Development of a novel strategy for semisynthesis of triantennary complex-type oligosaccharides and its application for glycopeptide synthesis

三分枝複合型糖鎖の新規半化学合成法の開発と
糖ペプチド合成への展開

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Abstract

Glycosylation of proteins is one of the major posttranslational modifications, and more than 50% proteins are estimated to be posttranslationally glycosylated. Complex-type oligosaccharides on proteins show characteristic branching structures including bi-, tri-, and tetraantennary forms. In the case of erythropoietin (EPO), which is glycoprotein hormone stimulating red blood cell production, tri- and tetra-antennary structures account for more than 90% of complex-type oligosaccharide, and furthermore, it is known that branched structures are often observed on cancer cells. However, relationship between oligosaccharide branching patterns and the behavior of proteins/cells has not yet well understood.

Chemical synthesis of oligosaccharides and glycoproteins provides homogenous molecules to understand the functions and necessities of oligosaccharides at molecular level, however syntheses of complex-type oligosaccharides unfortunately necessitate time-consuming protocols due to repetitive protection/deprotection and glycosylation steps. Prompt access to such highly complicated oligosaccharyl molecule would greatly contribute to reveal oligosaccharide functions.

Thus, I envisaged developing an unprecedented semisynthetic strategy for the synthesis of triantennary oligosaccharide **1** and **2** from the substrate of biantennary asialononasaccharide **3**, which can be isolated from a natural source in gram scale. Twenty-four hydroxy groups of the biantennary oligosaccharide were specifically and precisely manipulated based on rigorous NMR structural analysis. Selective introduction and deprotection of benzylidene acetals produced suitably protected oligosaccharyl acceptors, followed by glycosylation with lactosaminyl donor and stepwise deprotection reactions to give desired triantennary oligosaccharide **1** and **2**, respectively. The idea to use biantennary structure as a scaffold of triantennary oligosaccharides was critical for the synthesis, along with a finding of selective benzylidene deprotection in mannosides.

The obtained triantennary oligosaccharyl asparagine was converted into EPO-glycopeptide **33** by segment coupling reactions in solution, which was further ligated with (glyco-)peptides in order to synthesize an EPO glycoform having one triantennary sialyltetradecasaccharide and two biantennary sialylundecasaccharide **67**.

In conclusion, I developed a novel semisynthetic strategy toward triantennary complex-type oligosaccharides and demonstrated robustness and utility of the synthesis by getting access to EPO. The established synthetic protocols can be powerful platform for the insight into oligosaccharide branching on proteins.

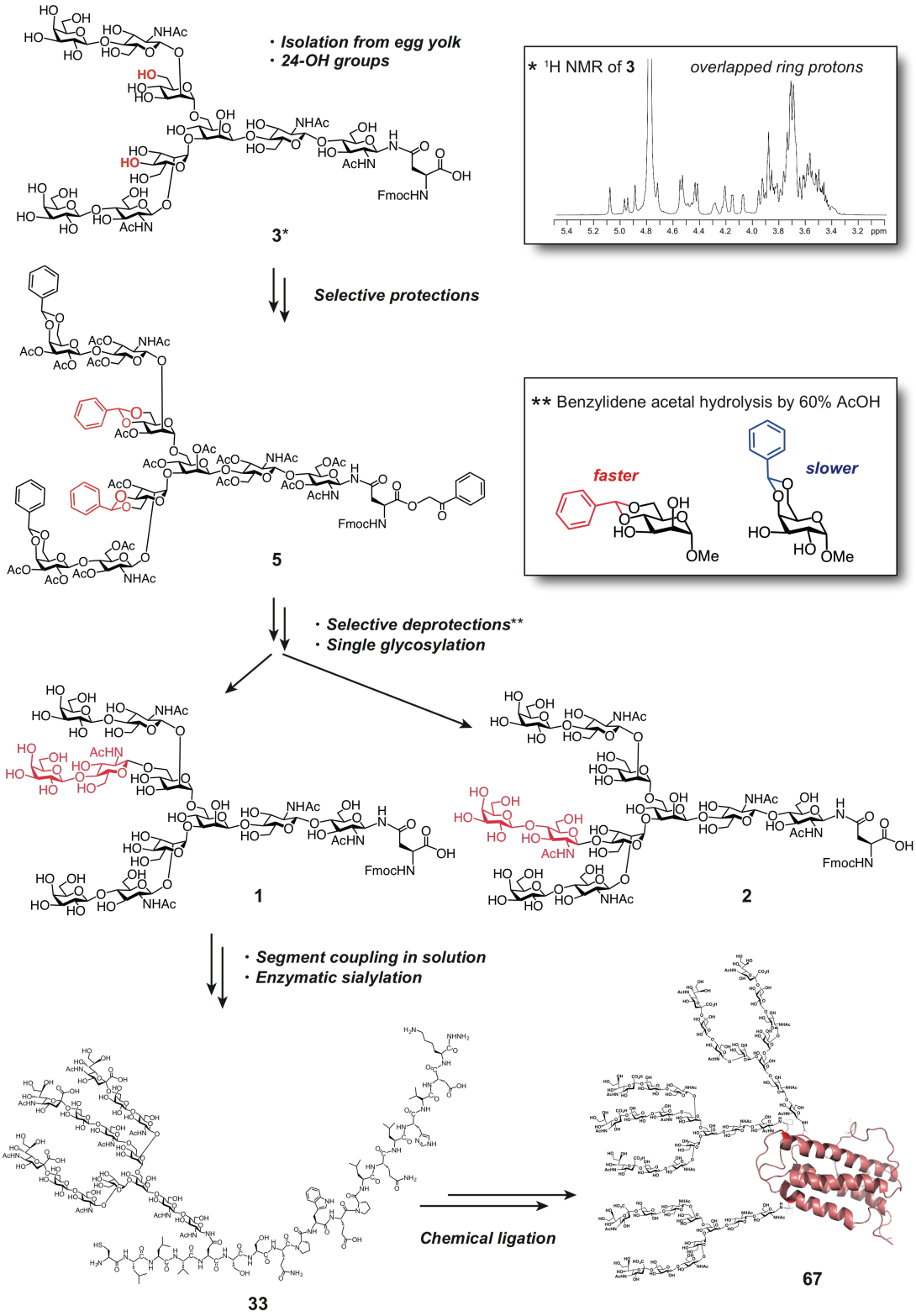

Figure. Outline of the semisynthesis and its application in this study.

Abbreviation

A: pre-exponential factor

Ac: acetyl

Acm: acetamidomethyl

AIBN: 2,2'-azobis(isobutyronitrile)

