAc IV	C1	100.5	99.8	99.7	99.5
	C2	64.5	55.5	54.9	53.6			C2	55.8	55.5	54.9	54.9
	C3	81.8	81.5	81.4	71.9			C3	71.5	73.1	74.8	72.2
	C4	77.4	78.4	78.5	79.8			C4	68	69.2	78.5	78.6
	C5	79.2	78	78.1	74.8			C5	71.4	76	71.8	74.7
	C6	68.4	68.2	68.3	60			C6	61.3	60.7	59.5	60.1
Man II	C1	101.2	101.4	102	100.5		GlcNAc IV'	C1	100	99.6	99.4	99.5
	C2	78.5	78.1	78.1	70.4			C2	55.8	55.4	54.9	54.9
	C3	80.4	79.9	79.9	80.6			C3	71.2	69.9	74.1	72.2
	C4	66.8	74.1	74.2	74.5			C4	68.4	73.1	77.9	78.6
	C5	73.9	66.2	66.1	65.8			C5	71.1	75.3	71.9	74.7
	C6	67. 6	65.2	65.2	65.8			C6	61.8	60	59.4	60.1
Man III	C1	97.1	99.3	99.3	99.6		Gal V	C1			103.1	103
	C2	74.7	76.7	76.4	76.4			C2			71.1	71.1
	C3	70.1	69.5	69.8	69.4			C3			72.1	72.7
	C4	65. 8	67.3	67.2	67.5			C4			68.6	68.7
	C5	69.1	73.8	73.8	73.6			C5			75.4	75.5
	C6	62.9	61.6	61.6	61.8			C6			60.9	60.9
Man III'	C1	97.9	96.4	96.4	97.1		Gal V'	C1			103.1	103
	C2	74.8	76.3	76.3	76.4			C2			71.1	71.1
	C3	70.4	69.6	69.8	69.5			C3			72.1	72.7
	C4	66.2	67.4	67.2	67.5			C4			68.6	68.7
	C5	69.2	73.1	72.6	73			C5			75.4	75.5
	C6	62.7	61.6	61.6	61.7			C6			60.9	61.1

3-2-3: Synthesis of tri-antennary *N*-glycan **7**



Scheme 3-2-3: Synthetic scheme of tri-antennary *N*-glycan.

My attempt for the synthesis of tri-antennary *N*-glycans was successful and achieved the expected product with high yield. For this purpose, acceptor **26** and donor **5** was glycosylated in the presence of boranetrifluoride dietherate afforded heptasaccharide **31** with 78% yield. The stereo- and regio selectivity of the intermediate **31** was confirmed by using 2 D NMR spectroscopy (Figure 3-2-3). Deprotection of NHTroc, azide and formation of acetamide using zinc in acetic acid followed by acetic anhydride, then deacetylation using sodium methoxide afforded partially protected free sugar **32**. Enzymatic galctosylation using Gal-T, and UDP Gal in the presence of tris-buffer at 25^{o}C produced partially benzylated decasaccharide **33** in almost quantitative yield. The galactosylation reaction was monitored and purified by using HPLC with column: Inertsil ODS-3 (250×20 mm), Eluent A: H_2O, Eluent B: CH_3CN, eluent (A/B = 97/3) was employed from 0 min, then the ratio of eluent B was increased linearly from 3% to 80% over 50 min with a flow rate of 5.0 ml/min (Figure 3-2-4). Dehydrogenation of decasaccharide **33** in the presence of palladium hydroxide afforded free tri-antennary *N*-glycan **7** in good yield. Due to the presence of thio group, the

replacement of fresh palladium is needed to complete the hydrogenation reaction. Synthesized compounds were completely assigned by using 2 D NMR spectroscopy and tabulated (Table 3-2-5 and 3-2-6).

Figure 3-2-3: HSQC spectrum of tri-antennary *N*-glycan intermediate **31**.

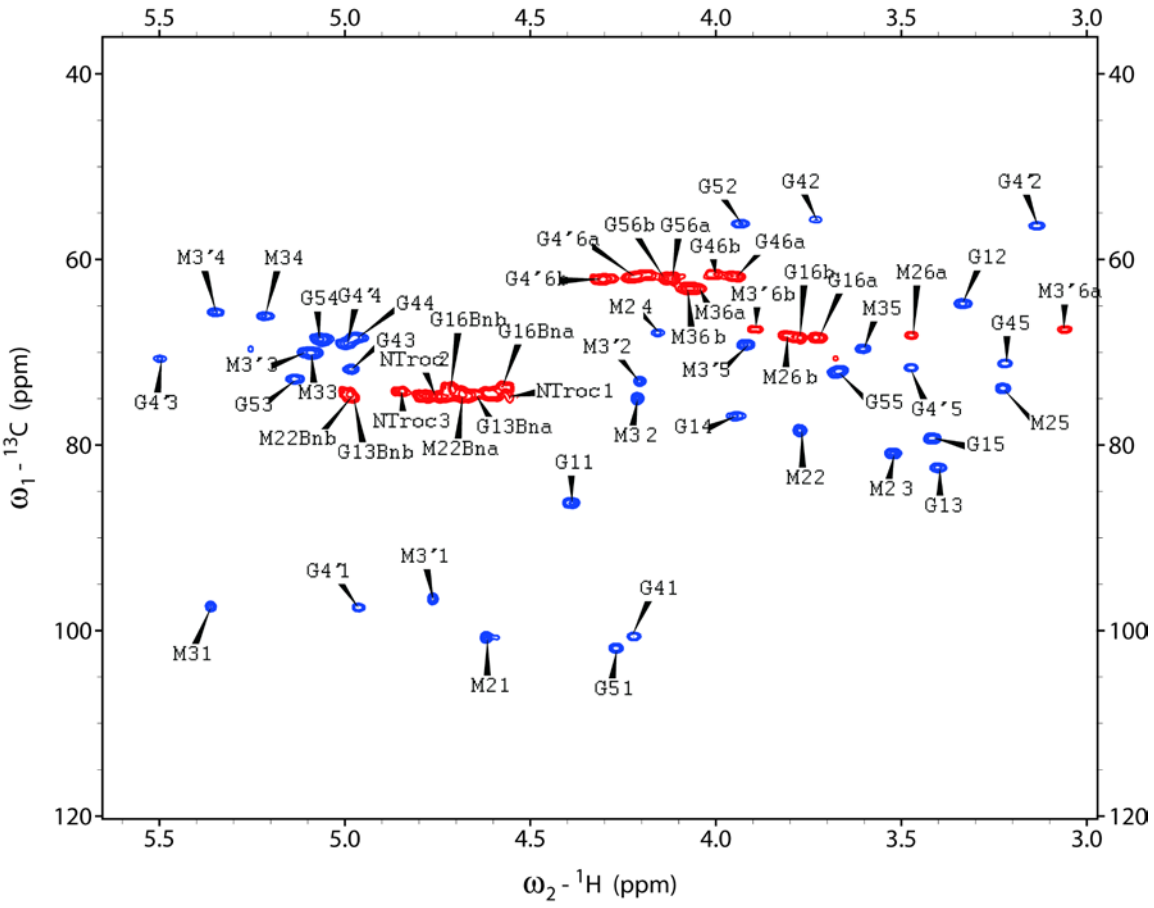
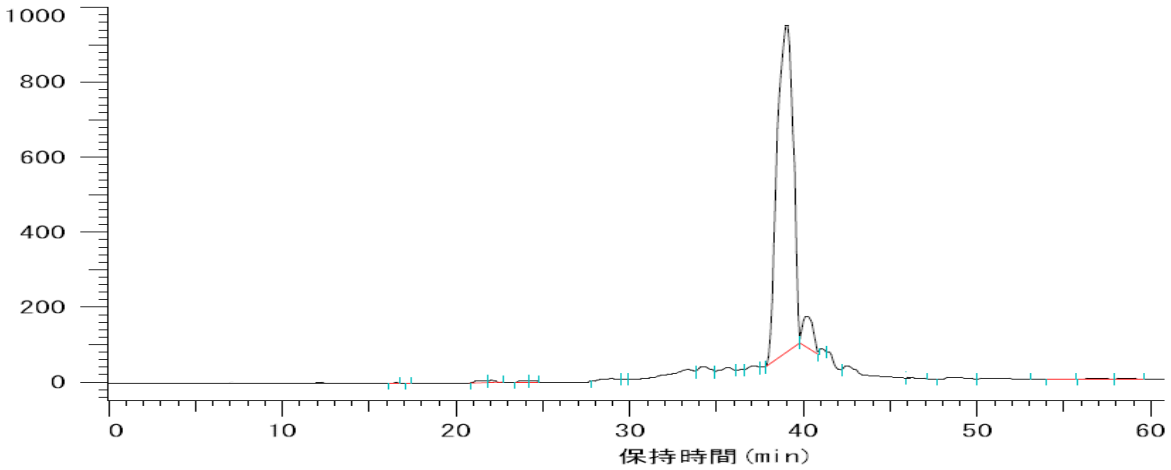


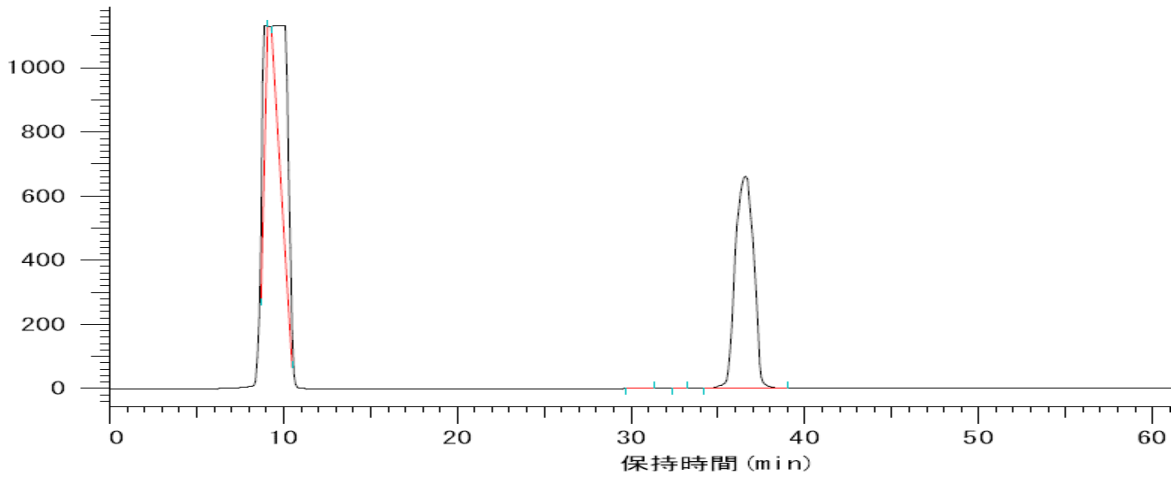
Figure 3-2-4: RP HPLC of (A) Heptasaccharide **32** and (B) Decasaccharide **33**.

Column: Inertsil ODS-3 (250×20 mm), Eluent A: H_2O, Eluent B: CH_3CN, eluent (A/B = 97/3) was employed from 0 min, then the ratio of eluent B was increased linearly from 3% to 80% over 50 min with a flow rate of 5.0 ml/min.

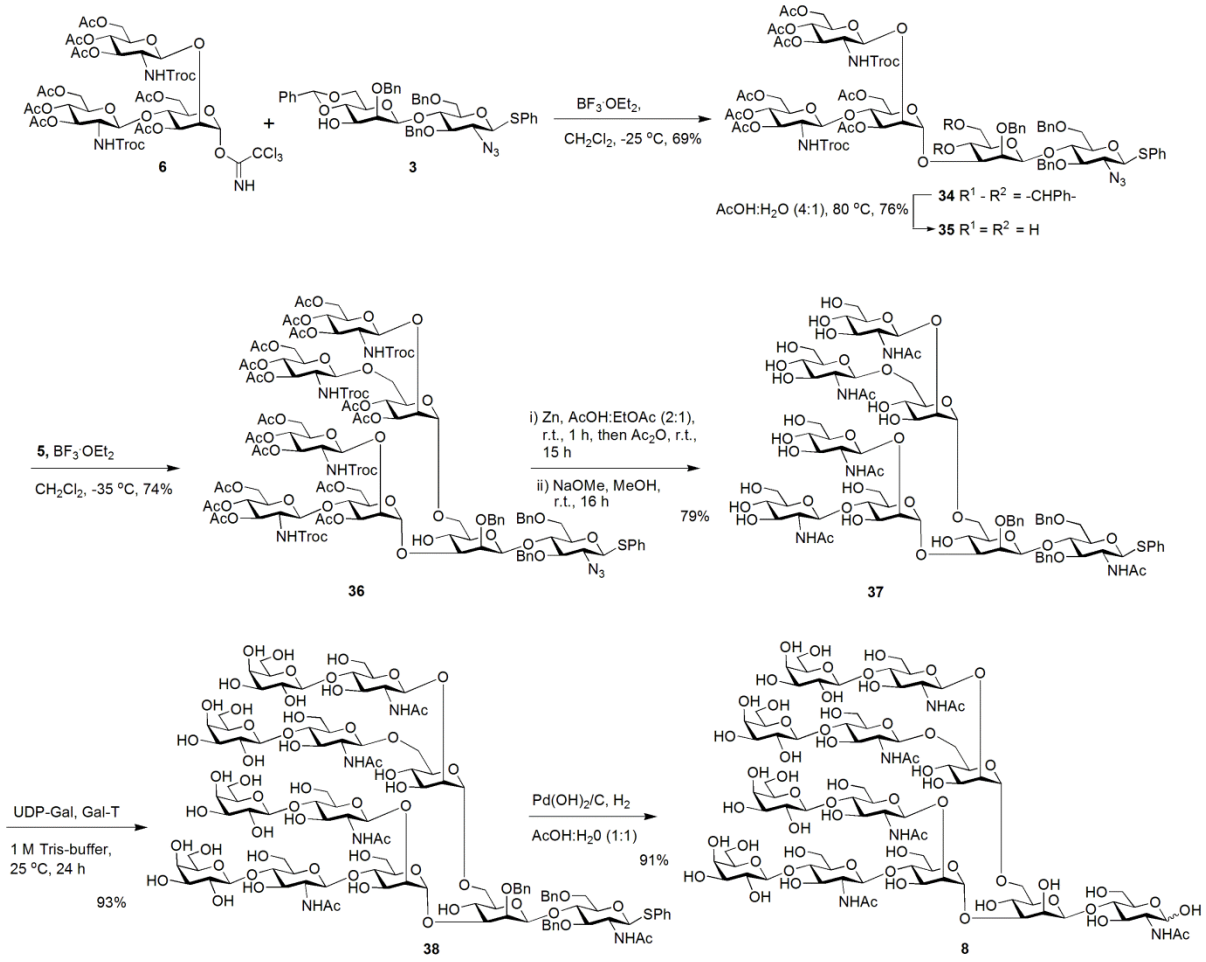
A.



B. Retention time



3-2-4: Synthesis of tetra-antennary *N*-glycan **8**



Scheme 3-2-4: Synthetic scheme of tetra-antennary *N*-glycan.

After completing the total synthesis of bi- and tri-antennary *N*-glycans, I switched to construct the tetra-antennary *N*-glycan structure by following same protocol. With the donors **5** and **6** in hand the glycosylation of the core disaccharide **3** and its coupling product was investigated. Starting with the building blocks **3** and **6** the pentasaccharide **35** was obtained after BF3·OEt2 mediated coupling and removal of the benzylidene acetal. Acceptor **35** was employed in a coupling reaction with the branched donor **5**. As desired a regio and stereo selective glycosylation occurred at the primary OH-6 to furnish the tetraantennary octasaccharide *N*-glycan **36** in good yield. Regio- and stereo selectivity were excellent in both cases and no signs of steric hindrance were found in these couplings (Figure 3-2-5).

Conversion of NHTroc and azide, to acetamide using zinc in acetic acid followed by acetic anhydride, then deacetylation using sodium methoxide afforded partially free sugar **37**. Enzymatic galctosylation using Gal-T, and UDP Gal in the presence of tris-buffer at 25^{o}C produced partially benzylated dodecasaccharide **38** in almost quantitative yield. The galactosylation reaction was monitored and purified by using HPLC with column: Inertsil ODS-3 (250×20 mm), Eluent A: H_2O, Eluent B: CH_3CN, eluent (A/B = 97/3) was employed from 0 min, then the ratio of eluent B was increased linearly from 3% to 90% over 50 min with a flow rate of 5.0 ml/min (Figure 3-2-6). Dehydrogenation of dodecasaccharide **38** in the presence of palladium hydroxide afforded free tetra-antennary *N*-glycan **8** in good yield. Due to the presence of thio group, the replacement of fresh palladium is needed to complete the hydrogenation reaction. Synthesized compounds were completely assigned by using 2 D NMR spectroscopy and tabulated (Table 3-2-5 and 3-2-6).

Figure 3-2-5: HSQC spectrum of tetra-antennary *N*-glycan intermediate **36**.