Ala: alanine

Asn: asparagine

Arg: arginine

Asp: aspartic acid

Boc: *tert*-butyloxycarbonyl

Boc-OSu: *N*-(*tert*-butoxycarbonyloxy)succinimide

Bn: benzyl

^{t}Bu: *tert*-butyl

Bz: benzoyl

CAN: Ammonium Cerium(IV) Nitrate

CMP: cytidine 5'-monophosphate

CD: circular dichroism

COSY: correlation spectrometry

CSA: (+)-10-camphorsulfonic Acid

Cys: cysteine

DBMP: 2,6-di-*tert*-butyl-4-methylpyridine

DBU: 1.8-diazabicyclo[5.4.0]-undec-7-ene

DCM: dichloromethane

DDQ: 2,3-dichloro-5,6-dicyanobenzoquinone

DEPBT: 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one

DIC: *N,N'*-diisopropylcarbodiimide

DIEA: *N,N'*-diisopropylethylamine

DMAP: 4-dimethylaminopyridine

DMF: *N,N'*-dimethylformamide

DMS: methyl sufide

DMSO: dimethyl sulfoxide

DMTST: dimethyl(methylthio)sulfonium trifluoromethanesulfonate

DNA: deoxyribonucleic acid

DNP: 2,4-dinitrophenyl

Dol: dolicol

DTT: D,L-dithiothreitol

E_a: activation energy

EDT: 1,2-ethanedithiol

EPO: erythropoietin

ER: endoplasmic reticulum

ESI: electrospray ionization

Fmoc: 9-fluorenylmethyloxycarbonyl

G: Gibbs energy

Gal: galactose

GalNAc: *N*-acetylgalactosamine

Glc: glucose

GlcNAc: *N*-acetyl-D-glucosamine

Gln: glutamine

Glu: glutamic acid

Gly: glycine

Gn-HCl: guanidine hydrochloride

GnT: *N*-acetylglucosaminyltransferase

H: enthalpy

h: Planck constant

HATU: *O*-(7-aza-1H-benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate

HBTU: *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate

hCG: human chorionic gonadotropin

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His: histidine

HMBC: heteronuclear multiple-bond correlation

HMPB: 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid

HOBt: 1-hydroxy-benzotriazole

HPLC: high performance liquid chromatography

HSQC: heteronuclear single-quantum coherence

IAD: intramolecular aglycon delivery

INF-β: interferon-β

κ: transmission coefficient

k_B: Boltzmann's constant,

LacNAc: *N*-acetyllactosamine

LC: liquid chromatography

Ile: isoleucine

Leu: leucine

Lys: lysine

NCL: native chemical ligation

MALDI: matrix assisted laser desorption/ionization

Man: mannose

MESNa: sodium 2-mercaptoethanesulfonate

Met: methionine

MPAA: 4-mercaptophenylacetic acid

MS: mass spectrometry

MSNT: 1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole

Mtr: 4-methoxy-2,3,6-trimethylbenzenesulfonyl

NIS: *N*-iodosuccinimide

NMR: nuclear magnetic resonance

Neu5Ac: *N*-acetylneuraminic acid

ODS: octa decyl silyl

OST: oligosaccharyl transferase

Pbf: 2,2,4,6,7-pentamethyldihydrobezofuran-5-sulfonyl

Phe: phenylalanine

Pro: proline

PTM: posttranslational modification

PyBOP: 1*H*-benzotriazol-1-yloxy-tri(pyrrolidino)phosphonium hexafluorophosphate

R: gas constant

RNA: ribonucleic acid

RDS: rate determining step

RP: reversed-phase

rt: room temperature

S: entropy

Seg 1: Ala^{1}-Cys^{28} of EPO sequence

Seg 2: Cys^{29}-Tyr^{49} of EPO sequence

Seg 3: Cys^{50}-Ala^{78} of EPO sequence

Seg 4: Cys^{79}-Lys^{97} of EPO sequence

Seg 5: Cys^{98}-Ala^{127} of EPO sequence

Seg 6: Cys^{128}-Arg^{166} of EPO sequence

Seg 23: Cys^{29}-Ala^{78} of EPO sequence

Seg 234: Cys^{29}-Lys^{97} of EPO sequence

Seg 56: Cys^{98}-Arg^{166} of EPO sequence

Seg 23456: Cys^{29}-Arg^{166} of EPO sequence

SGP: sialylglycopeptide

SPPS: solid phase peptide synthesis

Ser: serine

TBDMS: *tert*-butyldimethylsilyl

TBDPSCl: *tert*-Butyldiphenylchlorosilane

TCEP: tris(2-carboxyethyl)phosphine hydrochloride

TE: 2-(trimethylsilyl)ethyl

TFA: trifluoroacetic acid

TFE 2,2,2-trifluoroethanol

TfOH: trifluoromethanesulfonic acid

THF: tetrahydrofuran

Thr: threonine

Thz: thiazolizine

TIPS: Triisopropylsilane

TMSOTf : trimethylsilyl trifluoromethanesulfonate

TOCSY (total correlation spectroscopy)

Tris-HCl: 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride

Troc: 2,2,2-trichloroethyl

Trt: trityl

TST: transition state theory

Typ: tryptophan

Tyr: tyrosine

UDP: uridine diphosphate

UV: ultraviolet

VA-044: 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride

Val: valine

Xan: xanthyl

Z: benzyloxycarbonyl

Chapter 1

General Introduction

1-1. Introduction of oligosaccharide

Glycosylation of proteins on cell surfaces and in body fluids is one of the major posttranslational modifications (PTM). Approximately human has 30,000 protein-coding genes,[1-2] however the coding capacity is expanded by transcriptional diversification (eg. mRNA splicing) and the PTM of proteins.[3-6] This PTM of proteins includes phosphorylation, methylation, acetylation, ubiquitylation, and so on. Especially, an analysis of SWISS-PLOT protein sequence database estimated that more than half of proteins are glycosylated.[7]

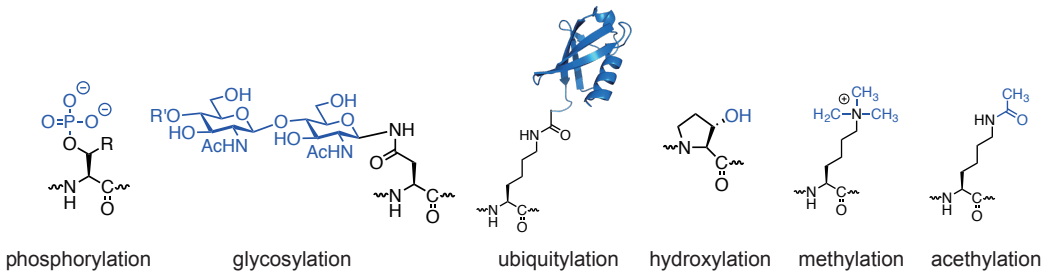

Figure 1-1. Examples of posttranslational modifications

Oligosaccharides on proteins play important roles in many biological events such as cell-cell interactions, protein folding, and immune systems.[8-11] Chemical properties of protein can be modified by attachment of oligosaccharides to alter the stability, protease resistance, and quaternary structures. In the biosynthetic pathway of glycoproteins, oligosaccharides are used as a tag, which allows interaction of the proteins with specific enzymes in defined order for maturation of proteins.[12-13]

Oligosaccharides on proteins are generally divided into two types: asparagine (*N*)-linked oligosaccharides and serine or threonine (*O*)-linked Oligosaccharides (Figure 1-2). *N*-linked oligosaccharides are attached to the nitrogen of asparagine side chain in consensus sequons: Asn-X(except for Pro)-Ser/Thr. They are further classified in 3 types: high-mannose type, hybrid-type, and complex-type oligosaccharides. All of them has a common core pentasaccharide: [Man-α-1,3-(Man-α-1,6)-Man-β-1,4-GlcNAc-β-1,4-GlcNAc-Asn], and additional carbohydrate moieties are linked to hydroxy groups of the core pentasaccharide. High-mannose type oligosaccharide consists of several mannose residues (9 residues in maximum) in addition to the core pentasaccharide, and some have Glc residues on an antenna. Complex-type oligosaccharides commonly have additional galactose (Gal), *N*-acetylglucosamine (GlcNAc), and *N*-acetylneuraminic acid (Neu5Ac). Further modifications of the oligosaccharide are observed such as fucosylation, phosphorylation, and sulfation. Hybrid-type oligosaccharides have some Man residues on the Man-α-1,6 arm of the core, and one or two antennae on the Man-α-1,3 arm. Representative structures are shown in Figure 1-2.

Modification of hydroxy groups of Ser or Thr with a GalNAc residue results in *O*-linked oligosaccharide. Carbohydrate part is usually elongated with other monosaccharides such as Gal and Neu5Ac residues, and the resulting structures can be divided from core-1 to core-8.

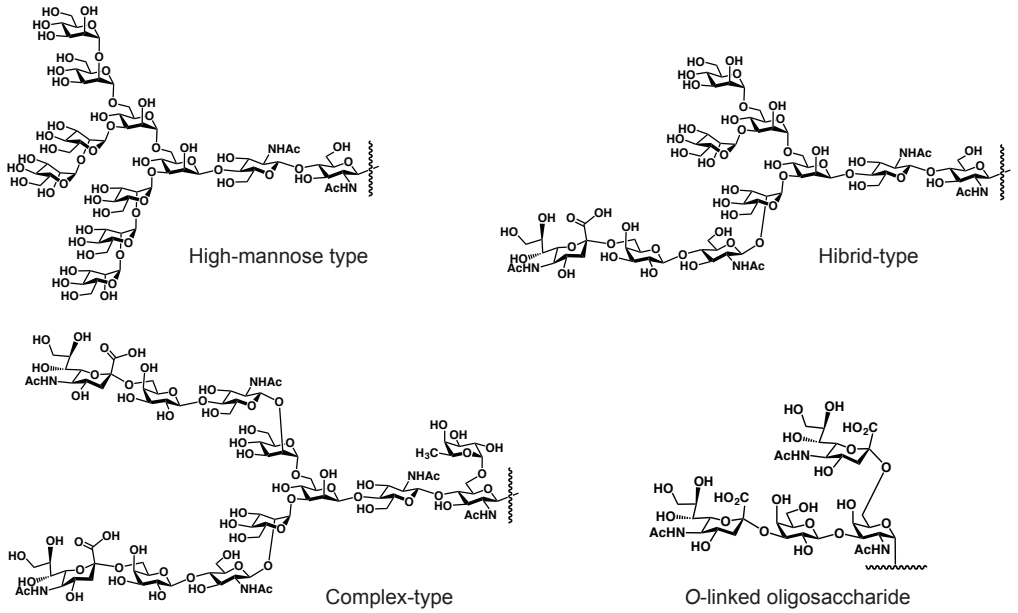

Figure 1-2. Representative structures of three types of *N*-linked oligosaccharides and *O*-linked oligosaccharide.

1-2. A biosynthetic pathway of complex-type oligosaccharides

$Glc_3Man_9GlcNAc_2$ (**F**, Figure 1-3) is assembled on dolicol-phosphate (Dol-P), and the oligosaccharide is transferred to an asparagine residue in selected Asn-X-Ser/Thr sequons of a nascent protein by an assistance of oligosaccharyltransferase (OST) in endoplasmic reticulum (ER) as shown in Figure 1-3. The biosynthesis of *N*-glycans begins with transferring GlcNAc-P from UDP (uridine-5'-diphospho)-GlcNAc to Dol-P (**A**) by a GlcNAc-1-phosphotransferase. Then, a GlcNAc and five Man residues are sequentially added to the GlcNAc-P-P-Dol (**B**) by corresponding glycosyltransferases and sugar-nucleotides, which results in a $Man_5GlcNAc_2$-P-P-Dol (**C**) in the cytosolic plasma membrane. Next, a flippase translocates the dolicol-linked oligosaccharide (**C**) to inside of ER across ER membrane bilayer, and the assembly of the precursor oligosaccharide is completed with addition of four Man and three Glc residues using Dol-P-Man and Dol-P-Glc as donors. The OST complex, which resides in the ER membrane and is comprised by several domains, transfers *en bloc* the synthesized tetradecasaccharide to a newly translated protein (**F** to **G**).

After enzymatic processing of an oligosaccharide on a protein in the ER, the glycosylated protein is transferred into *cis*-Golgi apparatus, and the oligosaccharide structure is further processed by trimming and addition of sugars by glycosidases and glycosyltransferases in Golgi apparatus (Figure 1-3). In ER, the $Glc_3Man_9GlcNAc_2$ (**G**) on a polypeptide/protein works as a tag to support protein folding through the interaction with chaperons.[13] During this glycoprotein quality control system, terminal Glc residues are removed. After the production of the $Man_9GlcNAc_2$-Asn structure (**H**) or additional trimming of terminal Man (**I**), the glycoprotein passes through the ER to *cis*-Golgi. Glycosidases and glycosyltransferases alter the high-mannose type to hybrid-type or complex-type structures in Golgi apparatus. First, Man residues are removed to give a $Man_5GlcNAc_2$ structure (**K**). In medial Golgi, *N*-acetylglucosaminyltransferase I (GnT-I) adds a GlcNAc to 2-OH of α-1,3 Man in the core pentasaccharide (**L**). Then, the majority of *N*-linked oligosaccharides are trimmed by α-mannosidases II to give a $GlcNAcMan_3GlcNAc_2$. Following addition of a GlcNAc to 2-OH of α-1,6 Man by GnT-II gives a precursor (**M**) for complex-type oligosaccharides. To the GlcNAc residues of the oligosaccharide, Gal residues are added in *medial*-Golgi (**N**).

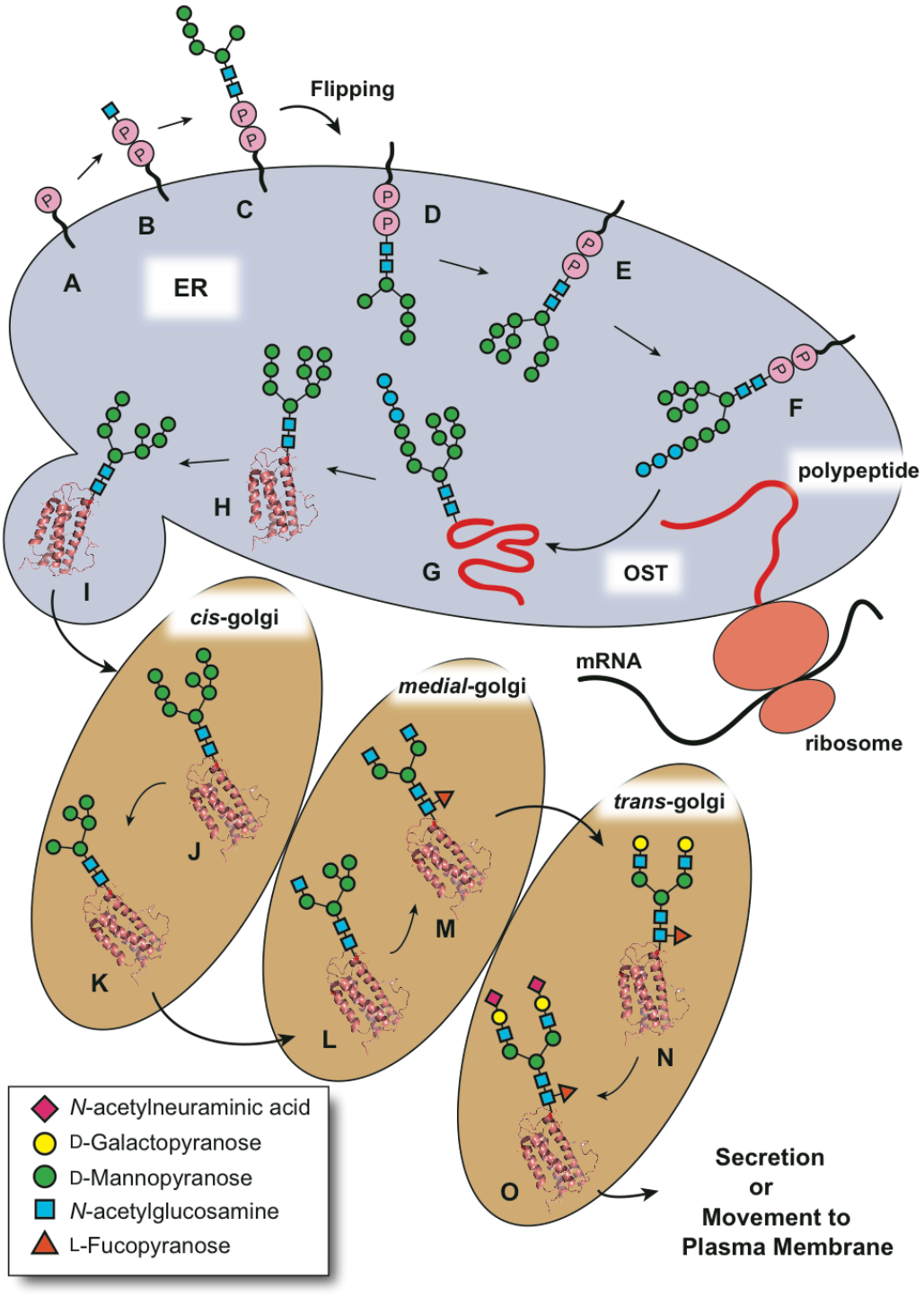

Figure 1-3. A biosynthetic pathway of *N*-linked oligosaccharides

1-3. Branching and maturation of *N*-glycans in Golgi apparatus

Some complex-type oligosaccharides extend their branching degree by a transfer of GlcNAc residues to the two mannoside ends of the common core pentasaccharide by GnT in *medial*-Golgi （Figure 1-4a）. GnT-V and GnT-IV individually transfer a GlcNAc residue to 6-OH of α1,6 Man and 4-OH of α1,3 Man of the core pentasaccharide to form tri- or tetra-antennary oligosaccharides. Moreover, GnT-III may add an antenna to 4-OH of β1,4 Man,[14] which is called bisecting GlcNAc. In birds and fishes, additional branches are found on 4-OH of α1,6 Man and some hydroxy groups of the core pentasaccharide.[15-16]

Repeating of *N*-acetyllactosamine (LacNAc) unit is observed on the non-reducing end of complex-type oligosaccharide (Figure 1-4b), and core fucosylation of 6-OH of GlcNAc-β-Asn is also a structural feature of complex-type oligosaccharides (Figure 1-4c).

In *trans*-Golgi, Gal residues on non-reducing end are capped with Neu5Ac via α2,3 or α2,6 sialyl linkages (Figure 1-4d),[17] and the resulting mature glycoproteins are secreted or transported to plasma membrane.

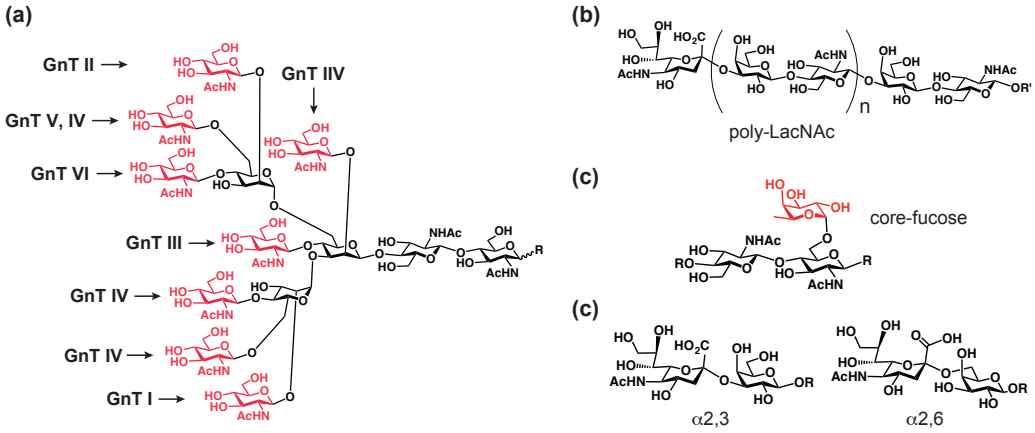

Figure 1-4. (a) Reported branched structures and related *N*-acetylglucosaminyltransferases (GnTs). (b) Structure of poly-*N*-acetyllactosamine (poly-LacNAc). (c) Structure of core-fucose linked to chitobiose. (d) Structures of two types of sialyl linkages.