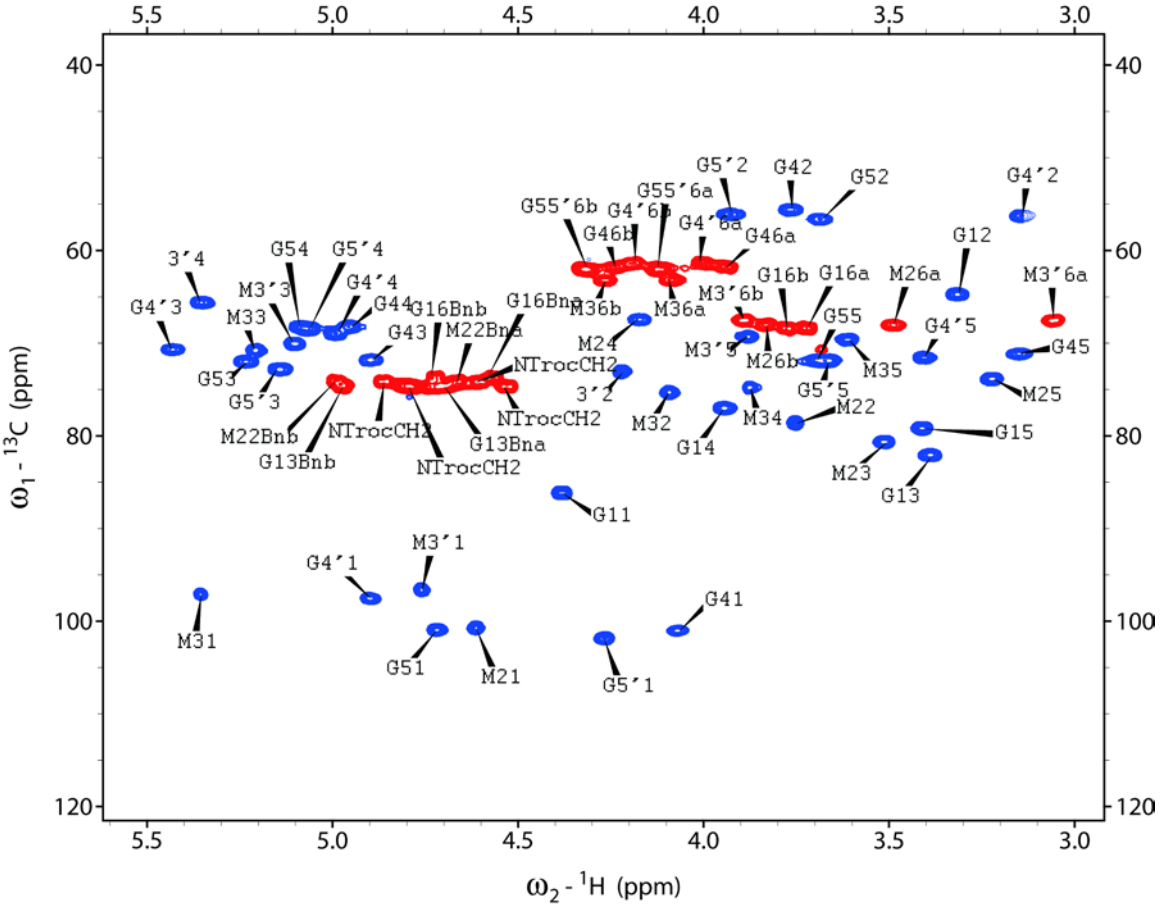
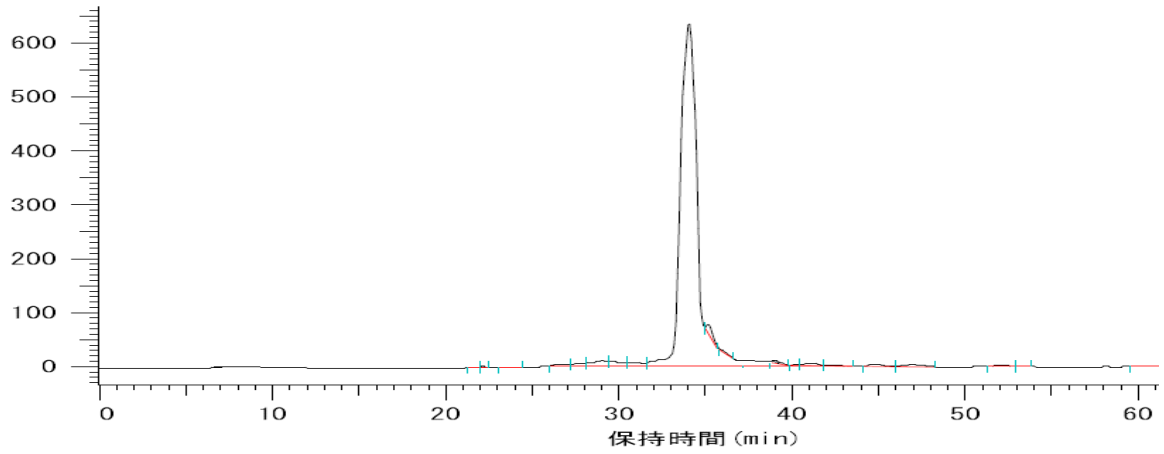


Figure 3-2-6: RP HPLC of (A) Decasaccharide **37** and (B) Dodecasaccharide **38**.

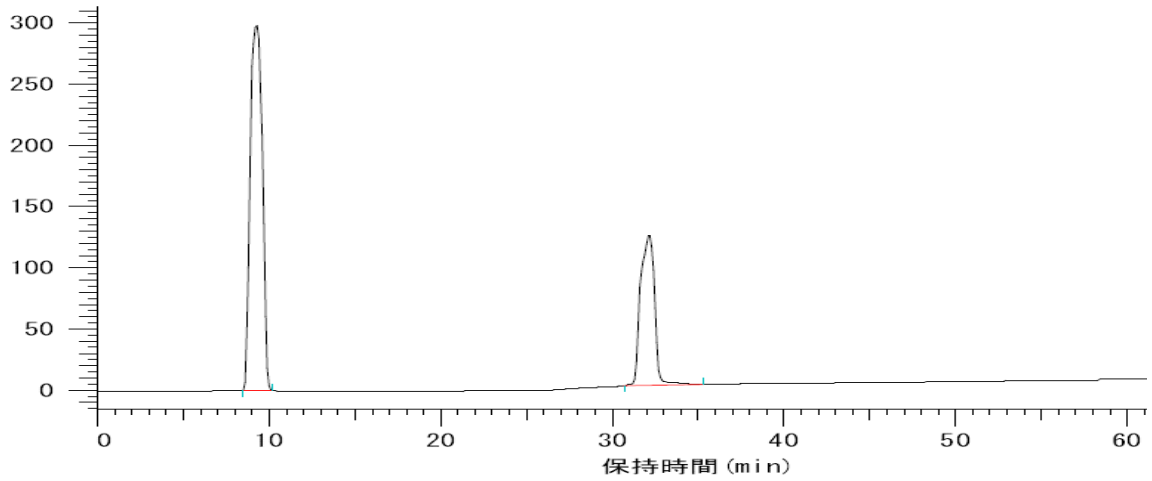
Column: Inertsil ODS-3 (250×20 mm), Eluent A: H_2O, Eluent B: CH_3CN, eluent (A/B = 97/3) was employed from 0 min, then the ratio of eluent B was increased linearly from 3% to 90% over 50 min with a flow rate of 5.0 ml/min.

A



B.

Retention time



Retention time

Table 3-2-5: ^{1}H-NMR chemical shift.

Residues		31	34	35	36	Residues		31	34	35	36
	$COCH_3$	1.95-2.13	2.01-2.15	1.96-2.13	1.98-2.15	NTrocGlc IV	H1	4.23	3.43	4.14	4.07
	PhCH	7.22-7.57	7.30-7.62	7.24-7.60	7.2-7.57		H2	3.73	3.62	3.68	3.76
N_3 Glc I	H1	4.39	4.41	4.39	4.38		H3	4.99	4.47	4.83	4.90
	H2	3.34	3.37	3.35	3.312		H4	4.97	4.88	4.97	4.95
	H3	3.40	3.51	3.40	3.385		H5	3.22	2.96	3.40	3.15
	H4	3.95	4.02	3.93	3.946		H6a	3.94	4.20	4.01	3.94
	H5	3.42	3.44	3.44	3.404		H6b	4.00	4.28	4.26	4.24
	H6a	3.72	3.75	3.73	3.719		$NTrocCH_2$	4.85	4.73	4.78	4.86
	H6b	3.77	3.85	3.80	3.774						
	$3PhCH_2$	4.65	4.69	4.56	4.701	NTrocGlc IV'	H1	4.96	4.61	4.74	4.89
		4.98	5.14	5.04	4.971		H2	3.13	3.74	3.66	3.14
	$6PhCH_2$	4.58	4.57	4.57	4.58		H3	5.49	5.19	5.23	5.43
		4.72	4.76	4.72	4.733		H4	4.99	5.09	5.07	4.99
Man II	H1	4.62	4.64	4.58	4.612		H5	3.48	3.63	3.73	3.40
	H2	3.77	3.73	3.82	3.751		H6a	4.22	3.90	4.17	4.01
	H3	3.52	3.69	3.50	3.506		H6b	4.31	4.35	4.29	4.18
	H4	4.15	4.08	4.03	4.174		$NTrocCH_2$	4.76	4.60	4.68	4.80
	H5	3.22	3.04	3.04	3.219						
	H6a	3.47	3.39	3.51	3.487	NTrocGlc V	H1				4.72
	H6b	3.81	4.02	3.67	3.829		H2				3.68
	$2PhCH_2$	4.68	4.69	4.65	4.665		H3				5.23
		4.99	4.81	5.03	4.99		H4				5.09
	46PhCH		5.43				H5				3.70
Man III	H1	5.36	5.03	5.29	5.354		H6a				4.12
	H2	4.21	4.07	4.04	4.093		H6b				4.31
	H3	5.09	5.05	5.27	5.21		$NTrocCH_2$				4.54
	H4	5.21	3.90	3.77	3.874	NTrocGlc V'	H1	4.27			4.27
	H5	3.60	3.63	3.87	3.61		H2	3.93			3.93
	H6a	4.04	4.06	4.03	4.093		H3	5.13			5.15
	H6b	4.08	4.23	4.22	4.265		H4	5.07			5.06
Man III'	H1	4.76			4.759		H5	3.67			3.66
	H2	4.21			4.216		H6a	4.12			4.12
	H3	5.11			5.105		H6b	4.14			4.31
	H4	5.35			5.356		$NTrocCH_2$	4.56			4.62
	H5	3.91			3.878						
	H6a	3.06			3.057						
	H6b	3.89			3.891						

Table 3-2-6: ^{13}C-NMR chemical shift.

Residues		31	34	35	36	Residues		31	34	35	36
	CO-H_3C	20.8-21.2	20.6-20.9	20.6-20.7	20.7-21.1	NTrocGlc IV	C1	100.6	101.8	100.9	101.1
	Ph-HC	126.9-133.4	126.8-134.4	127.2-134	126.5-133.4		C2	55.8	55.2	55.6	55.6
N_3 Glc I	C1	86.2	85.7	86.0	86.2		C3	72.0	72.0	72.0	71.9
	C2	64.7	64.2	64.4	64.9		C4	68.5	68.2	68.4	68.3
	C3	82.4	83.5	83.4	82.2		C5	71.2	71.2	71.6	71.1
	C4	76.9	76.2	76.5	77.1		C6a	61.8	62.4	61.9	61.9
	C5	79.3	79.5	79.4	79.3		C6b	61.6	62.3	61.9	61.8
	C6a	68.3	68.4	68.2	68.3		NTroc-H_2C	74.1	74.7	74.5	74.2
	C6b	68.4	68.4	68.3	68.4						
	3Ph-H_2C	74.6	75.6	75.1	74.6	NTrocGlc IV'	C1	97.7	101.1	101.2	97.7
		74.9	75.4	75.2	74.7		C2	56.4	56.6	56.4	56.3
	6Ph-H_2C	73.7	73.6	73.8	73.6		C3	70.7	72.4	72.0	70.7
		74.0	73.7	73.8	73.6		C4	69.1	68.2	68.3	69.0
Man II	C1	100.8	101.1	100.7	100.9		C5	71.8	71.7	72.0	71.5
	C2	78.4	77.8	79.2	78.7		C6a	61.9	61.7	61.9	61.3
	C3	81.0	75.7	81.3	80.7		C6b	62.1	61.8	61.9	61.2
	C4	67.9	78.9	66.5	67.3		NTroc-H_2C	74.7	74.8	74.6	74.7
	C5	74.0	66.8	75.8	73.9						
	C6a	68.3	68.5	62.4	68.0	NTrocGlc V	C1				101.0
	C6b	68.1	68.3	62.3	68.0		C2				56.6
	2Ph-H_2C	74.7	75.5	74.2	74.2		C3				72.0
		74.5	75.5	74.3	74.1		C4				68.3
	46PhCH		102.3				C5				72.0
Man III	C1	97.5	98.4	98.1	97.2		C6a				61.9
	C2	75.0	75.5	75.5	75.3		C6b				61.9
	C3	70.1	70.7	70.7	70.8		NTroc-H_2C				74.6
	C4	66.0	74.1	75.9	74.7	NTrocGlc V'	C1	102.0			102.0
	C5	69.7	69.4	69.3	69.6		C2	56.1			55.9
	C6a	63.1	62.0	63.5	63.2		C3	73.0			72.8
	C6b	63.1	61.7	63.4	63.3		C4	68.7			68.4
Man III'	C1	96.9			96.8		C5	72.2			71.9
	C2	73.2			73.1		C6a	61.9			61.9
	C3	70.1			70.1		C6b	62.0			61.9
	C4	65.7			65.6		NTroc-H_2C	74.8			74.3
	C5	69.2			69.2						
	C6a	67.5			67.4						
	C6b	67.3			67.5						

3-3. Conclusion.

In summary, we designed di and tri saccharide glycosyl donors **4**, **5**, and **6** as an essential key synthons for the synthesis of all types of complex *N*-glycans. To show the excellent versatility of acceptor **3**, the bi-antennary *N*-glycan was efficiently synthesized with glycosyl donor **4** in high yield. By the use of donors **4**, **5** and acceptor **3**, we developed efficient synthetic method for the synthesis of tri-antennary *N*-glycan. After constructing bi- and tri-antennary *N*-glycans, we developed a novel synthetic method for the synthesis of tetra-antennary *N*-glycan by using glycosyl donors **5**, **6** and acceptor **3.** We successfully developed novel synthetic protocol for the synthesis of hyper branched *N*-glycans. This new strategy should allow for the synthesis of a variety of glycopeptides and glycoproteins having homogeneous and highly complicated *N*-glycans.

3-4. Experimental Section.

All reactions were carried out under a nitrogen atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Proton and carbon NMR was recorded with Varian UnityInova 500 MHz (Agilent Inc., USA; ^{1}H: 500 MHz, ^{13}C: 125 MHz). Chemical shifts are given in ppm and referenced to internal TMS (δ_H 0.00 in $CDCl_3$), $CHCl_3$ (δ_H 7.26 in $CDCl_3$) or $CDCl_3$ (δ_C 77.00). Assignments in ^{1}H NMR were made by first-order analysis of the spectra by using ACD/NMR processor software (Advanced Chemistry Development, inc.) and were verified by H–H COSY and HSQC experiments. 2D NMR of compounds were recorded at 300 K with a Bruker Avance 600 spectrometer at 600.03 MHz for proton frequency equipped with cryoprobe. For the complete assignments and structural determination, two dimentional homonuclear DQF-COSY, TOCSY with MLEV-17 and NOESY spectra were recorded in the indirect dimension using States-TPPI phase cycling. Additionally two dimentional heteronuclear ^{13}C edited HSQC and HSQC-TOCSY measurements were also recorded with echo-antiecho mode for sensitive enhancement. All NMR data were processed by NMRPipe software and analysed using the Sparky program. A high/low resolution electrospray ionization mass spectra (ESI-MS) were recorded by JMS-700TZ (JEOL, Japan) and Bruker ultraflex-I. TLC was performed on Merck pre coated plates (20 cm × 20 cm; layer thickness, 0.25 mm; Silica Gel $60F_{254}$); spots were visualized by spraying a solution of 90:5:5 (v/v/v) MeOH-*p*-anisaldehyde-concentrated sulfuric acid and heating at 250°C for ca. $^{1}/_{2}$ min. Column chromatography was performed on Silica Gel N60 (spherical type, particle size 40–50 μm; Kanto Chemical Industry) with the solvent systems specified, and the ratio of solvent systems was given in v/v.

The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, br = broad.

Synthesis of disaccharide donor 4

4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-(1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose (20).

A glycosyl acceptor **16** (1.0g, 2.9 mmol) and glycosyl donor **15** (3.6g, 5.7 mmol) was dissolved and co-evaporated with toluene three times and dried under vacuum for 12 h. Dry DCM (0.1 M) was added and cooled to -20°C. After 10 min, TMSOTf (6 µL, 0.3 mmol) was added at -20^{o}C and the reaction mixture was stirred for 1 h at -20°C, and then quenched with triethylamine. The mixture was diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=1:1) to afford disaccharide **20** (1.9 g, 80%).

^{1}H NMR (500 MHz, $CDCl_3$, 25°C, TMS): δ 5.70 (s, 1 H; H-1), 5.35-5.23 (m, 2 H; H-4, H'-4), 5.09-5.00 (m, 2 H; H'-3, H-CTroc), 4.78-4.74 (m, 1 H; H-3), 4.73-4.64 (m, 2 H; H'-1, H-CTroc), 4.36 (d, *J* = 3.1 Hz, 1 H; H-2), 4.26 (dd, *J* = 5.5, 12.3 Hz, 1 H; H'-6b), 4.12-4.08 (m, 2 H; H-6ab), 4.02 (dd, *J* = 2.0, 12.5 Hz, 1 H; H'-6a), 3.76 (q, *J* = 9.1 Hz, 1 H; H'-2), 3.72-3.64 (m, 2 H; H-5, H'-5), 2.19-2.02 (s each, 3 H each, 6xCH_3CO).

^{13}C NMR (125 MHz, $CDCl_3$, 25°C, TMS): δ 170.7, 170.7, 170.6, 170.4, 169.4, 169.3, 168.3, 153.9, 95.1, 91.4, 91.0, 73.1, 72.9, 72.2, 71.9, 71.7, 71.5, 68.8, 68.2, 64.9, 62.1, 20.7, 20.6.

HRMS (ESI): *m/z* calcd for $C_{29}H_{38}Cl_3NNaO_{19}$, $[M+Na]^+$ 832.10013, found 832.10355.