1-4. Branching structures of complex-type oligosaccharide on specific proteins and cells

Frequently found on mature glycoproteins, a complex-type oligosaccharide exhibits unique antennary structures as described above, and the branching pattern is highly depends on glycoproteins and cell types.

Change of branching pattern is known to alter the physiological properties and biological activities of glycoproteins, as seen in the case of erythropoietin (EPO). EPO is a glycoprotein hormone regulating red blood cell production and has three complex-type oligosaccharides at 24^{th}, 38^{th}, and 83^{rd} Asn residues.[18-19] Contrary to other cytokines, majority of the EPO glycoforms have highly branched oligosaccharides, and the branching degree is thought to be crucial for the biological activity *in vivo*. Rahbek-Nielsen *et al.* reported the glycoprofile of urinary EPO by means of matrix assisted laser desorption/ionization (MALDI) mass spectrometry. Their analysis showed that ratio of biantennary, triantennary, and tetraantennary (including LacNAc repeating) was 8: 30: 62, indicating that 92% of the oligosaccharides are tri- or tetra-antennary forms (Figure 1-5).[20] In terms of another cytokine interferon-β1, which has one complex-type oligosaccharide and is secreted by fibroblast in response to viral infection, it is reported that 82% of oligosaccharide structure is biantennary forms and the other 18% is two types of triantennary structures.[21] In addition, human lactoferrin isolated from human milk has only biantennary structures, and more than 98% of oligosaccharides are biantennary forms in the case of natural serum transferrin.[22-23] These data excites our curiosity about branching difference observed on glycosylated proteins and their necessities/functions.

Regarding cancer cells, it is known that the number of branches increases depending on the tumor stage. Some groups reported that β1,6-*N*-acetyltranssferase V (GnT-V) is involved in angiogenesis and tumor metastasis.[24-25] Similarly, in the case of human chorionic gonadotropin (hCG), urinary hCG consists of solely biantennary structures with or without α2,3 sialyl linkages,[26] however, about half of oligosaccharides from hCG produced in choriocarcinoma are triantennary forms as previously reported by the Kobata group.[27]

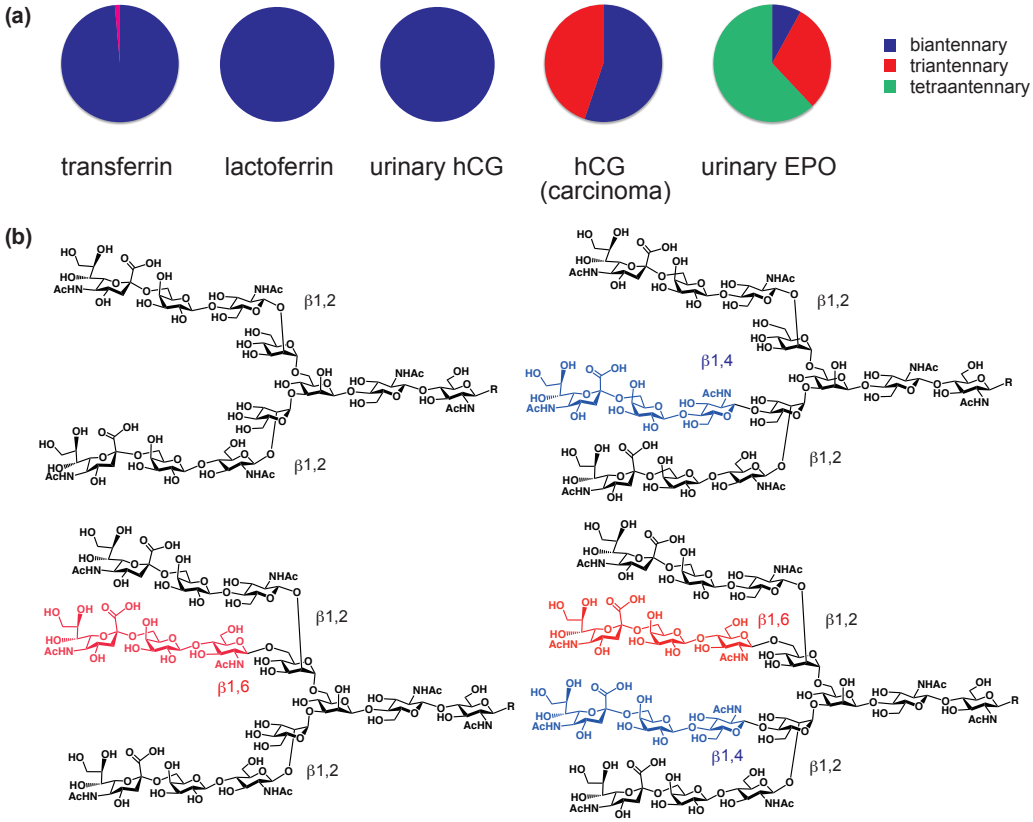

Figure 1-5. (a) Reported ratio of branched complex-type oligosaccharides of several secreted glycoproteins. (b) Typical branched structures of human complex-type oligosaccharides.

1-5. Isolation and structural analysis of oligosaccharides

Isolation and functional study of oligosaccharides has suffered from scarcity and considerable heterogeneity of oligosaccharides on naturally occurring glycoproteins. Oligosaccharide on the same position of a protein shows structural diversity, called glycoforms, because carbohydrate sequence is not genetically coded. Enzymatic glycosylation reactions in biosynthetic pathway are not 100%, thus resulting in truncation of oligosaccharides in non-reducing ends. These structural features are called microheterogeneity,[28-30] which makes separation of individual oligosaccharides less promising due to the physiological similarity. In addition to the heterogeneity, scarcity of natural glycoprotein causes a difficulty in isolating and structural determination of oligosaccharides. In the case of cytokines, which are clinically meaningful molecules, their abundance in plasma is less than 10^2 pg/mL.[31] Among the investigated proteins in plasma, serum albumin was measured to be 35~50 mg/mL at the high abundance end. In contrast, interleukin 6 was measured to be 0~5 pg/mL at the low abundance end.

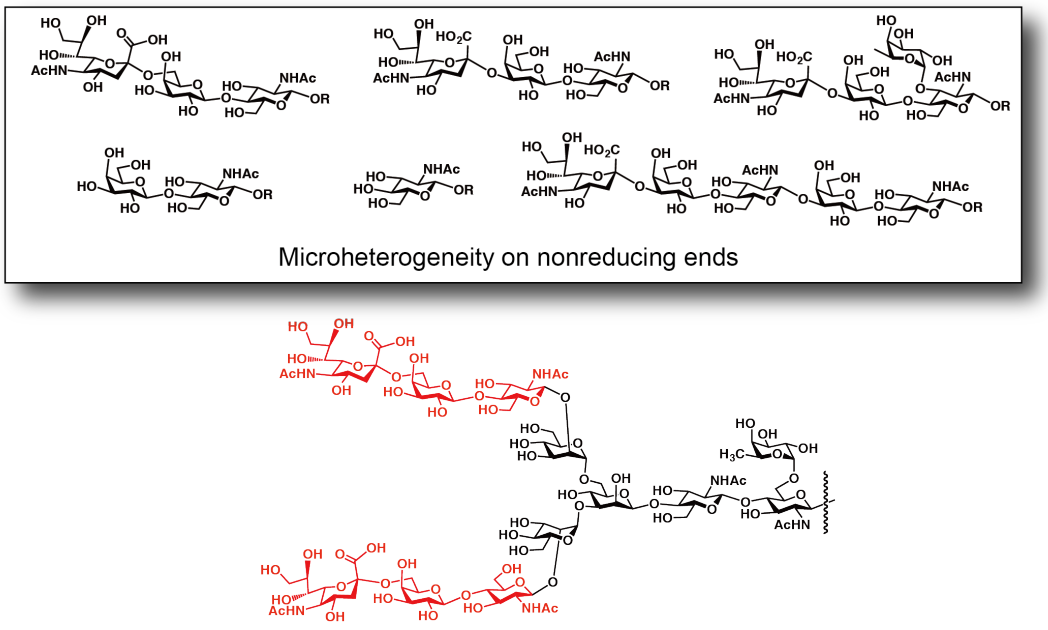

Figure 1-6. Frequently observed heterogeneity on nonreducing ends of oligosaccharides.

Hen egg yolk, however, contains sialylglycopeptide (SGP) including complex-type biantennary sialylundecasaccharide with relatively well-defined structure, and the Kajihara group established an isolation strategy of the oligosaccharide attached to Fmoc-Asn-OH in large scale (Figure 1-7a).[32] Seko *et al*. reported that one egg yolk contains 8 mg SGP (2.8 umol) and pure SGP can be isolated by phenol treatment of egg yolk along with some purification steps.[33] By using the SGP, the idea to introduce hydrophobic 9-fluorenylmethyloxycarbonyl (Fmoc) group on an asparagine residue enabled prompt preparation of a library of complex-type biantennary oligosaccharides in large scale. Thus, nowadays complex-type biantennary oligosaccharides are easily available for advancing glycoscience.[34-36]

In terms of highly branched oligosaccharides, one triantennary structure having a Gal-β-1,4-GlcNAc-β-1,4-Man antenna can be isolated from bovine fetuin in moderate scale as described by Meldal *et al.* as shown in Figure 1-7b.[37] The triantennary structure linked with Fmoc-Asn-O^{t}Bu (8 mg) was prepared from bovine fetuin or ribonuclease B (5 g) through hydrazinolysis reaction and HPLC chromatographic separation. In addition, they performed Fmoc SPPS using the acetylated triantennary oligosaccharyl asparagine as a building block. This isolating procedure yielded moderate quantities, however the hydrazinolysis reaction potentially has a risk because anhydrous hydrazine is highly toxic, flammable, and explosive.

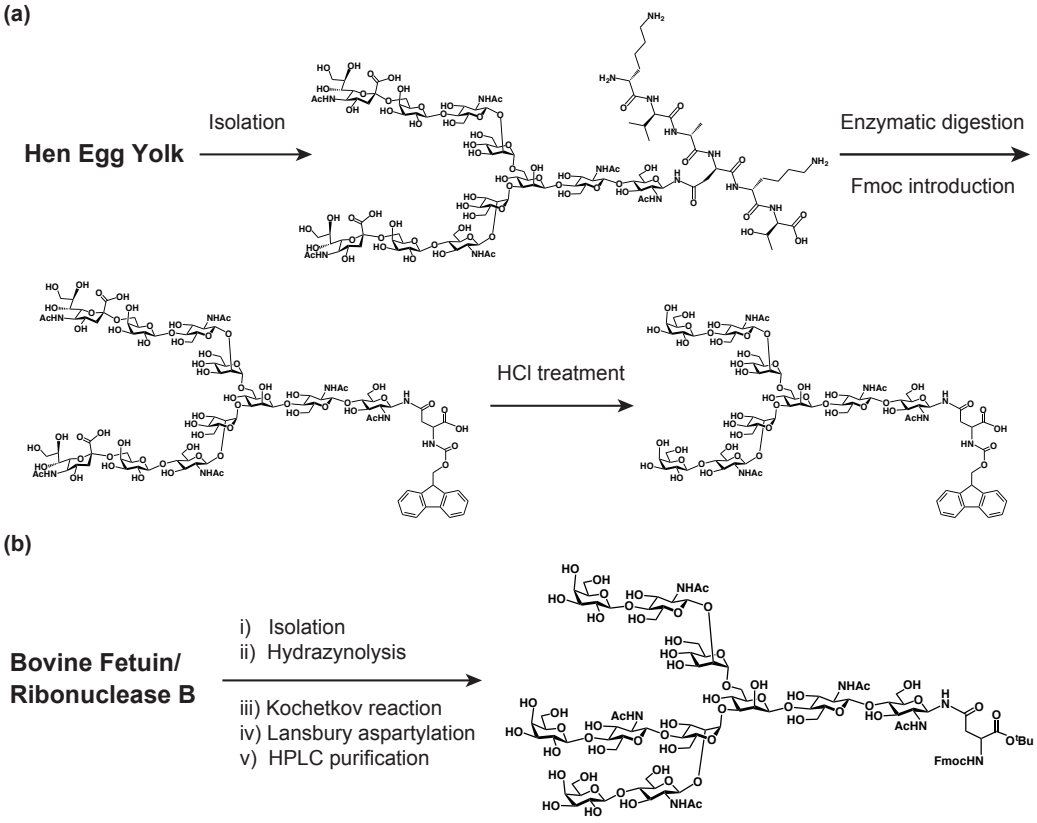

Figure 1-7. Outline of isolation steps of (a) biantennary oligosaccharides and (b) a triantennary oligosaccharide.

Structural determination of oligosaccharides is generally very difficult because regioselectivity and stereoselectivity make several possibilities of glycosyl linkages, and thus many methodologies have been developed to analyze oligosaccharide structures precisely and promptly. Oligosaccharides require to be cleaved from glycosylated proteins for structural determination. Glycopeptidase and peptide:*N*-glycanase (PNGase) specifically cleave GlcNAc-Asn linkages of *N*-linked oligosaccharides to afford free oligosaccharides.[38-41] Another method is the chemical hydrazinolysis reaction as mentioned above. Treatment of glycosylated peptides/proteins with anhydrous hydrazine cleaves amide bonds.[42] *N*-acetylation is usually conducted after hydrazinolysis because they are also cleaved during hydrazinolysis. After the isolation steps, oligosaccharides can be separated by high performance liquid chromatography (HPLC) including two-dimensional (2D) HPLC analysis. Introduction of 2-aminopyridine improves separation and detection as reported by Hase *et al*.[43] In this method, an aldehyde of GlcNAc on the reducing end makes Schiff base by the treatment with 2-aminopyridine, followed by $NaBH_3CN$ reduction to yield a pyridylaminated oligosaccharide, which can be separated by reverse-phased (RP), amino, or amide column chromatographies.

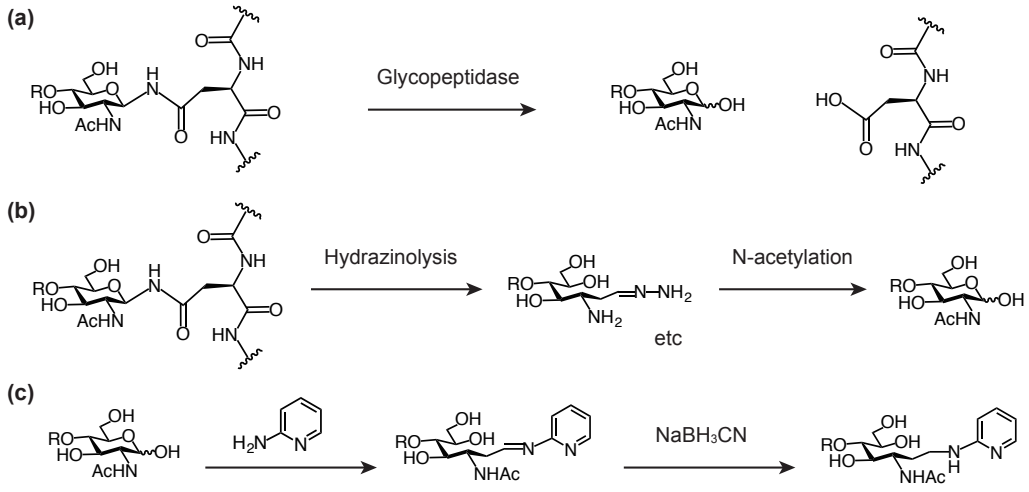

Figure 1-8. (a), (b) Cleavage of an oligosaccharide from peptide backbone by enzymatic and chemical reactions. (c) Introduction of 2-aminopyridine to a reducing sugar.

One of the major methods is methylation analysis of oligosaccharides, giving information of glycosylated positions of monosaccharides (Figure 1-9). Dimethylsulfoxide (DMSO) and sodium hydride (NaH) make methylsulfonylcarbanion, and this strong base catalyzes the alcoxide formation, followed by methylation with methyl iodide to give a permethylated oligosaccharide as reported by Hakomori.[44] Then, glycosyl linkages are hydrolyzed by the treatment with 0.5 N H_2SO_4 in 93% AcOH. The resulting partially methylated monosaccharides are reduced by $NaBH_4$ to produce methylated alditols, which are further acetylated and analyzed by a gas chromatograph-mass spectrometer to determine the glycosylation sites and monosaccharide composition.[26, 45]

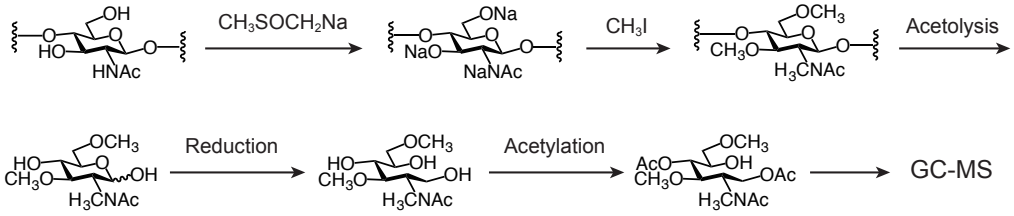

Figure 1-9. Scheme of methylation analysis.