***O*-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)]-(1→2)-(3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl trichloroacetimidate (4).**

A mixture of **20** (1.4 g, 1.7 mmol) and hydrazine acetate (239 mg, 2.6 mmol) was stirred in DMF (0.1 M). Upon completion (by TLC) the reaction mixture was concentrated under vacuo. The residue was diluted with ethyl acetate, washed with water, HCl (1 N), sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered, concentrated and purified by flash chromatography (hexane/EtOAc=1:1) to obtain the hemiacetal (916 mg, 69%). The obtained hemiacetal (916 mg, 1.2 mmol) was dissolved in dry DCM (0.1 M) and cooled to 0^oC followed by the addition of trichloroacetonitrile (603 µL, 6.0 mmol) and DBU (17 µL, 0.1 mmol). After completion of the reaction, concentrated in vacuum and purified by flash chromatography (hexane/EtOAc=1.5:1) to afford imidate **4** (660 mg, 72%) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 8.69 (s, 1 H; H-N), 6.18 (s, 1 H; H-1), 5.42-5.34 (m, 2 H; H-4, H'-3), 5.33-5.26 (m, 1 H; H-Troc), 5.12-5.03 (m, 2 H; H-3, H'-4), 4.89-4.78 (m, 2 H; H'-1, H-Troc), 4.64 (d, *J* = 11.8 Hz, 1 H; H-6b), 4.44 (br. s., 1 H; H-2), 4.30 (dd, *J* = 4.7, 12.2 Hz, 1 H; H'-6b), 4.15-4.09 (m, 3 H; H-5, H-6a, H-EA), 4.03 (dd, *J* = 1.9, 12.3 Hz, 1 H; H'-6a), 3.70 (dd, *J* = 2.7, 9.8 Hz, 1 H; H'-5), 3.67-3.59 (m, 1 H; H'-2), 2.09-2.02 (s each, 3 H each, 6xCH_3CO).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 170.6, 170.6, 170.5, 170.3, 169.4, 169.3, 162.9, 160.0, 153.8, 100.1, 95.2, 95.1, 90.6, 72.8, 71.9, 71.3, 69.9, 68.5, 65.0, 62.2, 61.8, 56.1, 20.7, 20.6, 20.6.

HRMS (ESI): *m/z* calcd for $C_{29}H_{36}Cl_6N_2NaO_{18}$, $[M+Na]^+$ 932.99865, found 932.99850.

Synthesis of trisaccahride donor (5).

Phenyl{*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→2)-*O*-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxy carbonylamino)-β-D-glucopyranosyl)-(1→6)]}-3,4-*O*-(2',3'-dimethoxybutane-2',3'-diyl)-1-thio-α-D-mannopyranoside (21).

A glycosyl acceptor **18** (1.0 g, 2.6 mmol) and glycosyl donor **15** (6.5 g, 10.4 mmol) was dissolved and co-evaporated with toluene three times and dried under vacuum for 12 h. Dry DCM (26.0 mL) was added and cooled to -20 °C. After 10 min, TMSOTf (50 µL, 0.3 mmol) was added at -20°C and the reaction mixture was stirred for 1 h at -20°C, and then quenched with triethylamine. The mixture was diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by column chromatography on silica gel (hexane/EtOAc=60:40) to give compound **21** (2.8 g, 83%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.47 (d, *J* = 7.3 Hz, 2 H; H-Ar), 7.39 (t, *J* = 7.6 Hz, 2 H; H-Ar), 7.34-7.30 (m, 1 H; H-Ar), 5.78 (br. s., 1 H; H'-3), 5.66 (br. s., 1 H; H-1), 5.24 (d, *J* = 7.8 Hz, 1 H; H'-1), 5.07 (t, *J* = 9.6 Hz, 1 H; H'-4), 5.02-4.95 (m, 2 H; H''-3, H''-4), 4.79 (d, *J* = 12.2 Hz, 2 H; H-Troc), 4.72-4.62 (m, 2 H; H-Troc), 4.58-4.45 (m, 2 H; H''-1, H'-6b), 4.33-4.18 (m, 5 H; H-2, H-3, H''-6b, EA), 4.10 (dd, *J* = 2.1, 12.1 Hz, 1 H; H'-6a), 4.05-3.99 (m, 2 H; H-5, H-6b), 3.94 (d, *J* = 10.0 Hz, 1 H; H-4), 3.77 (t, *J* = 9.9 Hz, 2 H; H-6a, H''-5), 3.65 (br. S., 1 H; H''-2), 3.58 (br. s., 1 H; H''-5), 3.45 (br. S., 1 H; H'-2), 3.33 (s, 3 H; H_3C-O), 3.21 (s, 3 H; H_3C-O), 2.08-1.99 (s each, 6 x $COCH_3$), 1.34 (s, 3 H; H_3C-C), 1.29 (s, 3 H; H3C-C).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 170.7, 170.6, 170.5, 169.9, 169.7, 169.4, 154.2, 133.8, 130.1, 129.4, 127.6, 100.5, 100.3, 99.8, 97.3, 95.6, 95.4, 85.6, 76.8, 74.5, 74.2, 72.5, 72.1, 71.6, 70.6, 68.9, 68.6, 67.8, 66.4, 63.1, 62.0, 61.8, 56.1, 55.9, 48.2, 47.9, 20.7, 20.7, 20.7, 20.6, 20.6, 17.7, 17.6.

HRMS (ESI): *m/z* calcd for $C_{48}H_{62}Cl_6N_2NaO_{25}S$, $[M+Na]^+$ 1331.13912, found 1331.13607.

Phenyl{*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→2)-*O*-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxy carbonylamino)-β-D-glucopyranosyl)-(1→6)]}-3,4-di-*O*-acetyl-1-thio-α-D-mannopyranoside (23).

To a solution of **21** (2.1 g, 1.6 mmol) in 90% TFA aq. (50 mL) was stirred at r.t., for 10 min. Then the solution was concentrated in *vacuo*. and co evaporated with toluene. To the residue, Ac_2O (5.0 mL) and pyridine (5.0 mL) was added and stirred for 15 h at r.t., Then the reaction mixture was concentrated and dissolved in EtOAc, washed with water, 1 N HCl, sat. $NaHCO_3$, bine, dried (Na_2SO_4) and concentrated. The crude residue was purified by column chromatography (hexane/EtOAc 58:42) to give **23** (1.7 g, 82%) as a white powder.

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.47-7.41 (m, 2 H; H-Ar), 7.34 (t, *J* = 6.7 Hz, 2 H; H-Ar), 7.31-7.23 (m, 1 H; H-Ar), 6.44 (d, *J* = 7.0 Hz, 1 H; HN), 5.84 (d, *J* = 8.7 Hz, 1 H; HN), 5.69-5.59 (m, 2 H; H-1, H'-3), 5.41 (t, *J* = 9.7 Hz, 1 H; H-4), 5.21 (d, *J* = 7.3 Hz, 1 H; H'-1), 5.15-5.01 (m, 3 H; H''-3, H''-4, H-3), 4.96 (t, *J* = 9.4 Hz, 1 H; H'-4), 4.83 (d, *J* = 11.6 Hz, 1 H; H-Troc), 4.74-4.66 (m, 2 H; H-Troc), 4.46-4.37 (m, 2 H; H-2, H-Troc), 4.31-4.23 (m, 3 H; H''-1, H''-6b, H'-6b), 4.19-4.07 (m, 3 H; H-5, H'-6a, EA), 4.02 (d, *J* = 11.9 Hz, 2 H; H-6b, H''-6a), 3.91 (d, *J* = 8.7 Hz, 1 H; H''-2), 3.75-3.69 (m, 1 H; H'-5), 3.65 (d, *J* = 9.3 Hz, 1 H; H''-5), 3.25 (d, *J* = 11.0 Hz, 1 H; H-6a), 3.21-3.14 (m, 1 H; H'-2), 2.09-2.01 (s each, 3 H each, 8xCH_3CO).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 170.7, 170.5, 170.5, 170.2, 169.9, 169.6, 169.3, 155.3, 154.5, 133.4, 130.6, 129.2, 127.6, 102.0, 97.1, 95.4, 95.2, 84.0, 75.0, 74.8, 74.3, 72.6, 72.0, 71.8, 70.4, 70.3, 70.0, 69.4, 68.4, 67.4, 65.9, 62.1, 61.9, 60.4, 56.3, 56.1, 21.0, 20.9, 20.8, 20.7, 20.6, 20.6, 20.6, 14.2.

HRMS (ESI): *m/z* calcd for $C_{46}H_{56}Cl_6N_2NaO_{25}S$, $[M+Na]^+$ 1301.09217, found 1301.08854.

***O*-{*O*-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→2)-*O*-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→6)]-3,4-di-*O*-acetyl-α-D-mannopyranosyl}-trichloroacetimidate (5).**

A mixture of **34** (1.2 g, 0.9 mmol) and NBS (670 mg, 3.7 mmol) was stirred in 4:1 CH_3CN-H_2O (0.05 M) for 30 min at r.t., upon completion (by TLC) the reaction mixture was concentrated under *vacuo*. The residue was diluted with ethyl acetate, washed with water, HCl (1 N), sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered, concentrated and purified by flash chromatography (hexane/EtOAc=60:40) to obtain the hemiacetal (800 mg, 72%). The obtained hemiacetal (800 mg, 0.7 mmol) was dissolved in dry DCM (0.02 M) and cooled to 0^oC followed by the addition of trichloroacetonitrile (679 µL, 6.7 mmol) and DBU (38 µL, 0.3 mmol). After completion of the reaction, the reaction mixture was concentrated in vacuum and purified by flash chromatography (hexane/EtOAc=1:1) to afford imidate **5** (745 mg, 83%) as white powder.

^{1}H NMR: Table 3-2-1.

^{13}C NMR: Table 3-2-2.

HRMS (ESI): *m/z* calcd for $C_{42}H_{52}Cl_9N_3NaO_{26}$, $[M+Na]^+$ 1351.99335, found 1351.99549.

II-5 Synthesis of trisaccharide donor (6).

Phenyl 3,6-di-*O*-allyl-1-thio-α-D-mannopyranoside (18).

A mixture of thiophenyl mannoside **17** (2.0 g, 4.5 mmol) and dibutyltin oxide (3.4 g, 13.6 mmol) was stirred under reflux in dry methanol (50 mL) for 5 h. The solvent was evaporated and dried under vacuum for 16 h. Dry dimethylformamide (23 mL) and cesium fluoride (2.1

g, 13.6 mmol) were added to the remainder and stirred at 0°C for 10 min and allyl bromide (3.8 mL, 45.4 mmol) was added at 0°C and stirred vigorously at r.t., for 4 days. The reaction was quenched with ethyl acetate (48 mL) and water (1 mL), filtered through celite, concentrated in vacuum and purified by flash chromatography (hexane/EtOAc=70:30) to afford **18** (961 mg, 60%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.45-7.55 (m, 2 H; H-Ar), 7.21-7.35 (m, 3 H; H-Ar), 5.82-6.03 (m, 2 H; 2 X HC=C-), 5.56 (s, 1 H; H-1), 5.35 (dd, *J* = 17.3, 1.4 Hz, 1 H; H_2C=C-), 5.21-5.30 (m, 2 H; H_2C=C-), 5.17 (dd, *J* = 10.3, 1.2, 1 H; H_2C=C-), 4.20-4.29 (m, 3 H; H-2, H-5, H_2C-O), 4.13-4.19 (m, 1 H; H_2C-O), 3.94-4.08 (m, 3 H; H-4, 2 X H_2C-O), 3.68-3.77 (m, 2 H; H-6ab), 3.62 (dd, *J* = 9.2, 3.3 Hz, 1 H; H-3), 2.96 (br. s., 1 H; OH), 2.91 (br. s., 1 H; OH)

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 134.4, 134.2, 133.9, 131.5, 129.0, 127.4, 118.2, 117.2, 87.6, 79.2, 72.5, 71.6, 70.8, 70.0, 69.4, 67.8

HRMS (ESI): *m/z* calcd for $C_{18}H_{24}NaO_5S$, $[M+Na]^+$ 375.1242, found 375.1263.

Phenyl{*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→2)-*O*-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxy carbonylamino)-β-D-glucopyranosyl)-(1→4)]}-3,6-di-*O*-allyl-1-thio-α-D-mannopyranoside (22).

A glycosyl acceptor **19** (950 mg, 2.7 mmol) and glycosyl donor **15** (5.9 g, 9.4 mmol) was dissolved and co-evaporated with toluene three times and dried under vacuum for 12 h. Dry DCM (26.0 mL) was added and cooled to -15°C. After 10 min, TMSOTf (52 µL, 0.3 mmol) was added at -15°C and the reaction mixture was stirred for 1 h at -15°C, and then quenched with triethylamine. The mixture was diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by gravity

column chromatography on silica gel (Toluene/EtOAc=70:30) to give compound **22** (2.5 g, 73%).

^{1}H NMR: Table 3-2-1.

^{13}C NMR: Table 3-2-2.

HRMS (ESI): *m/z* calcd for $C_{48}H_{60}Cl_6N_2NaO_{23}S$, $[M+Na]^+$ 1297.13309, found 1297.13421.

{*O*-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→2)-*O*-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→4)]}-1,3,6-tri-*O*-acetyl-D-mannopyranose (24).

To a solution of **22** (2.0 g, 1.6 mmol) in 95% AcOH aq. (157 mL) was added NaOAc (1.28 g, 15.7 mmol) and palladium chloride (1.39 g, 7.8 mmol) and stirred at 80^{o}C for 5 h. Subsequently, the precipitate was filtered through celite and the solvents were concentrated in *vacuo*. The remainder was dissolved in CH_2Cl_2 and extracted with water and sat. $NaHCO_3$, brine, dried (Na_2SO_4) and concentrated. To the residue, Ac_2O (5.0 mL) and pyridine (5.0 mL) was added and stirred for 15 h at r.t. Then the reaction mixture was concentrated and dissolved in EtOAc, washed with water, 1 N HCl, sat. $NaHCO_3$, bine, dried (Na_2SO_4) and concentrated. The crude residue was purified by column chromatography (hexane/EtOAc 60:40) to afford **24** (1.5 g, 78%, α/β=2/1) as a white powder.

^{1}H NMR: Table 3-2-1.

^{13}C NMR: Table 3-2-2.

HRMS (ESI): *m/z* calcd for $C_{42}H_{54}Cl_6N_2NaO_{27}$, $[M+Na]^+$ 1251.0937, found 1251.0943.

***O*-{*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→2)-O-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→4)]-3,4-di-*O*-acetyl-α-D-mannopyranosyl}-trichloroacetimidate (6).**

Hydrazine monohydrate (73 µL, 1.5 mmol) and AcOH (86 µL, 1.5 mmol) was added to a solution of trisaccharide **24** (1.47 g, 1.2 mmol) in dimethylformamide (5 mL). After complete reaction (by TLC), solvents were concentrated under *vacuo*. The residue was diluted with ethyl acetate, washed with water, HCl (1 N), sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered, concentrated and dried. The obtained hemiacetal was dissolved in dry DCM (60 mL) and trichloroacetonitrile (1.2 mL, 11.9 mmol) and DBU (84 µL, 0.6 mmol) were added. After completion of the reaction, the mixture was concentrated in vacuum and purified by flash chromatography (hexane/EtOAc=55:45) to afford imidate **6** (1.05 g, 66%).

^{1}H NMR: Table 3-2-1.

^{13}C NMR: Table 3-2-2.

HRMS (ESI): *m/z* calcd for $C_{42}H_{52}Cl_9N_3NaO_{26}$, $[M+Na]^+$ 1351.99335, found 1351.99645.

Synthesis of biantennary *N*-glycan octasaccharide (30).

Phenyl[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-4,6-*O*-benzylidine-β-D-mannopyranosyl]-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (15).

A glycosyl donor **4** (150 mg, 0.15 mmol) and glycosyl acceptor **3** (95 mg, 0.10 mmol) was dissolved and co-evaporated with toluene three times and dried under vacuum for 12 h. Dry DCM (1.2 mL) was added and cooled to -15°C. After 10 min, TMSOTf (2 µL, 0.01 mmol)

was added at -15°C and the reaction mixture was stirred for 1 h at -15°C, and then quenched with triethylamine. The mixture was diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by flash column chromatography on silica gel (Toluene/EtOAc=80:20) to give compound **25** (130 mg, 72%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.61-7.57 (m, 2 H), 7.55-7.50 (m, 6 H), 7.40 (d, J = 7.5 Hz, 2 H), 7.42 (d, J = 6.9 Hz, 3 H), 7.37-7.31 (m, 13 H), 7.30-7.27 (m, 4 H), 5.42 (s, 1 H), 5.11 (d, J = 9.9 Hz, 1 H), 5.03-4.98 (m, 2 H), 4.86-4.80 (m, 3 H), 4.72 (d, J = 12.0 Hz, 2 H), 4.67-4.61 (m, 4 H), 4.53 (d, J = 12.0 Hz, 1 H), 4.38 (d, J = 10.2 Hz, 1 H), 4.06-3.98 (m, 5 H), 3.86-3.78 (m, 2 H), 3.76 (d, J = 3.0 Hz, 1 H), 3.73-3.60 (m, 5 H), 3.48 (t, J = 9.1 Hz, 1 H), 3.43-3.33 (m, 3 H), 3.08-3.00 (m, 1 H), 2.75 (d, J = 8.1 Hz, 1 H), 2.07-2.00 (s each, 3 H each, 6xCH_3CO).

^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 170.1, 169.6, 169.5, 169.3, 169.3, 168.3, 168.2, 152.5, 137.1, 136.9, 136.7, 133.2, 129.7, 129.4, 128.1, 128.0, 127.8, 127.6, 127.4, 127.2, 127.1, 126.9, 126.7, 125.7, 101.3, 99.9, 97.7, 94.4, 84.7, 82.4, 78.5, 77.7, 76.6, 75.3, 75.0, 74.4, 74.4, 74.3, 73.6, 72.7, 72.7, 72.6, 70.8, 70.1, 68.8, 67.6, 67.4, 67.3, 65.8, 65.0, 63.1, 61.6, 60.5, 59.4, 54.3, 30.6, 21.6, 20.0, 19.7, 19.7, 19.6, 13.2, 13.1.

HRMS (ESI): *m/z* calcd for $C_{73}H_{81}Cl_3N_4NaO_{26}S$, $[M+Na]^+$ 1589.38230, found 1589.38113.

Phenyl[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-β-D-mannopyranosyl]-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (26).