Nuclear magnetic resonance (NMR) spectroscopy is the predominant method to understand the structure of oligosaccharides, and we are able to identify the chemical structure of an oligosaccharide including stereochemistry and the position of a glycosyl linkage. Proton NMR analysis gives us important information of molecules: i) chemical shifts (δ), ii) integration, and iii) coupling constants (*J*). Combination of carbon NMR and other mutidimensional measurement enables structural determination of even complicated molecules. In the case of carbohydrate, deprotected oligosaccharides usually show heavily overlapped ring proton signals in the region of 3-4 ppm in ^{1}H NMR, thus resulting in a difficulty in assigning chemical shifts. However, anomeric proton signals appear in 4.5-5.5 ppm region due to the distinct acetal structures. Similarly, in the case of *N*-linked oligosaccharides, the downfield shifted H-1 signals show distinctive chemical shifts and coupling pattern depending on monosaccharide structures. Furthermore, COSY (COrrelation SpectroscopY) and TOCSY (TOtal Correlation SpectroscopY) make use of the non-overlapped H-1 signals to determine other ring proton signals in same monosaccharide residue.[46] To improve unambiguous structural analysis of oligosaccharides, some NMR techniques have been developed as reported by Sato *et al*.[47-48] Contrary to X-ray crystallography, NMR data offer information about dynamics of molecules usually based on NOE (nuclear magnetic resonance) experiments.[49-51]

1-6. Synthesis of oligosaccharides

Chemical and enzymatic syntheses have greatly contributed to provide homogeneous oligosaccharides for understanding their functions at molecular level. Isolation of oligosaccharides mostly results in heterogeneous and scarce materials as described above, however, on the other hand, synthetic methodologies enables on demand preparation of not only natural but also unnatural oligosaccharides.

Structural feature of monosaccharides needs to be mentioned prior to the chemical reactions. Monosaccharides employing six-membered ring are known as aldoses (eg. D-glucose, D-galactose, and D-ribose) and ketoses (eg. D-fructose). Their structures are drawn as their Fischer projections in Figure 1-10a, and carbohydrates usually form cyclic hemiacetals and hemiketals by intramolecular nucleophilic attack of a hydroxy group to the carbonyl function of a monosaccharide, thus resulting in preferable 5- or 6-membered rings (furanoses and pyranoses). Each aldose and ketose has two enantiomers, and they are distinguished by the prefix D (*dexter* = right) and L (*laevus* = left), which is determined by the configuration of the highest-numbered chiral center of a monosaccharide (Figure 1-10b). For example, in the case of glyceraldehyde depicted in its Fischer projection, when the hydroxy group at the chiral center takes left position, the glyceraldehyde is called the L-enantiomer. In addition, hemiacetal ring formation produce a new chiral center, which is called the anomeric center. This new chiral center results in two diastereomers. The stereochemical relationship between an anomeric center and a configuration of most distant chiral center designates α- and β-anomers (Figure 1-10b). When the hydroxy group at anomer position is bound to the most distant chiral center in the same position (*cis*), it is named the α-anomer. On the other hand, it is called β-anomer when the anomeric hydroxy group is bound in opposite direction (*trans*).

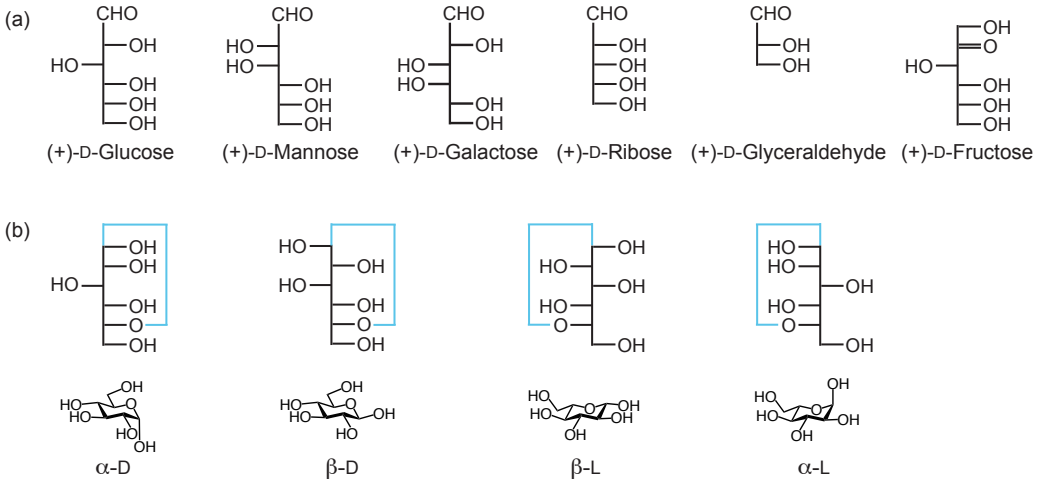

Figure 1-10. (a) Acyclic forms of aldoses and ketoses drawn in the Fischer projections. (b) Cyclic forms of glucopyranoses drawn in Fischer projections and chair conformations.

1-6-1. Chemical glycosylation

A suitably protected monosaccharide (glycosyl acceptor) is coupled with a fully protected monosaccharide equipped with a leaving group (glycosyl donor) at its anomeric center to make an oligosaccharide (Figure 1-11a).[52-55] A monosaccharide has several hydroxy groups, and furthermore, coupling of two monosaccharides (glycosylation) yields potentially two anomers. Thus, regio- and stereoselective reaction is essential to make a desired oligosaccharide. To gain the regioselectivity, hydroxy groups of glycosyl acceptor requires protecting group except for a hydroxy group that is used to make a new glycosyl linkage. For this purpose, enormous protecting groups have been developed such as acyl protecting groups (acetyl, benzoyl, levulinoyl), ether protecting groups (benzyl, allyl, silyl), and acetal protecting groups (benzylidene, isopropylidene). On the other hand, a glycosyl donor needs a leaving group at its anomeric position in addition to protecting groups for other hydroxy groups. Nucleophilic attack of a hydroxy group of glycosyl acceptor to the anomeric center of glycosyl donor *via* S_N2 pathway or to the oxocarbenium cation formed from the donor *via* S_N1 type reaction allow a transfer of glycosyl moiety of donor to glycosyl acceptor (Figure 1-11a).[56-57]

The first glycosylation reaction was reported by Fischer in 1893, and then many glycosylation methods have been developed such as Koenigs-Knorr, glycosyl imidate,

thioglycoside, and phosphate methods. The Fischer glycosylation involves protonation of the hydroxy group at anomeric position (hemiacetal), and following formation of acetal with alcohol *via* oxocarbenium ion intermediate gives a glycoside.[58] However, excess of alcohol is required to shift the equilibrium toward the product. In 1901, Wilhelm Koenigs and Eduard Knorr developed a 1,2-trans glycosylation, which is called Koenigs-Knorr reaction and a widely used classic reaction.[59] This reaction uses glycosyl bromides and glycosyl chlorides as donors and silver salts such as Ag_2O, silver triflate (AgOTf), and $AgClO_4$ as promoters. Glycosyl trichloroacetimidate is one of the most popular glycosyl donor because of its easy preparation by trichloroacetonitrile-base (K_2CO_3, NaH, or DBU) along with its high reactivity.[60-61] Lewis acid such as $BF_3 \cdot Et_2O$ and trimethylsilyl trifluoromethanesulfonate (TMSOTf) catalyze the activation of the imidate donors. In addition, *N*-phenyltrifluoroacetimidate is also known as an effective donor.[62] Thioglycosides are quite often used as glycosyl donors, which are commonly prepared by fully acetylated monosaccharides and thiols such as thiophenol and alkylthiol in the presence of Lewis acid.[63] Moreover, thioglycosides also work as protecting groups in the absence of thiophilic promoters even under basic or acidic conditions and can be converted into other glycosyl donors in one or two steps. In contrast to imidate donors, thioglycoside is activated by at least an equimolar amount of thiophilic promoters such as *N*-iodosuccinimide (NIS)/TfOH, MeOTf, and dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST).

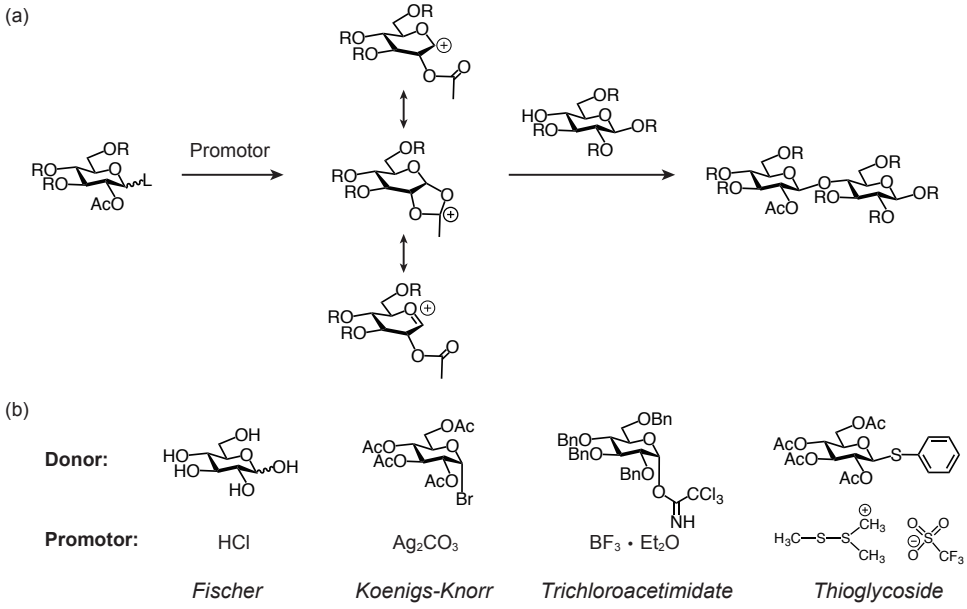

Figure 1-11. (a) A general glycosylation mechanism with a participating group at 2-position. (b) Examples of donors and their promoters.