Compound **25** (130 mg) in acetic acid: water: ethanol (4:1:1) (4.0 mL) was stirred at 80^{o}C. After reaction completion by TLC, the reaction mixture was evaporated. The crude residue was purified by flash column chromatography on silica gel (hexane/EtOAc=40:60) to give diol **26** (98 mg, 80%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ 7.62-7.55 (m, 2 H), 7.44-7.36 (m, 6 H), 7.36-7.27 (m, 10 H), 7.25-7.21 (m, 2 H), 5.33 (br. s., 1 H), 5.16-5.11 (m, 1 H), 5.10-4.99 (m, 3 H), 4.92 (d, *J* = 9.7 Hz, 2 H), 4.71-4.62 (m, 4 H), 4.59-4.52 (m, 3 H), 4.37 (d, *J* = 9.7 Hz, 1 H), 4.22-4.16 (m, 2 H), 4.14-4.10 (m, 1 H), 4.08-4.01 (m, 3 H), 4.00-3.94 (m, 2 H), 3.89 (t, *J* = 9.3 Hz, 1 H), 3.81-3.76 (m, 2 H), 3.74-3.69 (m, 1 H), 3.64 (d, *J* = 11.2 Hz, 1 H), 3.56 (d, *J* = 7.4 Hz, 1 H), 3.50 (dd, *J* = 2.8, 9.9 Hz, 1 H), 3.47-3.40 (m, 2 H), 3.39-3.34 (m, 1 H), 3.31 (d, *J* = 9.4 Hz, 2 H), 3.04 (ddd, *J* = 4.1, 5.0, 12.7 Hz, 1 H), 2.09-2.01 (s each, 3 H each, 6xCH_3CO).

^{13}C-NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ 170.7, 170.5, 170.4, 170.2, 169.4, 153.8, 138.6, 138.1, 137.7, 133.8, 131.8, 130.8, 129.1, 128.7, 128.6, 128.6, 128.3, 128.1, 128.0, 127.8, 127.7, 127.3, 126.7, 100.8, 100.3, 97.1, 95.4, 85.9, 83.3, 81.0, 79.3, 78.9, 76.6, 75.7, 75.2, 74.8, 74.5, 74.2, 73.7, 71.4, 69.9, 69.2, 68.5, 68.3, 66.3, 66.2, 64.5, 63.2, 62.4, 61.9, 55.6, 20.8, 20.7, 20.7, 20.6, 20.6.

HRMS (ESI): *m/z* calcd for $C_{66}H_{77}Cl_3N_4NaO_{26}S$, $[M+Na]^+$ 1501.35100, found 1501.35236.

Phenyl{(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-mannopyranosyl]}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (27).

A glycosyl donor **4** (69 mg, 0.08 mmol) and glycosyl acceptor **26** (80 mg, 0.05 mmol) was co-evaporated with toluene three times and dried under vacuum for 12 h. Dry DCM (1.0 mL) was added and cooled to -15°C. After 10 min, TMSOTf (1 µL, 0.01 mmol) was added at -15°C and the reaction mixture was stirred for 1 h at -15°C, and then quenched with triethylamine. The mixture was diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by flash column chromatography on silica gel (hexane/EtOAc=50:50) to give hexasaccharide **27** (84 mg, 70%).

^{1}H NMR: Table 3-2-3.

^{13}C NMR: Table 3-2-4.

HRMS (ESI): *m/z* calcd for $C_{93}H_{111}Cl_6N_5Na_2O_{43}S$, $[M+2Na]^+$ 2273.43041, found 2273.43443.

Phenyl-{(2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-[(2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→6)] -2-*O*-benzyl -β-D-mannopyranosyl}-(1→4)-2-acetamido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (28).

To a solution of compound **27** (70.0 mg, 0.03 mmol) in 1:2 AcOH: EtOAc (7.0 mL) was added zinc (614 mg, 9.39 mmol) and the solution was stirred at r.t., for 2 h. The reaction mixture was filtered through celite and washed with EtOAc. The filtrate was concentrated to 2.0 mL level, then added Ac_2O (700 µL), pyridine (700 µL) and the resulting mixture were stirred at r.t., for 16 h. The reaction mixture was diluted with EtOAc, washed with water, 1 N HCl, sat. $NaHCO_3$ (aq), and brine, dried over Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash chromatography on silica gel

(hexane/EtOAc=20:80). The resulting compound (48.0 mg) was dissolved in MeOH (1.5 mL) was added NaOMe (1.3 mg, 23.7 μmol) and the solution was stirred at room temperature for 15 h. The reaction mixture was neutralized using Dowex 50W-X8[H^+] resin, filtered and concentrated. The residue was dissolved in water and lyophilized, later purified by RP HPLC to give compound **28** (33 mg, 72%).

^{1}H NMR: Table 3-2-3.

^{13}C NMR: Table 3-2-4.

HRMS (ESI): *m/z* calcd for $C_{69}H_{93}N_3NaO_{30}S$, [*M*+2Na]$^+$ 1498.54623, found 1498.53901.

Phenyl-{(β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3))-[(β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)]-2-*O*-benzyl-β-D-mannopyranosyl}-(1→4)-2-acetamido-2-deoxy-3,6-di-*O*-benzyl-β-D-glucopyranoside (29).

To a solution of compound **28** (22 mg, 14.9 μmol) in 745 μL of water (20 mM; theoretical concentration) was added to the mixture of 1 M Tris buffer pH-7.5 (147 μL), 1M $MnCl_2$ (29.4 μL), 200 mM UDP-galactose (740 μL), 4 units/mL β1-4-Galacosyl transferase (GalT) (210 μL) and water (1084 μL). After incubation at 25 ^oC for 24 h, purified by RP-HPLC (column: Inertile ODS-3 (20x250 mm) eluted with H_2O and CH_3CN to afford Octasaccharide **29** (25.2 mg, 94%).

^{1}H NMR: Table 3-2-3.

^{13}C NMR: Table 3-2-4.

HRMS (ESI): *m/z* calcd for $C_{81}H_{113}N_3Na_2O_{40}S$, [*M*+2Na]$^+$ 1845.64164, found 1845.63841.

β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-{β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)}-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranoside (30).

To a solution of compound **29** (23 mg, 12.7 μmol) in 50% AcOH (aq.) (5.0 mL) was added 20% $Pd(OH)_2/C$ (20 mg) and stirred for 24 h under H_2 atmosphere. The reaction mixture was filtered through a celite pad. The filtrate was concentrated and then dissolved in water and lyophilized. The crude product was purified on a Sephadex G-15 column by elution with H_2O. Fractions containing the product were pooled and lyophilized to give the free octasaccharide **30** (15.4 mg, 94%) as white solid.

^{1}H NMR: Table 3-2-3.

^{13}C NMR: Table 3-2-4.

HRMS (ESI): *m/z* calcd for $C_{54}H_{91}N_3Na_2O_{41}$, $[M+2Na]^+$ 1483.49234, found 1483.49731.

Synthesis of Triantennary decasaccharide (7).

Phenyl {(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→6)]-3,4-di-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-mannopyranosyl}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (31).

A solution of glycosyl acceptor **26** (42 mg, 28.4 μmol) and glycosyl donor **5** (76 mg, 56.7 μmol) was co-evaporated with toluene three times, added molecular sieves 4 Å (42 mg) and

dried under vacuum for 12 h. Dry DCM (5.7 mL) was added and cooled to -35°C. After 30 min, BF_3Et_2O (1.4 µL, 11.4 µmol) was added at -35°C and the reaction mixture was stirred for 2 h at -35°C, and then the mixture was filtered, diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by column chromatography on silica gel (toluene/EtOAc=65:35) to give compound **7** (59 mg, 78%).

^{1}H NMR: Table 3-2-5.

^{13}C NMR: Table 3-2-6.

HRMS (Maldi): *m/z* calcd for $C_{106}H_{127}Cl_9N_6NaO_{51}S$, $[M+Na]^+$ 2674.432, found 2674.430.

Phenyl {(2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl)-(1→3)-[(2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-mannopyranosyl}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (32).

To a solution of compound **31** (145 mg, 0.05 mmol) in EtOAc (5.0 mL) was added zinc (1.5 g, 23.6 mmol), acetic acid (810 µL, 14.2 mmol) and the solution was stirred at r.t., for 1 h. The reaction mixture was filtered through celite and washed with EtOAc. To the filtrate was added Ac_2O (890 µL) and the resulting mixture were stirred at r.t., for 15 h. The reaction mixture was concentrated and co- evaporated with toluene. The residue (100 mg) was dissolved in MeOH (1.5 mL). To the solution was added NaOMe (2.6 mg, 0.05 mmol) and the mixture was stirred at room temperature for 16 h. The reaction mixture was neutralized using 20% acetic acid and concentrated. The residue was dissolved in water and lyophilized, and purified by RP HPLC to give compound **32** (70 mg, 79%).

^{1}H NMR (500 MHz, D_2O, 25°C, TMS): δ 7.42 - 7.22 (m, 18 H), 7.03 (d, *J* = 7.0 Hz, 2 H), 5.09 (s, 1 H), 4.81 (s, 2 H), 4.56 - 4.48 (m, 2 H), 4.48 - 4.39 (m, 3 H), 4.36 (d, *J* = 13.2 Hz, 1 H), 4.12 - 4.03 (m, 2 H), 4.03 - 3.88 (m, 4 H), 3.86 - 3.78 (m, 4 H), 3.73 - 3.56 (m, 16 H), 3.51 - 3.41 (m, 8 H), 3.40 - 3.30 (m, 9 H), 2.01 - 1.93 (m, 9 H), 1.58 (s, 3 H).

HRMS (Maldi): *m/z* calcd for $C_{85}H_{119}N_5NaO_{40}S$, $[M+Na]^+$ 1904.704, found 1904.707.

Phenyl {(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl)-(1→3)-[(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-manno pyranosyl}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (33).

To a solution of compound **32** (22 mg, 11.7 μmol) in 584 μL of water (20 mM; theoretical concentration) was added to the mixture of 1 M Tris buffer (pH 7.5) (234 μL), 1 M $MnCl_2$ (23 μL), 200 mM UDP-galactose (1168 μL), 4 units/mL human recombinant β1,4-galactosyltransferase (GalT) (234 μL) and water (93 μL). After incubation at 25^{o}C for 24 h, the crude product was purified by RP-HPLC (column: Inertile ODS-3 (20x250 mm) eluted with H_2O and CH_3CN to afford decasaccharide **33** (27.5 mg, 96%).

^{1}H NMR (500 MHz, D_2O, 25°C, TMS): δ 7.42 - 7.22 (m, 18 H), 7.03 (d, *J* = 7.0 Hz, 2 H), 5.10 (s, 1 H), 4.82 (s, 1 H), 4.72 (s, 1 H), 4.54 (s, 1 H), 4.51 - 4.34 (m, 8 H), 4.12 - 4.00 (m, 3 H), 3.99 - 3.87 (m, 5 H), 3.87 - 3.81 (m, 5 H), 3.76 (d, *J* = 5.3 Hz, 1 H), 3.73 - 3.55 (m, 36 H), 3.52 - 3.39 (m, 12 H), 3.34 - 3.22 (m, 2 H), 2.61 (d, *J* = 9.6 Hz, 1 H), 2.01 - 1.92 (s each, 9 H), 1.58 (s, 3 H).

HRMS (Maldi): *m/z* calcd for $C_{109}H_{159}N_5NaO_{61}S$, $[M+Na]^+$ 2552.916, found 2552.915.

β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl)-(1→3)-[(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-

glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-manno pyranosyl}-(1→4)-2-azido-2-deoxy-D-glucopyranoside (7).

To a solution of compound **33** (9 mg, 3.6 µmol) in 50% AcOH (aq.) (4.0 mL) was added 20% $Pd(OH)_2/C$ (5 mg) and stirred for 4 h under H_2 atmosphere. The reaction mixture was filtered through a celite pad and replace the fresh pd. Continued the process until the reaction was completed by maldi. Finally the filtrate was concentrated and then dissolved in water and lyophilized. The crude product was purified on a Sephadex G-15 column by elution with H_2O. Fractions containing the product were pooled and lyophilized to give the free decasaccharide **7** (7.3 mg, 95%) as white solid.

^{1}H NMR (500 MHz, D_2O, 25°C, TMS): δ 5.03 (s, 1 H), 4.60 (d, *J* = 8.1 Hz, 4 H), 4.53 - 4.43 (m, 4 H), 4.40 - 4.32 (m, 3 H), 4.15 - 4.06 (m, 2 H), 3.99 (m, 1 H), 3.95 (d, *J* = 9.3 Hz, 1 H), 3.93 - 3.85 (m, 3 H), 3.83 - 3.77 (m, 5 H), 3.73 (s, 3 H), 3.75 (s, 3 H), 3.70 - 3.60 (m, 27 H), 3.60 - 3.41 (m, 16 H), 2.01 - 1.91 (m, 12 H).

HRMS (Maldi): *m/z* calcd for $C_{109}H_{159}N_5NaO_{61}S$, $[M+Na]^+$ 2552.916, found 2552.915

Synthesis of tetraantennary dodecasaccharide (8).

Phenyl [(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-4,6-*O*-benzylidine-β-D-mannopyranosyl]-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (34).

A solution of glycosyl donor **6** (342 mg, 0.26 mmol) and glycosyl acceptor **3** (150 mg, 0.18 mmol) was co-evaporated with toluene three times, added molecular sieves 4 Å (350 mg) and

dried under vacuum for 12 h. Dry DCM (37 mL) was added and cooled to -25°C. After 30 min, BF_3OEt_2 (5 µL, 0.04 mmol) was added at -25°C and the reaction mixture was stirred for 1.5 h at -25°C, and then quenched with triethylamine. The mixture was diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by flash column chromatography on silica gel (hexane/EtOAc=55:45) to give pentasaccharide **34** (252 mg, 69%).

^{1}H NMR: Table 3-2-5.

^{13}C NMR: Table 3-2-6.

HRMS (Maldi): *m/z* calcd for $C_{86}H_{97}Cl_6N_5NaO_{34}S$, $[M+Na]^+$ 2010.3735, found 2010.3740.

Phenyl [(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-β-D-mannopyranosyl]-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (35).

Compound **34** (210 mg) in acetic acid: water (4:1) (6.0 mL) was stirred at 80°C for 2 h. Then, the reaction mixture was evaporated. The crude residue was purified by gravity column chromatography on silica gel (hexane/EtOAc=46:54) to give diol **35** (152 mg, 76%).

^{1}H NMR: Table 3-2-5.

^{13}C NMR: Table 3-2-6.

HRMS (Maldi): *m/z* calcd for $C_{79}H_{93}Cl_6N_5NaO_{34}S$, $[M+Na]^+$ 1922.3422, found 1922.343.

Phenyl {(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-

trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→4)-(3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→6)]-3,4-di-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-mannopyranosyl}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (36).

A solution of glycosyl donor **5** (158 mg, 0.12 mmol) and glycosyl acceptor **35** (150 mg, 0.08 mmol) was co-evaporated with toluene three times, added molecular sieves 4 Å (200 mg) and dried under vacuum for 10 h. Dry DCM (16 mL) was added and cooled to -35°C. After 30 min, BF_3OEt_2 (4 µL, 0.03 mmol) was added at -35^{o}C and the reaction mixture was stirred for 1 h at -35°C, then quenched with triethylamine, and filtered through celite. The residual mixture was diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by gravity column chromatography on silica gel (toluene/EtOAc=60:40) to give compound **36** (179 mg, 74%).

^{1}H NMR: Table 3-2-5.

^{13}C NMR: Table 3-2-6.

HRMS (Maldi): *m/z* calcd for $C_{119}H_{143}Cl_{12}N_7NaO_{59}S$, $[M+Na]^+$ 3094.420, found 3094.426.

Phenyl {(2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→4)-α-D-mannopyranosyl)-(1→3)-[(2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-mannopyranosyl}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (37).

To a solution of compound **36** (145 mg, 0.05 mmol) in EtOAc (5.0 mL) was added zinc (1.5 g, 23.6 mmol), acetic acid (810 µL, 14.2 mmol) and the solution was stirred at r.t., for 1 h. The reaction mixture was filtered through celite and washed with EtOAc. To the filtrate was added Ac_2O (890 µL) and the resulting mixture were stirred at r.t., for 15 h. The reaction mixture was concentrated and co- evaporated with toluene. The residue (100 mg) was dissolved in MeOH (1.5 mL). To the solution was added NaOMe (2.6 mg, 0.05 mmol) and the mixture was stirred at room temperature for 16 h. The reaction mixture was neutralized using 20% acetic acid and concentrated. The residue was dissolved in water and lyophilized, and purified by RP HPLC to give compound **37** (70 mg, 79%).

^{1}H NMR (500 MHz, D_2O, 25°C, TMS): δ 7.36 (d, *J* = 6.8 Hz, 3 H), 7.34 - 7.25 (m, 12 H), 7.24 (m, 3 H), 7.08 (d, *J* = 6.8 Hz, 2 H), 5.07 (s, 1 H), 4.74 (m, 1 H), 4.71 (m, 2 H), 4.63 (m, 2 H), 4.51 (s, 1 H), 4.45 - 4.37 (m, 6 H), 4.07 (m, 1 H), 4.04 - 3.97 (m, 2 H), 3.91 - 3.85 (m, 2 H), 3.82 - 3.78 (m, 4 H), 3.72 - 3.59 (m, 12 H), 3.59 - 3.51 (m, 8 H), 3.47 - 3.38 (m, 11 H), 3.36 - 3.25 (m, 9 H), 2.57 (d, *J* = 8.7 Hz, 1 H), 1.96 (s, 3 H), 1.93 (s, 3 H), 1.92 (s, 3 H), 1.92 (s, 3 H)

HRMS (Maldi): *m/z* calcd for $C_{85}H_{119}N_5NaO_{40}S$, $[M+Na]^+$ 1904.704, found 1904.707.

Phenyl {(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→4)-α-D-mannopyranosyl)-(1→3)-[(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-manno pyranosyl}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (38).

To a solution of compound **37** (22 mg, 11.7 µmol) in 584 µL of water (20 mM; theoretical concentration) was added to the mixture of 1 M Tris buffer (pH 7.5) (234 µL), 1 M $MnCl_2$

(23 µL), 200 mM UDP-galactose (1168 µL), 4 units/mL human recombinant β1,4-galactosyltransferase (GalT) (234 µL) and water (93 µL). After incubation at 25^{o}C for 24 h, the crude product was purified by RP-HPLC (column: Inertile ODS-3 (20x250 mm) eluted with H_2O and CH_3CN to afford dodecasaccharide **38** (27.5 mg, 93%).

^{1}H NMR (500 MHz, D_2O, 25°C, TMS): δ 7.36 (d, *J* = 4.6 Hz, 3 H), 7.32 - 7.27 (m, 11 H), 7.24 (m, 4 H), 7.07 (d, *J* = 6.0 Hz, 2 H), 5.07 (s, 1 H), 4.74 - 4.70 (m, 3 H), 4.51 (s, 1 H), 4.47 - 4.39 (m, 7 H), 4.38 - 4.32 (m, 5 H), 4.07 (m, 1 H), 4.05 - 4.00 (m, 2 H), 3.93 - 3.85 (m, 6 H), 3.83 - 3.80 (m, 6 H), 3.77 - 3.70 (m, 7 H), 3.69 - 3.60 (m, 33 H), 3.58 - 3.52 (m, 17 H), 3.49 - 3.43 (m, 11 H), 3.42 m, 4 H), 3.31 - 3.23 (m, 2 H), 2.66 (d, *J* = 9.2 Hz, 1 H), 1.96 (s, 3 H), 1.93 (s, 6 H), 1.91 (s., 3 H)

HRMS (Maldi): *m/z* calcd for $C_{109}H_{159}N_5NaO_{61}S$, $[M+Na]^+$ 2552.916, found 2552.915.

Phenyl {(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→4)-α-D-mannopyranosyl)-(1→3)-[(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-manno pyranosyl}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (8).

To a solution of compound **37** (9 mg, 3.6 µmol) in 50% AcOH (aq.) (4.0 mL) was added 20% $Pd(OH)_2/C$ (5 mg) and stirred for 4 h under H_2 atmosphere. The reaction mixture was filtered through a celite pad and replace the fresh pd. Continued the process until the reaction was completed by maldi. Finally the filtrate was concentrated and then dissolved in water and lyophilized. The crude product was purified on a Sephadex G-15 column by elution with H_2O. Fractions containing the product were pooled and lyophilized to give the free dodecasaccharide **38** (7.3 mg, 95%) as white solid.

^{1}H NMR (500 MHz, D_2O, 25°C, TMS): δ 7.36 (d, *J* = 4.6 Hz, 3 H), 7.32 - 7.27 (m, 11 H), 7.24 (m, 4 H), 7.07 (d, *J* = 6.0 Hz, 2 H), 5.07 (s, 1 H), 4.74 - 4.70 (m, 3 H), 4.51 (s, 1 H), 4.47 - 4.39 (m, 7 H), 4.38 - 4.32 (m, 5 H), 4.07 (m, 1 H), 4.05 - 4.00 (m, 2 H), 3.93 - 3.85 (m, 6 H), 3.83 - 3.80 (m, 6 H), 3.77 - 3.70 (m, 7 H), 3.69 - 3.60 (m, 33 H), 3.58 - 3.52 (m, 17 H), 3.49 - 3.43 (m, 11 H), 3.42 m, 4 H), 3.31 - 3.23 (m, 2 H), 2.66 (d, *J* = 9.2 Hz, 1 H), 1.96 (s, 3 H), 1.93 (s, 6 H), 1.91 (s., 3 H)

HRMS (Maldi): *m/z* calcd for $C_{109}H_{159}N_5NaO_{61}S$, $[M+Na]^+$ 2552.916, found 2552.915.

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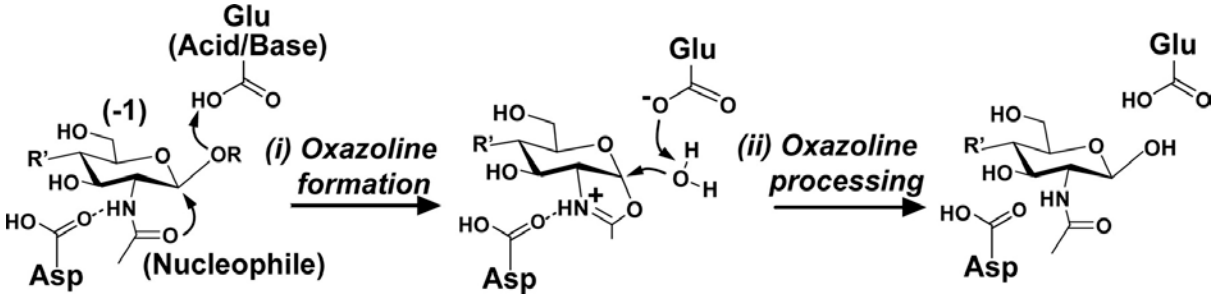
Chapter 4

Endoglycosidases-catalyzed transglycosylation for the synthesis of glycopeptides.

4.1 Introduction.

Endo-β-*N*-acetylglucosaminidase (ENGase) (EC 3.2.1.96) is a glycoside hydrolase that acts on the β1,4-glycosidic linkage between the *N,N*'-diacetylchitobiose core of *N*-glycans. This type of enzymes is classified into two glycoside hydrolase (GH) families, GH18 and GH85, in the CAZY data base. GH18 includes bacterial endo-β-*N* acetylglucosaminidases, such as Endo-H[1], Endo-F1[2], Endo-F2, and Endo-F3[3] as well as many chitinases. On the other hand, GH85 is exclusively composed of endo-β-*N*-acetylglucosaminidases from both prokaryotes and eukaryotes, such as Endo-M from *Mucor hiemalis*[4–7], Endo-A from *Arthrobacter protophormiae*[8], Endo-D from *Streptococcus pneumoniae*[9], Endo-CE from *Caenorhabditis elegans*[10,11], and ENGase from human[12]. Notably, unlike GH18 enzymes, most of the GH85 enzymes possess transglycosylation activity, *i.e.* the ability to transfer oligosaccharide *en bloc* from a donor substrate to a GlcNAc-containing backbone as an acceptor to form new glycoconjugates[13–18]. This enzymatic method is one of the most promising approaches to synthesize homogeneous glycoproteins[19]. Structural and mechanistic studies with some GH18 ENGases and GH18 chitinases[20] and GH20 β-*N*-acetylhexosaminidases[21] have indicated that the catalysis of these GH18 and GH20 enzymes proceeds in a substrate-assisted mechanism involving the participation of the 2-acetamide group in the substrate. In this mechanism (Fig 4-1-1), a catalytic residue (Asp or Glu) acts as a general acid to protonate the glycosidic oxygen. Upon activation of the glycosidic bond, the 2-acetamide group of the (-1)GlcNAc acts as a nucleophile to substitute the leaving group at the anomeric center, resulting in the formation of an oxazolinium ion intermediate. The catalytic carboxylate residue then acts as a general base to activate a water molecule at the catalytic center to facilitate the hydrolysis of the oxazolinium ion intermediate to form the hydrolytic product. The double *sn*-2 type

displacement results in a retaining of the anomeric configuration. In addition, several structural analyses on GH18 chitinases and GH20 β-*N*-acetylhexosaminidases revealed that the proper orientation of the acetamide group may be aided by another key carboxylate residue (usually Asp) located at 1 or 2 amino acid residues upstream from the general acid/base catalytic residue (Fig 4-1-1). The GH85 ENGases, most of which show transglycosylation activity.



Scheme 4-1-1: substrate-assisted mechanism of ENGases.

The novel glycosynthase, EndoM-N175A, that previously created has demonstrated a great potential for the synthesis of homogeneous glycoproteins carrying natural complex-type and high mannose-type *N*-glycans. Despite the lack of the product hydrolysis, however, the specific activity of the glycosynthase for transglycosylation was much lower than that of the wild type Endo-M. The enzymatic reactions catalyzed by the EndoM-N175A mutant are usually slow, requiring relatively large amounts of the mutant enzyme and/or extended incubation time. To improve the catalytic efficiency of the glycosynthase for practical use, a systematic mutagenesis at the critical Asn-175 site of Endo-M, as well as site-directed mutations at other conserved Glu and Asp residues was performed to probe whether those carboxylate residues are critical in the catalysis. The mutagenesis and subsequent enzymatic evaluation have led to the identification of an array of glycosynthase mutants that showed enhanced catalytic activity. In particular, the N175Q mutant was found to possess significantly enhanced transglycosylation activity for activated sugar oxazoline, whereas its

hydrolysis activity for the product was diminished. Surprisingly, this mutant was also capable of efficiently transglycosylating *N*-glycan while having a significantly diminished product hydrolysis activity behaving as a typical "transglycosidase."

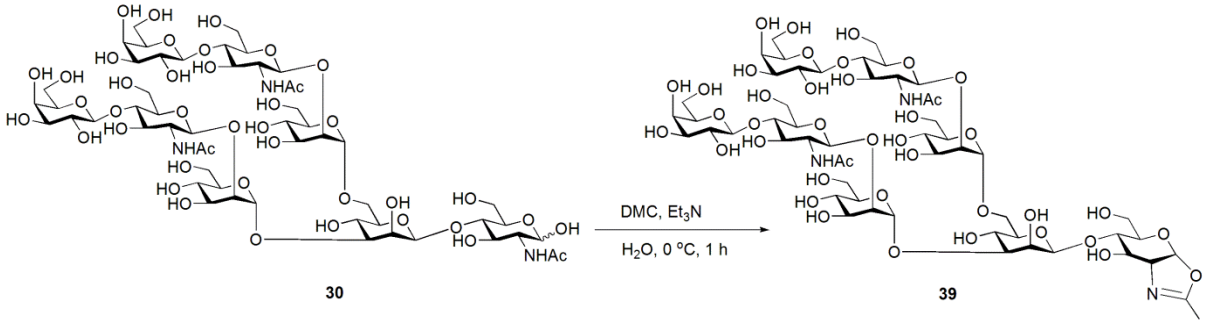
In contrast to common glycosyltransferases and exoglycosidases that transfer only monosaccharides, mutated Endo-M can transfer a large intact oligosaccharide to a GlcNAc-peptide acceptor in a single step to form a new glycopeptide, thus allowing a highly convergent glycopeptides synthesis without the need of protecting groups. A number of large *N*-glycopeptides were synthesized by the chemoenzymatic method for structural and functional studies.[22-25]

Best of our knowledge, Prof. Kajihara group and Prof. Lai-Xi Wang group are majorly working in this area. Up to now they concentrated on bi-antennay glycans containing glycopeptides and high mannose glycans conataining glycopeptides synthesis. They didn't focus on hyper branched complex type glycopeptides synthesis.

In this chapter, we focused on endoglycosidase (Endo-M-75Q) catalyzed transglycosilation with our synthesized N-glycans of bi-, tri- and tetra- antennary structures. Firstly, we tried to synthesize oxazoline of each glycans and then transglycosilation reaction to obtain Immunoglobuline-G (IgG-1) glycopeptide with bi- antennary glycans and Erythropoitein (EPO) related glycopeptides with tri- and tetra- antennary glycans.

4.2 Results and discussion.

4-2-1: Synthesis of bi-antennary *N*-glycan oxazoline **39**



Scheme 4-2-1: Synthesis of bi-antennary N-glycan oxazoline 39.

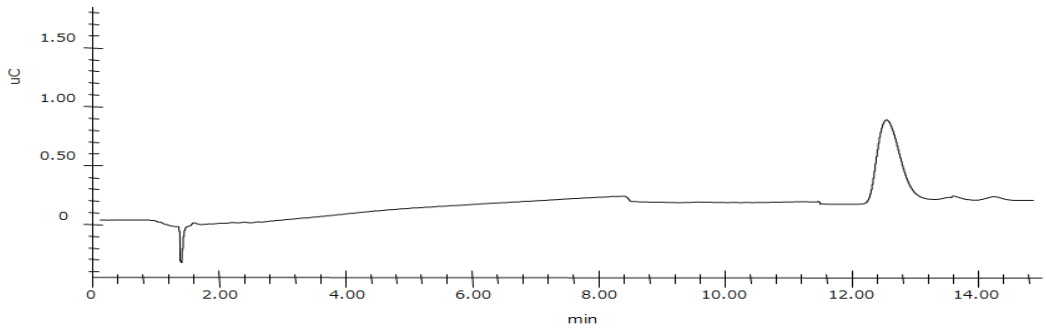
In this section, firstly I tried to test the transglycosylation reaction with synthesized biantennary glycan and synthesized IgG1 glycopeptide using Endo-M-N175Q. For that attempt, I synthesized octasaccharide oxazoline **39** from free octasaccharide **30** with DMC and triethyl amine. The oxazoline formation was confirmed by analyzing with DIONEX. The newly formed oxazoline compound was eluted earlier than free sugar with the eluting conditions (Fig 4-2-2). The formation of oxazoline was almost quantitative.

HPAEC (DIONEX) monitoring of oxazoline formation.

A. Free octasaccharide **30**, B. Octasaccharide oxazoline **39**

HPEAC Conditions: Column, CarboPac PA-1 (4x250 mm); elution, 10% 1 M NaOAc for 20 min. In 30-60 min, the newly formed oligosaccharide was eluted earlier than free sugar in HPEAC (DIONEX) condition.

A.



B.

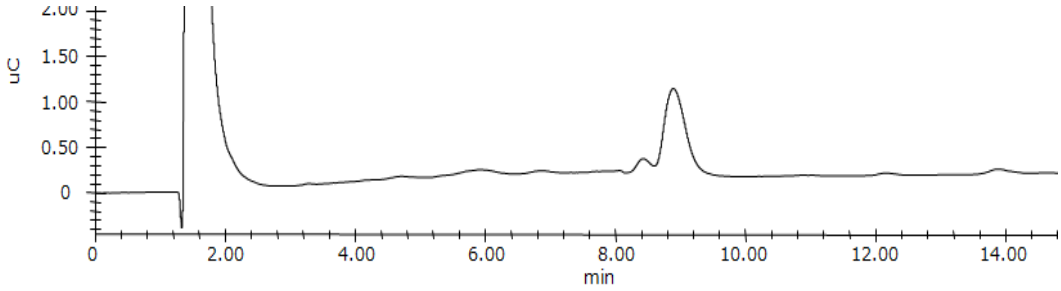
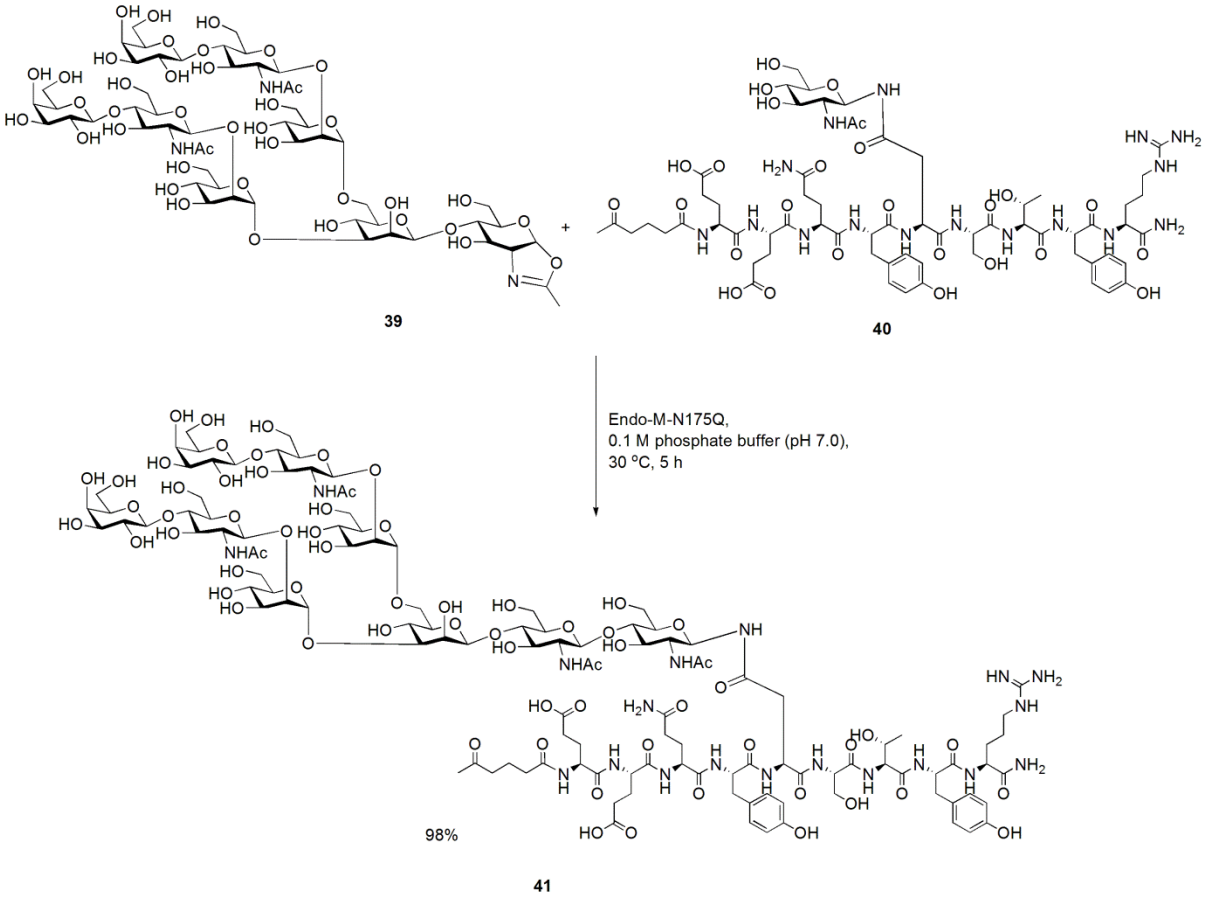


Fig 4-2-1: HPAEC (DIONEX) monitoring of oxazoline formation.