α-sialylation, β-mannosylation, and *N*-glycosylation are known as challenging glycosylations due to their low stereoselectivity and low yield. With respect to sialylation reactions, neighboring participating group, which is most common way to construct 1,2-trans-glycosides, is not available because C-3 has no hydroxy group in sialic acid.[64] Combination of the deoxy moiety and C-1 carboxylic acid often results in elimination to give a glycal, and the tertiary oxocarbenium ion at the sterically hindered position also decrease the reactivity and yield. However, not only high yield but also highly stereoselective sialylation have been investigated such as solvent (acetonitrile) participation (Figure 1-12a),[65-67] 3-thio or -seleno modification (Figure 1-12b),[68-69] and C-5 modifications.[70] β-mannosylation is also known as one of the difficult glycosylation reactions because anomeric effect favorably produces α-mannosides. The Hindsgaul, Stork, and Ogawa groups independently developed intermolecular aglycon delivery (IAD) reactions (Figure 1-12c).[71-73] In 1996, Crich *et al.* reported the direct β-mannosylation using 4,6-*O*-benzylidene system through glycosyl triflate intermediate (Figure 1-12d).[74-76]

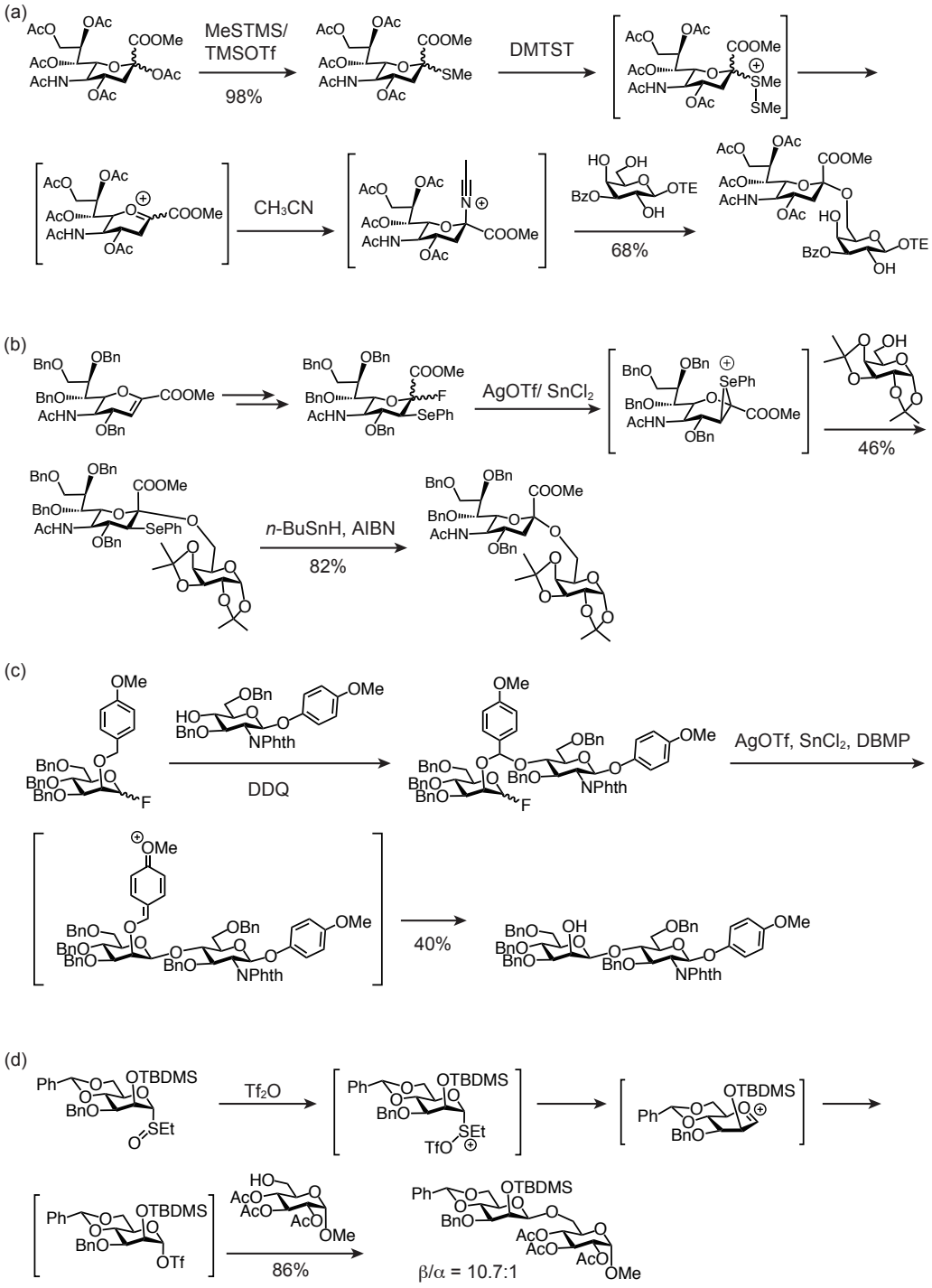

Figure 1-12. (a) Sialylation with acetonitrile participation by the Kiso-Hasegawa group.[66-67, 77-78] (b) Sialylation with 3-seleno (or -thio) auxiliary by Ito and Ogawa.[68, 79-80] (c) IAD β-mannosylation by Ito and Ogawa.[73] (d) 4,6-*O*-benzylidene assisted β-mannosylation by the Crich group.[74] TE: 2-(trimethylsilyl)ethyl, DDQ: 2,3-dichloro-5,6-dicyanobenzoquinone, AIBN:

2,2'-azobis(isobutyronitrile), DBMP: 2,6-di-*tert*-butyl-4-methylpyridine, TBDMS: *tert*-butyldimethylsilyl.

1-6-2. Enzymatic glycosylation

Another way to make a glycosidic bond is to use an enzyme, glycosyltransferase, which catalyzes a transfer of a monosaccharide from a sugar-nucleotide to a specifically recognized acceptor. On the contrary to the difficulties of regioselectivity and stereoselectivity in chemical glycosylation reactions, glycosyltransferases can transfer a sugar from sugar-nucleotides to unprotected sugar substrates in buffer, and they have been used for a variety of oligosaccharide syntheses.[81-85] A lot of efforts have been dedicated to improve availability of sugar-nucleotides and glycosyltransferases, and expand substrate specificity.

1-6-3. Synthesis of asparagine linked oligosaccharides

In terms of complex-type biantennary oligosaccharides, the Ogawa group reported the first total chemical synthesis of an α2,6-sialylundecasaccharide in 1986 (Figure 1-13a).[86] Then, the Ito group accomplished total chemical synthesis of an α2,3-sialylated complex-type undecasaccharide in 2000 by a similar convergent strategy where αMan-2,3 and αMan-2,6 glycosides were constructed in the latest step.[87] Moreover, the Danishefsky group made a biantennary sialyloligosaccharide having core-fucose and bisecting-GlcNAc (Figure 1-13b).[88-89]

Syntheses of complex-type tri- and tetra-antennary oligosaccharides have been reported by some groups such as the Danishefsky, Wong, Boons, and Unverzagt groups. Though synthesized oligosaccharides lack Neu5Ac-Gal at the non-reducing ends, the Unverzagt group established a synthetic strategy toward multiantennary oligosaccharides including pentaantennary strucutres.[90-91] In 2012 and 2013, the Danishefsky group reported the syntheses of two types of triantennary sialyloligosaccharides (Figure 1-14a).[92-93] In addition to the fully chemical strategy, Wong *et al.* synthesized a library of bi- to tetra-antennary α2,3- or α2,6-sialylated oligosaccharide library in combination with chemical synthesis and enzymatic sialylation reactions.[94-95] Moreover, the Boons group reported the chemoenzymatic synthesis of asymmetrically branched complex-type oligosaccharide library (Figure 1-14b).[96]