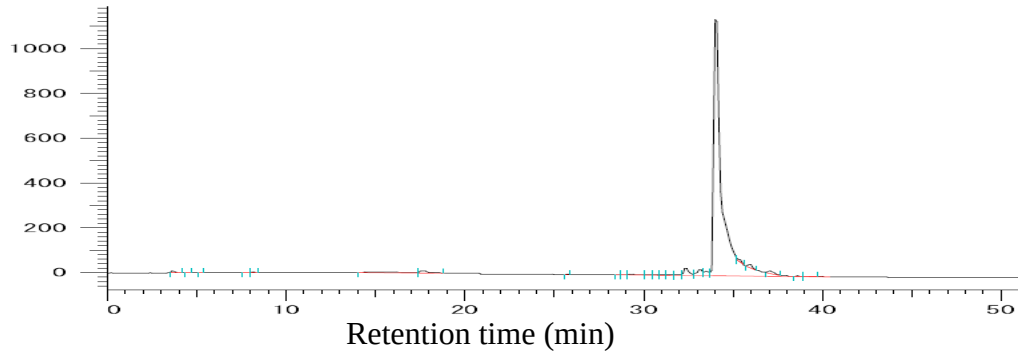
4-2-2: Synthesis of IgG1 glycopeptide **41**



Scheme 4-2-2: Synthesis of IgG1 glycopeptide **41**

Then, we assessed the efficacy of the Endo-M-N175Q for transglycosylation using chemically synthesized GlcNAc-nanopeptide derived from Immunoglobulin-G (IgG1). Expectedly, the mutant gave the transglycosylation product in high yield and did not show the rehydrolysis of the transglycosylation product. When the ratio of donor to acceptor was 10:1, Endo-M-N175Q gave about 97% yield of the transglycosylation product in 5 h. Transglycosylation reaction was analyzed by reversed-phase (RP)-HPLC. Analytical RP-HPLC was performed by a Inertsil ODS-3 (250×4.6 mm) (Fig 4-2-3).

A.



B.

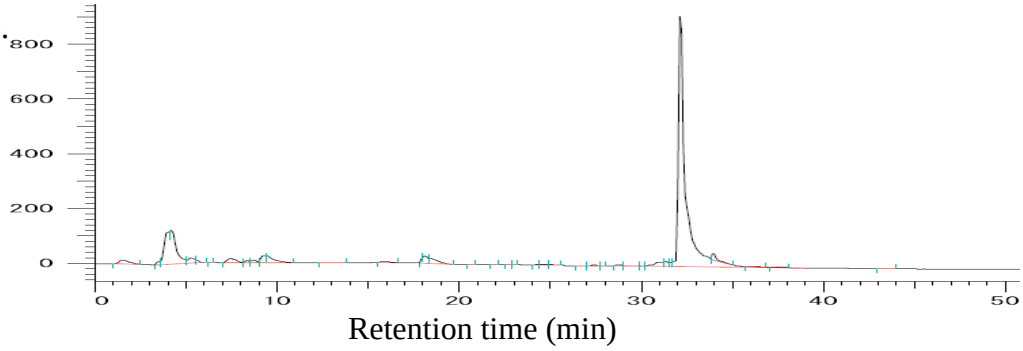


Figure 4-2-3. RP-HPLC anlalysis of transglycosilation reaction.

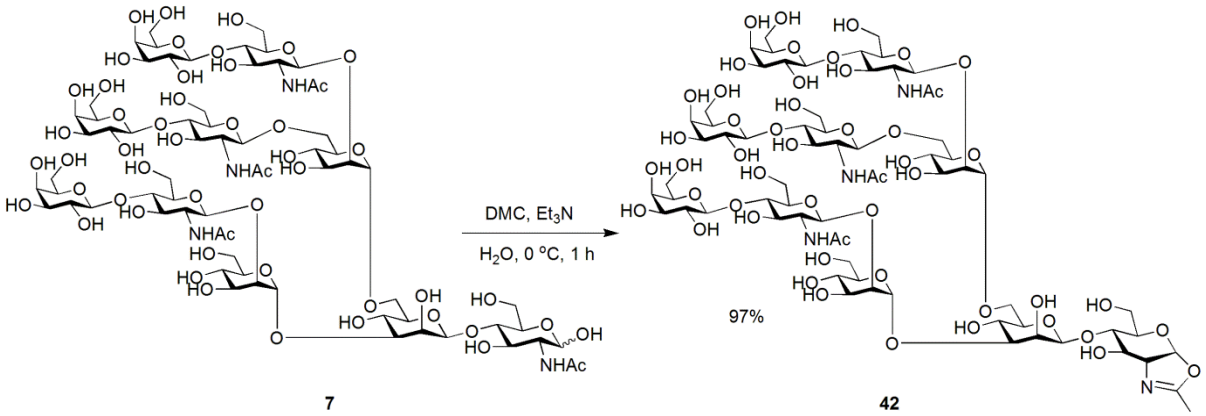
A. IgG1 GlcNAc peptide **40** and B. IgG1 Glycopeptide **41**.

Column: Inertsil ODS-3 (250×4.6 mm), Eluent A: 25 mM ammonium acetate, pH 5.8,

Eluent B: acetonitrile containing 10% eluent A, eluent (A/B = 98/2) was employed from

0 to 5 min, then the ratio of eluent B was increased lineally from 2% to 30% over 60 min with a flow rate of 0.7 ml/min.

4-2-3: Synthesis of triantennary N-glycan oxazoline **42**



Scheme 4-2-3: Synthesis of triantennary *N*-glycan oxazoline **42**.

After the success of synthesizing Immunoglobuline G (IgG1) glycopeptides with almost quantitative yield, I am very curious about the tri- and tetra antennary glycans for transglycosylation reaction, because upto now there is no report with respect to these hyper branched glycans. Firstly, I choose tri antennary *N*-glycans to test the feasibility towards Endo-M-N175Q catalyzed transglycosylation. I synthesized decasaccharide oxazoline **42** from free decasaccharide **7** with excess of DMC and triethyl amine. The oxazoline formation was confirmed by analyzing with DIONEX. The newly formed oxazoline compound was eluted earlier than free sugar with the eluting conditions. The formation of oxazoline was almost quantitative.

HPEAC (DIONEX) monitoring of oxazoline formation.

A.

B.

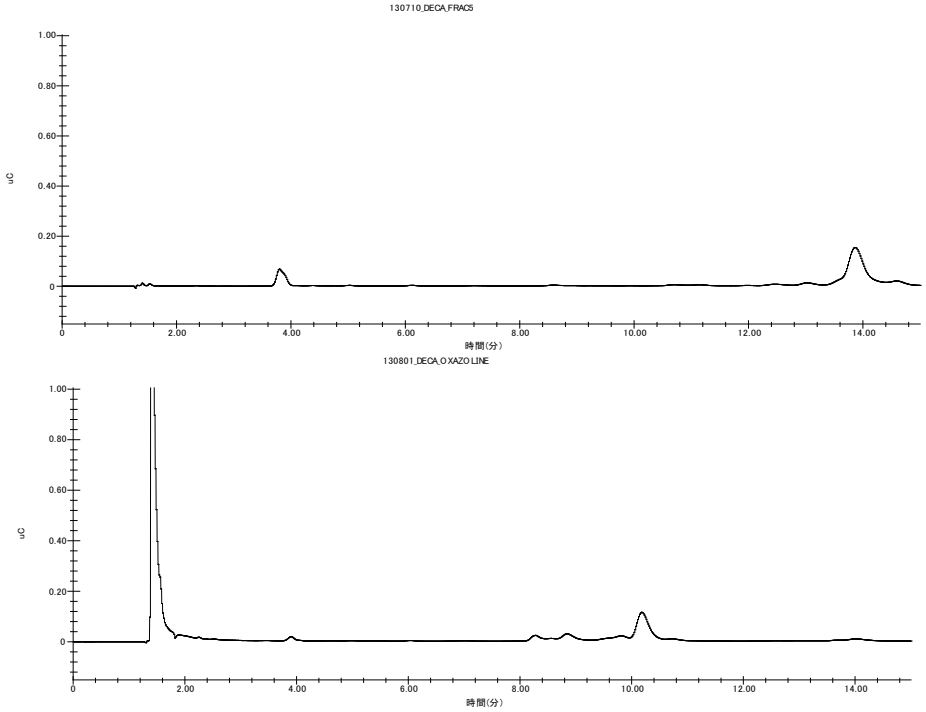
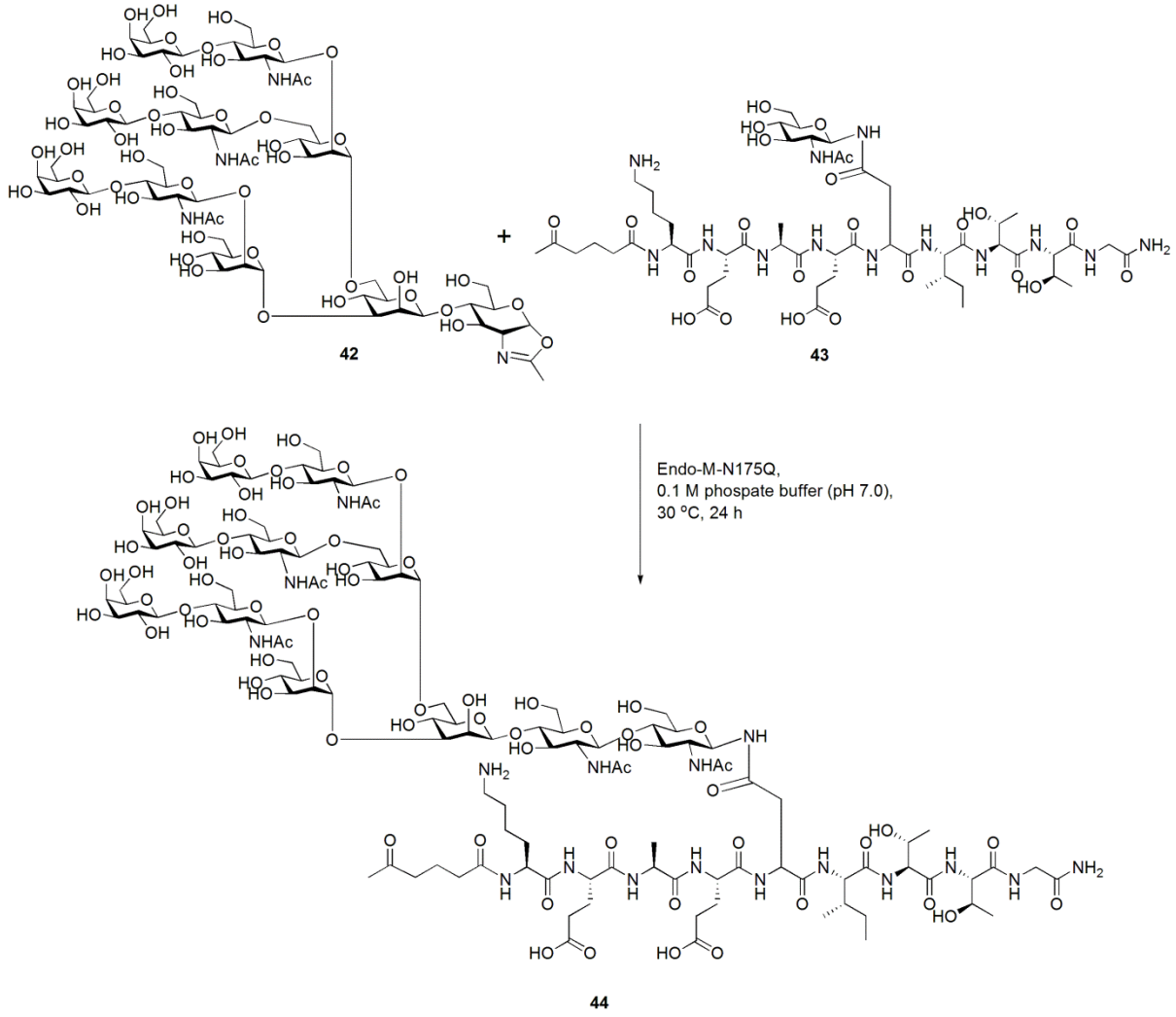


Figure 4-2-3: HPAEC (DIONEX) monitoring of oxazoline formation.

(A) Free dodecasaccharide **7**, (B) dodecasaccharide oxazoline **42**

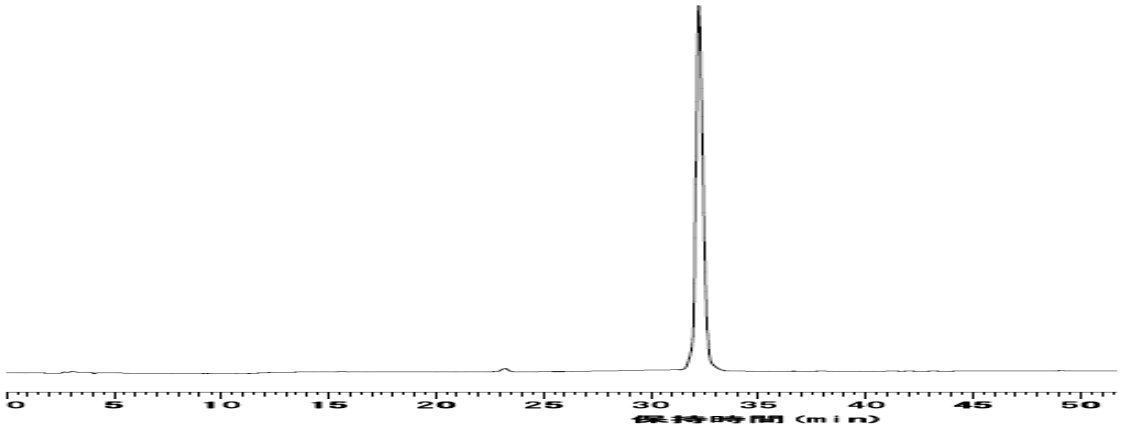
HPEAC Conditions: Column, CarboPac PA-1 (4x250 mm); elution, 10% 1 M NaOAc for 20 min. In 30-60 min, the newly formed oligosaccharide was eluted earlier than free sugar in HPAEC (DIONEX) conditions.

4-2-4: Synthesis of IgG-1 glycopeptide **46**



To examine whether the endoglycosidase Endo-M-N175Q is able to recognize the synthetic decasaccharide sugar oxazoline for trans-glycosylation, a model reaction was carried out with a small GlcNAc-peptide related to Erythropoitein **43** as acceptor. The enzymatic reaction was monitored by reverse phase HPLC. It was observed that the reaction between **42** and **43** (molar ratio 10:1) occurred and produced moderate yield in the presence of Endo-M-N175Q. Transglycosylation reaction was analyzed by reversed-phase (RP)-HPLC. Analytical RP-HPLC was performed by an Inertsil ODS-3 (250×4.6 mm) (Fig 4-2-5). MALDI-TOF MS analysis confirmed the formation of expected product (Fig 4-2-6).

A.



B.

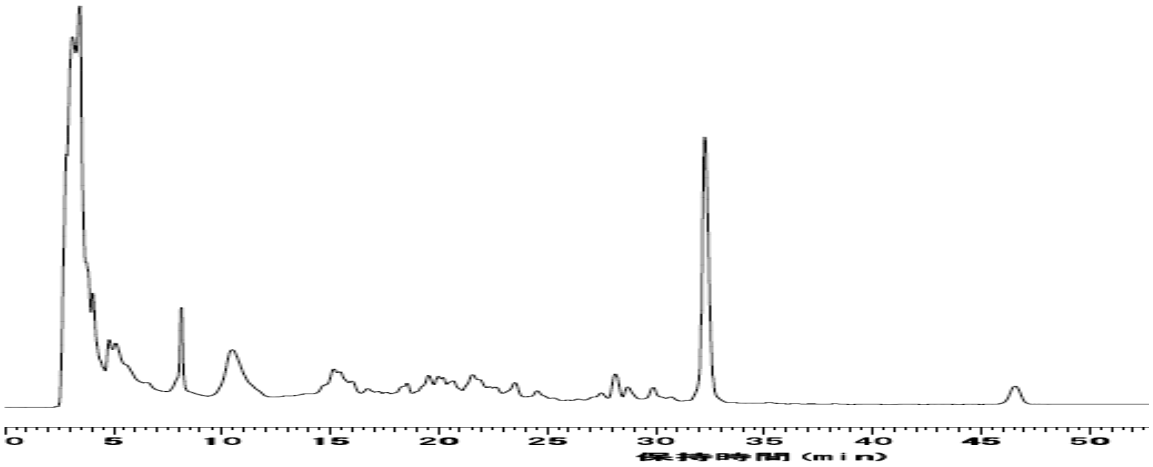
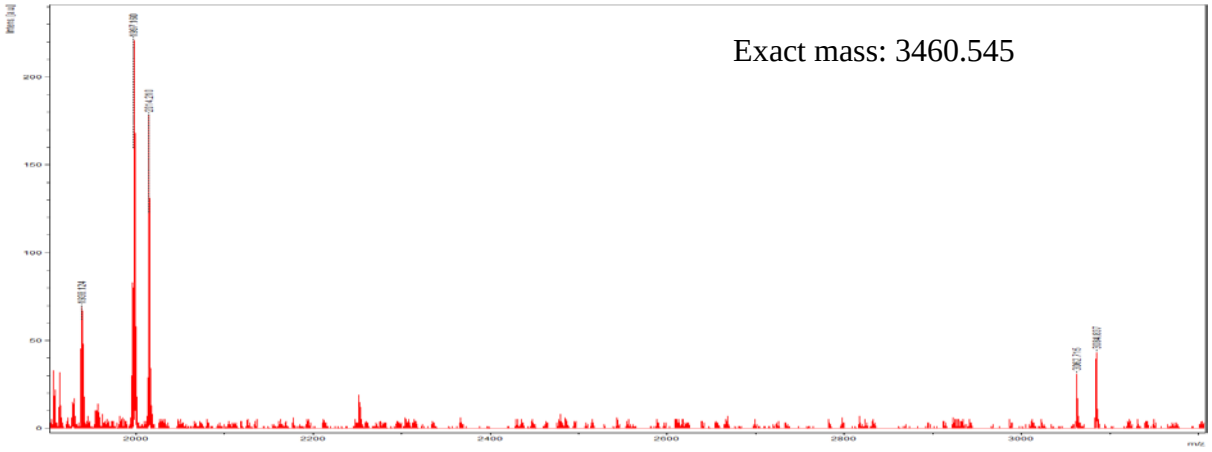


Figure 4-2-5. RP-HPLC anlalysis of transglycosilation reaction.

A. EPO GlcNAc peptide **43** and B. EPO Glycopeptide **44**.

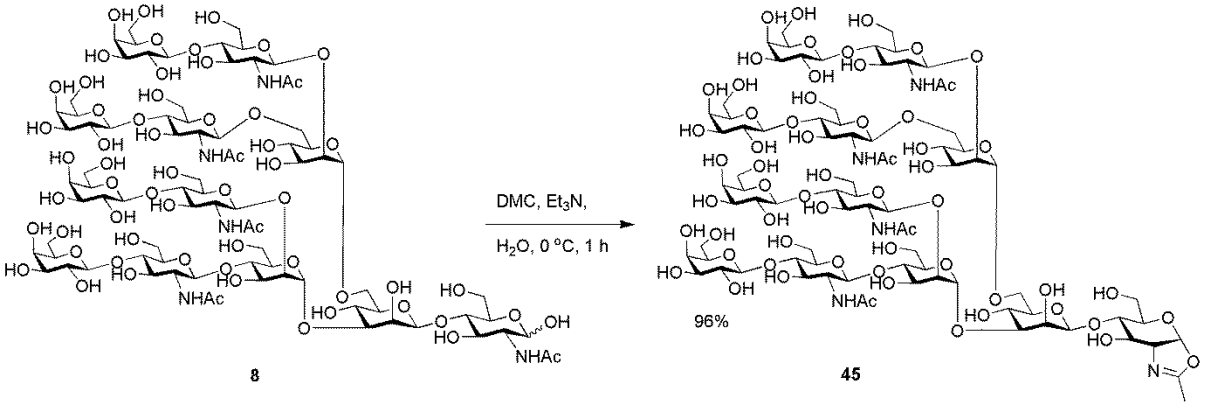
Column: Inertsil ODS-3 (250×4.6 mm), Eluent A: 0.1% TFA in water, Eluent B: acetonitrile containing 0.1% TFA, eluent (A/B = 98/2) was employed from0 min, then the ratio of eluent B was increased lineally from 2% to 30% over 50 min with a flow rate of 1.0 ml/min.

Fig 4-2-6: MALDI-TOF MS of EPO-related triantennary glycopeptide **44**. (Bruker ultrflex-I)



Signals observed around m/z: 2000 are supposed to be generated from oxazoline derivative **42** by hydrolysis.

4-2-5: Synthesis of tetraantennary *N*-glycan oxazoline **45**



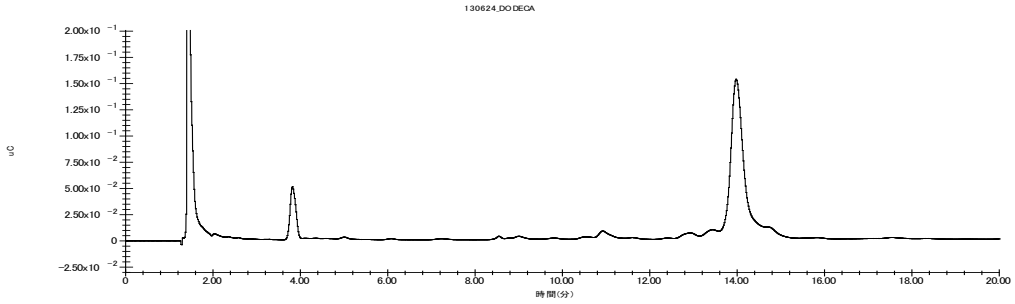
Scheme 4-2-5: Synthesis of tetraantennary *N*-glycan oxazoline **45**

After the success of synthesizing Erythropoitein (EPO) related glycopeptide with moderate yield, I am interested about the tetra antennary glycan for transglycosylation reaction, because upto now there is no report with respect to these hyper branched glycans. TO check the feasibility of tetra antennary *N*-glycan towards Endo-M-N175Q catalyzed transglycosylation, I synthesized dodecasaccharide oxazoline **45** from free dodecasaccharide **8** with excess of DMC and triethyl amine. The oxazoline formation was confirmed by

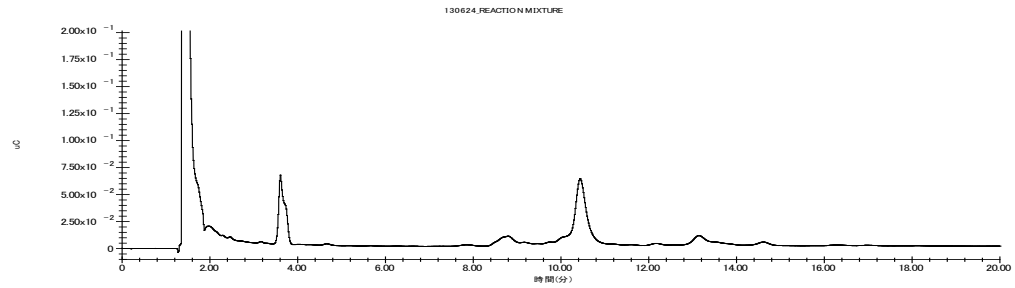
analyzing with DIONEX. The newly formed oxazoline compound was eluted earlier than free sugar with the eluting conditions. The formation of oxazoline was almost quantitative (Fig 4-2-7).

Figure 4-2-7: HPAEC (DIONEX) monitoring of oxazoline formation.

A



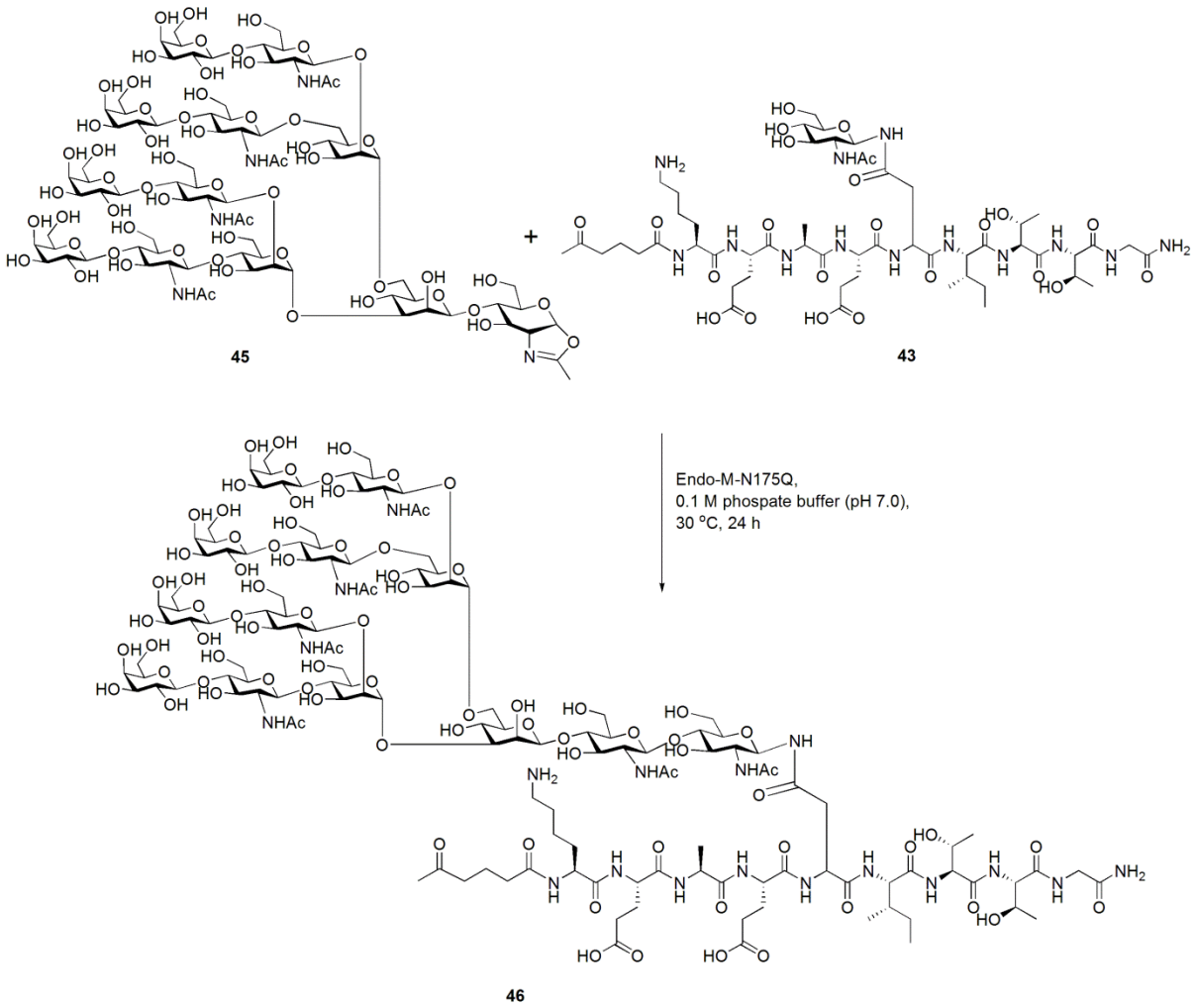
B



(A) Free dodecasaccharide **8**, (B) dodecasaccharide oxazoline **45**

HPAEC Conditions: Column, CarboPac PA-1 (4x250 mm); elution, 10% 1 M NaOAc for 20 min. In 30-60 min, the newly formed oligosaccharide was eluted earlier than free sugar in HPAEC (DIONEX) conditions.

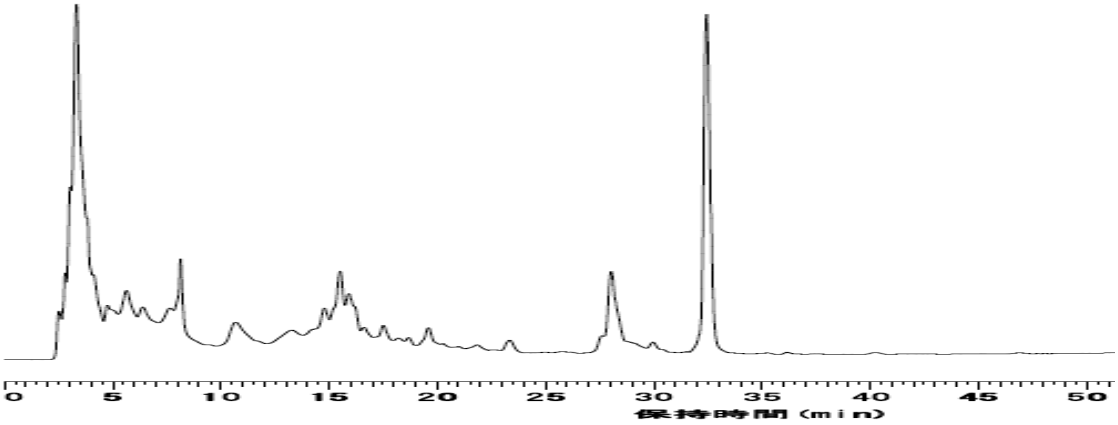
4-2-4: Synthesis of IgG-1 glycopeptide **46**



Scheme 4-2-4: Synthesis of IgG-1 glycopeptide **46.**

To examine whether the endoglycosidase Endo-M-N175Q is able to recognize the synthetic dodecasaccharide sugar oxazoline **45** for trans-glycosylation as like decasaccharide oxazoline **42**, a model reaction was carried out with a small GlcNAc-peptide related to Erythropoitein **43** as an acceptor. The enzymatic reaction was monitored by reverse phase HPLC. It was observed that the reaction between **45** and **43** (molar ratio 10:1) occurred and produced moderate yield in the presence of Endo-M-N175Q. Transglycosylation reaction was analyzed by reversed-phase (RP)-HPLC. Analytical RP-HPLC was performed by an Inertsil ODS-3 (250×4.6 mm).

A.



B.

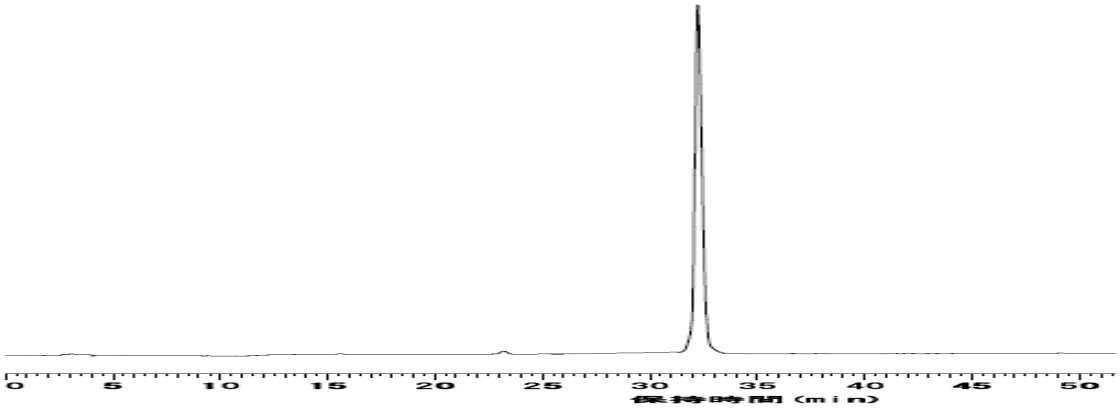
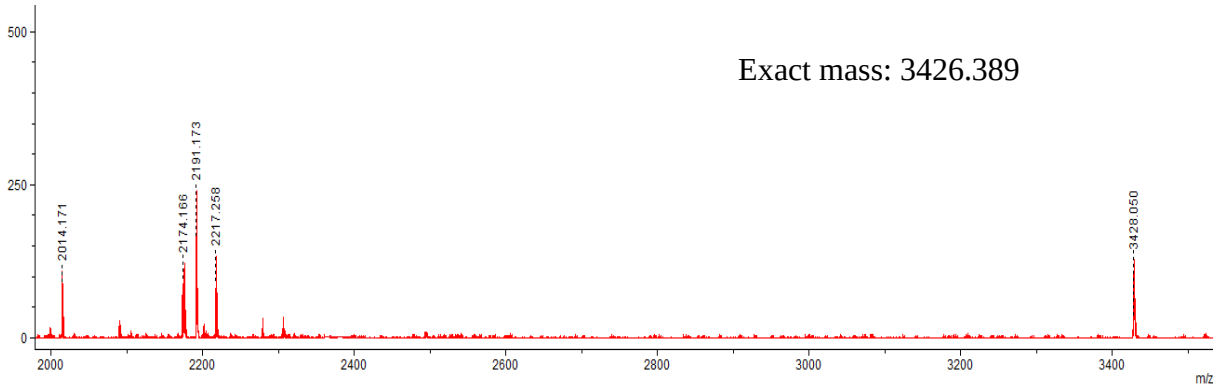


Figure 4-2-8. RP-HPLC anlalysis of transglycosilation reaction.

A. EPO GlcNAc peptide **43** and B. EPO Glycopeptide **46**.

Column: Inertsil ODS-3 (250×4.6 mm), Eluent A: 0.1% TFA in water, Eluent B: acetonitrile containing 0.1% TFA, eluent (A/B = 98/2) was employed from0 min, then the ratio of eluent B was increased lineally from 2% to 30% over 50 min with a flow rate of 1.0 ml/min.

Figure 4-2-9: MALDI-TOF-MS of EPO-related glycopeptide **46**. (Bruker ultrflex-I)



Signals observed around m/z: 2200 are supposed to be generated from oxazoline derivative **43** by hydrolysis.

4-3. Conclusion.

In summary, we have described a highly efficient chemoenzymatic synthesis of IgG 1 glycopeptide by a combined use of the newly developed method for synthesis of biantennary complex-type sugar oxazoline and the glycosynthase like N175Q mutant of Endo-M. We have demonstrated that when a sufficient amount of biantennary complex-type sugar oxazoline was used, the corresponding glycopeptides could be synthesized in high yield by the Endo-M-N175Q-catalyzed transglycosylation, without hydrolysisof the product. After, it was demonstrated, for the first time, that the reaction between donors **42**, **45** and acceptor **43** conducted by recombinant endo-M-N175Q proceeded significantly and afforded the desired EPO-related glycopeptides **44** and **46** in moderate yield.

4-4. Experimental Section.

All reactions were carried out under a nitrogen atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Proton and carbon NMR was recorded with Varian UnityInova 500 MHz (Agilent Inc., USA; ^{1}H: 500 MHz, ^{13}C: 125 MHz). Chemical shifts are given in ppm and referenced to internal TMS (δ_H 0.00 in $CDCl_3$), $CHCl_3$ (δ_H 7.26 in $CDCl_3$) or $CDCl_3$ (δ_C 77.00). Assignments in ^{1}H NMR were made by first-order analysis of the spectra by using ACD/NMR processor software (Advanced Chemistry Development, inc.) and were verified by H-H COSY and HSQC experiments. 2D NMR of compounds were recorded at 300 K with a Bruker Avance 600 spectrometer at 600.03 MHz for proton frequency equipped with cryoprobe. For the complete assignments and structural determination, two dimentional homonuclear DQF-COSY, TOCSY with MLEV-17 and NOESY spectra were recorded in the indirect dimension using States-TPPI phase cycling. Additionally two dimentional heteronuclear ^{13}C edited HSQC and HSQC-TOCSY measurements were also recorded with echo-antiecho mode for sensitive enhancement. All NMR data were processed by NMRPipe software and analysed using the Sparky program. A high/low resolution electrospray ionization mass spectra (ESI-MS) were recorded by JMS-700TZ (JEOL, Japan) and Bruker ultraflex-I. TLC was performed on Merck pre coated plates (20 cm × 20 cm; layer thickness, 0.25 mm; Silica Gel $60F_{254}$); spots were visualized by spraying a solution of 90:5:5 (v/v/v) MeOH-*p*-anisaldehyde-concentrated sulfuric acid and heating at 250°C for ca. $^{1}/_{2}$ min. Column chromatography was performed on Silica Gel N60 (spherical type, particle size 40-50 μm; Kanto Chemical Industry) with the solvent systems specified, and the ratio of solvent systems was given in v/v.

The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, br = broad.

4-3-1: synthesis of IgG 1 *N*-glycopeptide containing biantennary *N*-glycan 41.

2-Methyl{β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)- α-D-mannopyranosyl-(1→3)-[β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)] -β-D-manno pyranosyl-(1→4)-1,2-dideoxy -D-glucopyrano}-(2,1-d)-2-oxazoline (39).

To a solution of compound **30** (14 mg, 9.7 µmol) in H_2O (1.4 mL) was added Et_3N (122 µL, 876 µmol) and 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (58 mg, 340 µmol) at 0 ^oC. The reaction was monitored by DIONEX HPAEC-PAD. In 30-60 min, the HPEAC analysis indicated that the free oligosaccharide was converted into a new oligosaccharide that was eluted earlier than the reducing sugar under the HPAEC conditions. The product was purified by gel filtration on a Sephadex G-10 column that was eluted with 0.5% aq. NH_3 to afford sugar oxazoline **39** (14.0 mg, quant.) as a white solid after lyophilisation.

^{1}H NMR (500 MHz, D_2O, 25 °C, TMS): δ 6.01 (d, *J* = 7.0 Hz, 1 H), 5.03 (s, 1 H), 4.86 (s, 1 H), 4.66 (s, 3 H), 4.51 (dd, *J* = 7.5, 16.2 Hz, 3 H), 4.38 (d, *J* = 7.0 Hz, 3 H), 4.30 (s., 1 H), 4.10 (m, 3 H), 4.07 (m, 3 H), 3.89 (s, 3 H), 3.90 (s, 2 H), 3.85 - 3.80 (m, 8 H), 3.76 (dd, *J* = 3.9, 11.8 Hz, 4 H), 3.73 - 3.61 (m, 29 H), 3.60 - 3.41 (m, 21 H), 3.34 (t, *J* = 6.6 Hz, 2 H), 2.01 - 1.92 (m, 12 H).

HRMS (ESI): *m/z* calcd for $C_{54}H_{89}N_3Na_2O_{40}$, $[M+2Na]^+$ 1465.48177, found 1465.47689.

Synthesis of IgG1 Glycopeptide 40.

Oxohexanoyl-*L*-Glu-*L*-Glu-*L*-Gln-*L*-Tyr-(*N*^{4}-2-acetamido-2-deoxy-β-D-glucopyranosyl)-*L*-Asn-*L*-Ser-*L*-Thr-*L*-Tyr-*L*-Argininamide (40)

TentaGel S RAM resin (0.24 mmol/g, 100mg, 0.024 mmol), Fmoc-amino acids (96 μmol, 4.0 equiv), and Fmoc-Asn(Ac3AcNH-β-Glc)-OH (36 μmol, 1.5 equiv) were used. Fmoc aminoacids used are Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Tyr(tBu). TentaGel S RAM resin was placed in a 10 mL Libra Tube and allowed to swell in DMF for a period of 30 min. removed the DMF and the Fmoc-removal reaction was conducted under microwave irradiation. Piperidine (20%)/DMF (2 mL) was added and the mixture was shaken under microwave irradiation for 3 min. Following filtration and washing with DMF (3 mL, three times) and DCM (3 mL, three times). The corresponding Fmoc aminoacid (96 μmol, 4.0 equiv) dissolved in 0.4 M HBTU, HOBt/DMF (96 μmol, 4.0 equiv), and DIEA (144 μmol, 6.0 equiv) in DMF (Final concentration of amino acid 0.2 M) was added to the resin, and the mixture was shaken under microwave irradiation for 10 min. For introduction of the Fmoc-glycosylated amino acid, Fmoc-Asn(Ac_3AcNH-β-Glc)-OH (36 μmol, 1.5 equiv) dissolved in 0.4 M PyBOP, HOBT/DMF (36 μmol, 1.5 equiv), and DIEA (72 μmol, 3 equiv) in DMF (Final concentration of amino acid 0.2 M) was treated to the resin under microwave irradiation for 15 min. Again activated using 0.4 M PyBOP, HOBT/DMF (1.5 eq). Following filtration and washing with DMF and DCM (3 mL, three times each), the unreacted amino groups on the resin were acetylated with a solution of Ac_2O:DIEA:DMF (1.0:0.5:8.5). After shaking for 5 min at r.t., the resin was filtered and washed with DMF and DCM (Three times each). Fmoc-removal, coupling, and capping procedures as described above were carried out repeatedly. After completion of the synthesis, the glycopeptidyl-resin was treated with TFA:H_2O:TIS(95.0:2.5:2.5) (2.0 mL) at r.t., for 2 h, and the resin was filtered. The resin was washed twice with the same cocktail and the filtrates were combined and concentrated by streaming of nitrogen gas. The glycopeptides was precipitated by adding cold tert-butyl methyl ether, dried by streaming of nitrogen gas, dissolved in 50% aq.

acetonirile and lyophilized. Then dissolved in methanol (5.0 mL) and pH was adjusted to 12.8 with 1 N sodium hydroxide and stirred for 2 h at r.t., the mixture was neutralized by addition of 1 N acetic acid and evaporation. The crude material was purified by RP-HPLC (tR=46.8 min) to give **40** (7.5 mg, 21%).

HRMS (ESI): *m/z* calcd for $C_{64}H_{95}N_{16}O_{26}$, $[M+1H]^+$ 1503.66034, found 1503.65752.

Synthesis of IgG1 Glycopeptide containing biantennary *N*-glycan (41):

A solution of 25 mM IgG1 GlcNAc-peptide **40** (1.9 mg, 1.26 μmol, 75 μL) and 125 mM octasaccharide oxazoline **39** (13.5 mg, 9.48 μmol, 50 μL) in a 1 M phosphate buffer (50 mM, pH 7.0, 25 μL) and 300 μL H_2O was incubated with 500 mU/mL mutant Endo-M-N175Q (2.5 μL, 1.25 mU) at 30 ^oC. The reaction was monitored by reverse-phase HPLC (Fig 4-2-3). After 5 h, the product was purified by RP-HPLC to afford glycopeptide **41** (3.6 mg, 98%).

MALDI-TOF MS: *m/z* calcd for: $C_{118}H_{183}N_{19}O_{66}$, $[M+1H]^+$ 2924.1659, found 2924.166.

4-3-2. Synthesis of EPO-related triantennary glycopeptide 44

{β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl)-(1→3)-[(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-β-D-mannopyranosyl-(1→4)-1,2-dideoxy -D-glucopyrano}-(2,1-d)-2-oxazoline (42).

Decasaccharide **7** (6 mg, 3.3 μmol) in H_2O (1.0 mL) was added Et_3N (42 μL, 300 μmol) and 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (22 mg, 117 μmol) at 0^oC. The reaction was monitored by DIONEX HPAEC-PAD. In 30~60 min, the HPEAC analysis indicated that the free dodecasaccharide (hemiacetal) was converted into an oxazoline derivative that was eluted earlier than the corresponding reducing carbohydrate under the HPAEC conditions.

The isolated product was purified by gel filtration on a Sephadex G10 column eluted with 0.5% aq. NH_3 to afford decasaccharide oxazoline **42** (5.7 mg, 97%) as a white solid after lyophilisation.

Synthesis of EPO-related glycopeptide (43).

TentaGel S RAM resin (0.24 mmol/g, 100mg, 0.024 mmol), Fmoc-amino acids (96 μmol, 4.0 equiv), and Fmoc-L(Ac_3AcNH-β-Glc)-OH (36 μmol, 1.5 equiv) were used. Fmoc amino acids used are Fmoc-Lys(Boc)-OH, Fmoc-Glu(t-Bu)-OH, Fmoc-Ala-OH, Fmoc-Ile-OH, Fmoc-Thr(t-Bu)-OH, and Fmoc-Gly(t-Bu). TentaGel S RAM resin was placed in a 10 mL Libra Tube and allowed to swell in DMF for a period of 30 min, removed the DMF and the Fmoc-removal reaction was conducted under microwave irradiation. Piperidine (20%)/DMF (2 mL) was added and the mixture was shaken under microwave irradiation for 3 min. Following filtration and washing with DMF (3 mL, three times) and DCM (3 mL, three times). The corresponding Fmoc amino acid (96 μmol, 4.0 equiv) dissolved in 0.4 M HBTU, HOBt/DMF (96 μmol, 4.0 equiv), and DIEA (144 μmol, 6.0 equiv) in DMF (final concentration of amino acid, 0.2 M) was added to the resin, and the mixture was shaken under microwave irradiation for 10 min. For introduction of the Fmoc-glycosylated amino acid, Fmoc-Asn(Ac_3AcNH-β-Glc)-OH (36 μmol, 1.5 equiv) dissolved in 0.4 M PyBOP, HOBT/DMF (36 μmol, 1.5 equiv), and DIEA (72 μmol, 3 equiv) in DMF (final concentration of amino acid, 0.2 M) was treated with the resin under microwave irradiation for 15 min. Again the resins were activated by using 0.4 M PyBOP, HOBT/DMF (1.5 eq). Following filtration and washing with DMF and DCM (3 mL, three times each), the unreacted amino groups on the resin were acetylated with a solution of Ac_2O:DIEA:DMF (1.0:0.5:8.5). After shaking for 5 min at r.t., the resins were filtered and washed with DMF and DCM (three times each). Fmoc-removal, coupling, and capping procedures as described above were carried out repeatedly. After completion of the synthesis, the glycopeptidyl-resin was treated

with TFA:H_2O:TIS (95.0:2.5:2.5) (2.0 mL) at r.t., for 2 h, and the resin was filtered. The resin was washed twice with the same cocktail and the filtrates were combined and concentrated by streaming of nitrogen gas. The glycopeptides was precipitated by adding cold *tert*-butyl methyl ether, dried by streaming of nitrogen gas, dissolved in 50% aq. acetonitrile and lyophilized. Then, the residue was dissolved in methanol (5.0 mL) and pH was adjusted to 12.8 with 1 N sodium hydroxide, and stirred for 2 h at r.t. The mixture was neutralized by addition of 1 N acetic acid and evaporated. The crude material was purified by RP-HPLC (t=32.3 min) to give **43** (20.5 mg, 67%).

HRMS (Maldi): *m/z* calcd for $C_{53}H_{89}N_{13}NaO_{23}$, $[M+Na]^+$ 1298.609, found 1298.612.

Reaction between 42 and 43 catalyzed by recombinant endo-M (N175Q).

A solution of 25 mM glycopeptide **43** (12.6 µL) and 75 mM decasaccharide oxazoline **42** (42 µL) in a 500 mM phosphate buffer (pH 7.0, 12.6 µL) and 46.2 µL of H_2O was incubated with 500 mU/mL mutant Endo-M-N175Q (12.6 µL) at 30^oC. The reaction was monitored by reverse-phase HPLC as indicated in the text as Figure 4-2-5.

MALDI-TOFMS: *m/z* calcd for: $C_{135}H_{224}N_{18}O_{83}$, $[M+1H]^+$ 3084.245, found 3084.550.

4-3-3: Synthesis of EPO-related tetraantennary glycopeptide 46

{β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→4)-α-D-mannopyranosyl)-(1→3)-[(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-β-D-mannopyranosyl-(1→4)-1,2-dideoxy -D-glucopyrano}-(2,1-d)-2-oxazoline (45).

Dodecasaccharide **8** (7.3 mg, 3.4 μmol) in H_2O (1.0 mL) was added Et_3N (43 μL, 305 μmol) and 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (22 mg, 119 μmol) at 0^oC. The reaction was monitored by DIONEX HPAEC-PAD. In 30~60 min, the HPEAC analysis indicated that the free dodecasaccharide (hemiacetal) was converted into a oxazoline derivative that was eluted earlier than the corresponding reducing carbohydrate under the HPAEC conditions. The isolated product was purified by gel filtration on a Sephadex G10 column eluted with 0.5% aq. NH_3 to afford dodecasaccharide oxazoline **45** (7.0 mg, 96%) as a white solid after lyophilisation.

^{1}H NMR (500 MHz, D_2O, 25 °C, TMS): δ 5.99 (d, *J* = 7.3 Hz, 1 H, H-1 of Oxazoline), 5.02 (s, 1 H), 4.79 (s, 1 H), 4.59 (s, 1 H), 4.54 - 4.42 (m, 6 H), 4.40 - 4.33 (m, 5 H), 4.25 (m, 1 H), 4.10 (d, *J* = 14.7 Hz, 4 H), 4.05 (m, 1 H), 4.01 (m, 1 H), 3.96 (dd, *J* = 2.9, 9.3 Hz, 1 H), 3.92 - 3.86 (m, 6 H), 3.82 (d, *J* = 2.7 Hz, 6 H), 3.72 (s, 4 H), 3.75 (s, 4 H), 3.66 (m, 7 H), 3.65 (m, 6 H), 3.63 (m, 9 H), 3.57 (d, *J* = 3.2 Hz, 3 H), 3.55 (d, *J* = 2.7 Hz, 4 H), 3.50 - 3.47 (m, 5 H), 3.46 (m, 3 H), 3.44 (m, 3 H), 3.42 (s, 1 H), 3.37 - 3.30 (m, 2 H), 1.97 (s, 6 H), 1.96 (s, 3 H), 1.95 (s, 3 H), 1.94 (s, 3 H).

Reaction between 43 and 45 catalyzed by recombinant endo-M (N175Q).

A solution of 25 mM glycopeptide **43** (4.5 μL) and 74 mM dodecasaccharide oxazoline **45** (15 μL) in a 500 mM phosphate buffer (pH 7.0, 4.5 μL) and 16.5 μL of H_2O was incubated with 500 mU/mL mutant Endo-M-N175Q (4.5 μL) at 30^oC. The reaction was monitored by reverse-phase HPLC as indicated in the text as Figure 4-2-8.

MALDI-TOFMS: *m/z* calcd for: $C_{135}H_{224}N_{18}O_{83}$, $[M+1H]^+$ 3426.392, found 3428.05.

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Chapter 4

Concluding Remarks

Asparagine-linked type oligosaccharides (*N*-glycans) of glycoproteins play essential roles of the maintenance and regulation of a variety of biological functions such as cell differentiation, cell adhesion, and homeostatic immune balance. Recent extensive efforts on large-scale *N*-glycan analysis revealed the importance of structural alteration of human serum/cellular glycoprotein *N*-glycans during disease progression in the potent biomarkers for early diagnosis and new class targets for the development of therapeutic antibodies. However, relationship between structures and functions of *N*-glycans of glycoproteins remains mostly unclear because of the structural complexity and heterogeneity in the dynamic posttranslational glycosylation of proteins at the potential *N*-glycosylation sites. Recently, chemical and enzymatic synthesis of glycopeptides and glycoproteins having *N*-glycans has been accelerated conspicuously by means of various endo-β-*N*-acetyl-D-glucosaminidases with oligosaccharide oxazolines as sophisticated donor substrates. However, it is clear that feasibility of this potential approach depends strongly on the availability of complex *N*-glycan structures found ubiquitously in nature. Although extensive efforts have been devoted to the construction of such complicated hyper-branched *N*-glycan structures the syntheses generally entail tedious procedures for the preparation of many designated monosaccharide synthons and multistep stereoselective glycosidation reactions. Especially, it should be noted that the formation of Manβ(1→4)GlcNAc unit involved definitely in *N*-glycan core moiety is one of the most difficult steps among the synthesis of glycoside linkages found in nature. The reason comes mainly from the facts that both the anomeric effect on and the neighboring group participation in the mechanism of the glycosidation reactions using D-mannosyl donors are not beneficial for the stereo selective generation of the β1,4-mannoside linkage. In addition, it is well documented that an extremely low reactivity of the secondary hydroxyl group at C-4 position of the acceptor

GlcNAc derivatives makes large-scale preparation of this disaccharide unit impossible. From a view point of the practical protocols for versatile and large-scale synthesis, it is considered that advent of a facile and more efficient strategy for the synthesis of such *N*-glycan core structures is strongly needed while many pioneering approaches have been reported.

In this thesis, the author focused on the synthesis of β1,4-mannobiose from abundant polysaccharide, development of novel synthetic methodology for the preparation of Manβ(1→4)GlcNAc related disaccharide core structure and these findings largely contribute for the practical synthesis of varieties of *N*-glycans. It should be emphasized that the present strategy should allow for the synthesis of a variety of glycopeptides and glycoproteins having homogeneous and highly complicated *N*-glycans when combined with endoglycosidase-catalyzed transglycosylation protocols.

In chapter 2, I established highly efficient synthetic strategy towards *N*-glycan core structure by means of β1,4-mannobiose octaacetate **2** obtained efficiently by combined enzymatic digestion and chemical modification from abundant locust bean gum **1** as a key starting material. Versatility of the intermediate **2** was demonstrated by developing the new synthetic approach for the preparation, Phenyl-(2-*O*-benzyl-4,6-*O*-benzylidine-*β*-D-mannopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside **3** by using the β1,4-mannobiose octaacetate **2.** We optimized the best condition to isolate β1,4-mannobiose from the abundant polysaccharide locust bean gum, then we developed the novel synthetic methodology for the synthesis of very important key compound **3**, which is very essential for the practical synthesis of *N*-glycans and glycopeptides.

In chapter 3, I have developed a modular set of building blocks **4**, **5** and **6** that can be used in a generally applicable double regio- and stereoselective glycosylation approach for multiantennary *N*-glycans from two to four antennae. The yields of the convergent glycosylation reactions were high and in all cases only the formation of desired stereo- and

regioisomers was found. In the case of the tri- and tetra-antennary products the glycosylation reactions were free of steric hindrance. This demonstrates a high compatibility of the donors with the corresponding acceptors. After, I performed enzymatic galactosylation of partially protected sugars **28**, **32** and **37** by Gal-T with UDP-Gal; the galctosylation went smoothely even with protection at reducing end. After one-pot deprotection the *N*-glycans can be further modified and incorporated into glycoconjugates.

In chapter 4, we have described a highly efficient chemoenzymatic synthesis of IgG 1 glycopeptide by a combined use of the newly developed method for synthesis of biantennary complex-type sugar oxazoline and the glycosynthase like N175Q mutant of Endo-M. We have demonstrated that when a sufficient amount of biantennary complex-type sugar oxazoline was used, the corresponding glycopeptides could be synthesized in high yield by the Endo-M-N175Q-catalyzed transglycosylation, without hydrolysisof the product. After, it was demonstrated, for the first time, that the reaction between donors **42**, **45** and acceptor **43** conducted by recombinant endo-M-N175Q proceeded significantly and afforded the desired EPO-related glycopeptides **44** and **46** in moderate yield.

As mentioned above, our new approach for the synthesis of hyper branched *N*-glycans from abundant polysaccharide locust bean gum is a useful methodology for practical synthesis of varieties of *N*-glycans. It will open new door for the scientists, who wish to synthesize varieties of *N*-glycans and glycopeptides for the drug discovery. It should be emphasized that the present strategy should allow for the synthesis of a variety of glycopeptides and glycoproteins having homogeneous and highly complicated *N*-glycans when combined with endoglycosidase-catalyzed transglycosylation protocols.

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