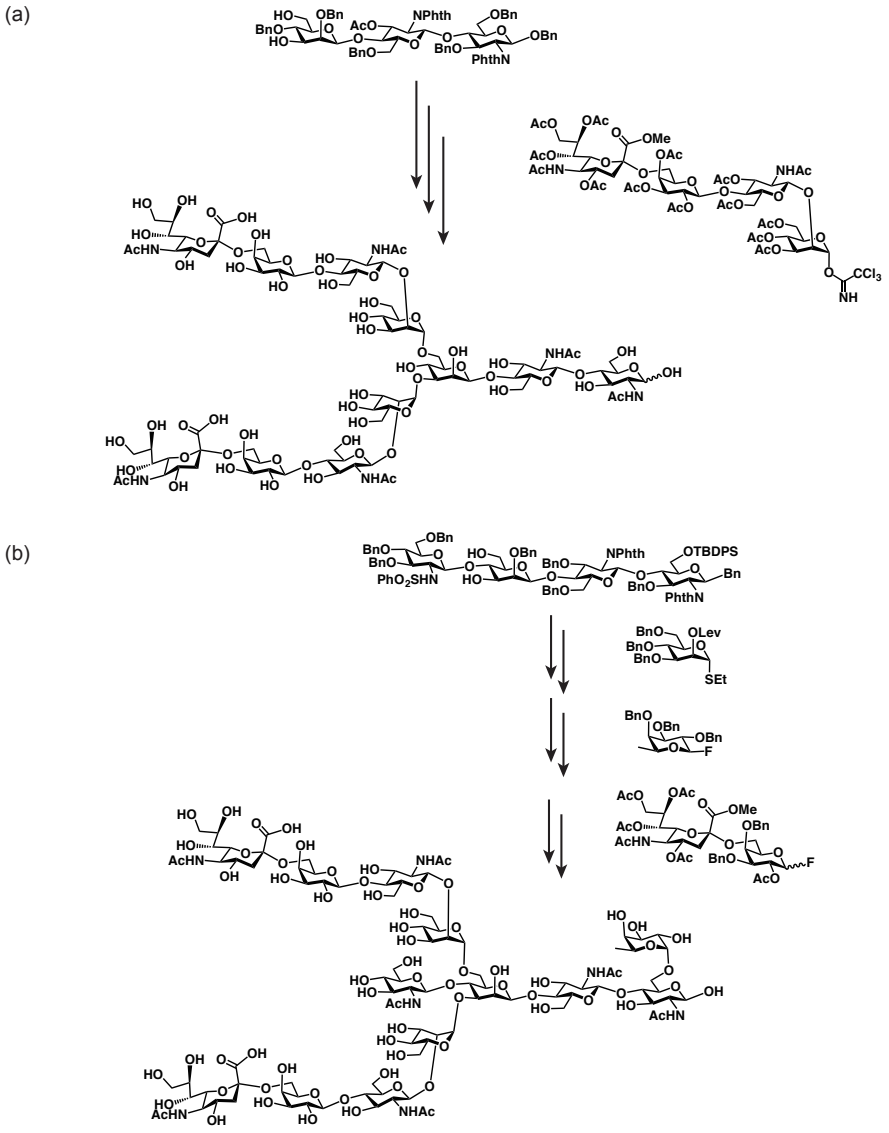

Figure 1-13. (a) Synthesis of biantennary sialylundecasaccharide by the Ogawa group.[86] (b) Synthesis of biantennary oligosaccharide having core-fucose and bisecting-GlcNAc by the Danishefsky group.[89]

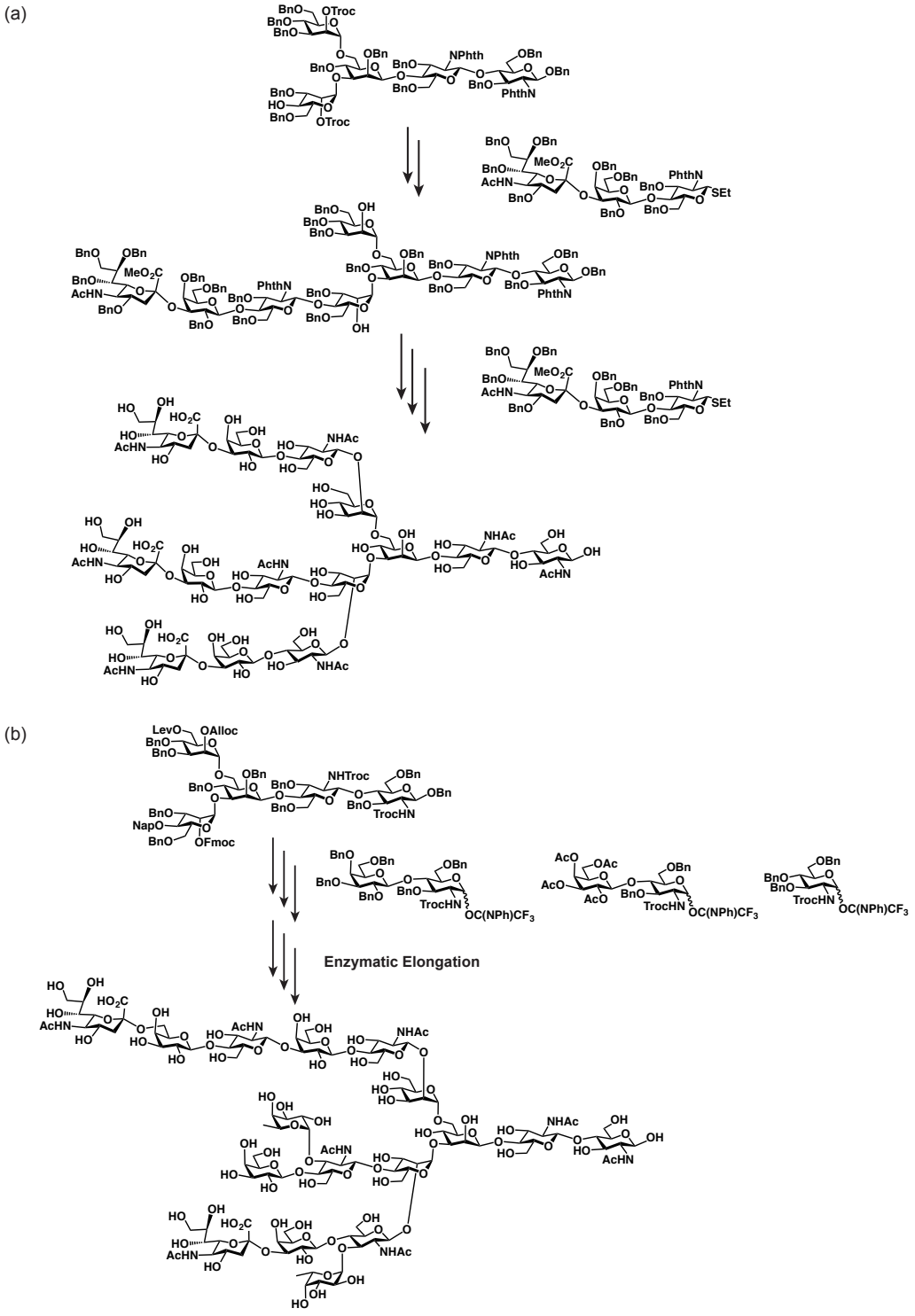

Figure 1-14. (a) Synthesis of a complex-type triantennary sialyloligosaccharide by the Danishefsky group.[92] (b) One example of chemoenzymatically synthesized asymmetry oligosaccharides by the Boons group.[96]

1-7. Problems in oligosaccharide synthesis

The chemical synthesis of an oligosaccharide requires an efficient synthetic strategy, as it is unfortunately a time-consuming protocol because of the need for repetitive protection/deprotection of hydroxy groups as well as the stepwise construction of stereoselective glycosidic linkages. After each synthetic step, recrystallization or silica gel purification steps are usually indispensable to obtain a pure material for the next reaction. Even the synthesis of biantennary oligosaccharides has not been accomplished within 20 steps, and the Danishefsky group mentioned that total synthesis of triantennary oligosaccharide was reached in total 71 steps from commercially available materials.[92] These long synthetic steps in addition to the necessity of sophisticated synthetic skills hamper the easy and prompt oligosaccharide synthesis, thus resulting in slow advance in glycoscience.

1-8. Glycoprotein and glycopeptide

In general, *N*-linked oligosaccharides including complex-type oligosaccharide are naturally attached to protein or peptide, and thus research of not only oligosaccharides but also glycopeptides/glycoproteins is essential to investigating the functions of oligosaccharides. In the case of human urinary EPO, which has three complex-type oligosaccharides at 24th, 38th, and 83rd Asn, ratio of bi-, tri-, and tetra-antennary structures is apparently distinct (Figure 1-15).[20] Oligosaccharides at 24th Asn consists of three types of branching almost equally, however, 38th and 83rd Asn have more than 70% tetraantennary forms in addition to ca. 30% triantennary structures. In the case of mucin type *O*-glycosylation, *in vitro* experiments show that glycosylation and its pattern depended on amino acid sequence including hydrophobicity and hydrophilicity of the peptide.[97] These data suggest that glycosylation is strongly related with peptide/protein scaffold properties.

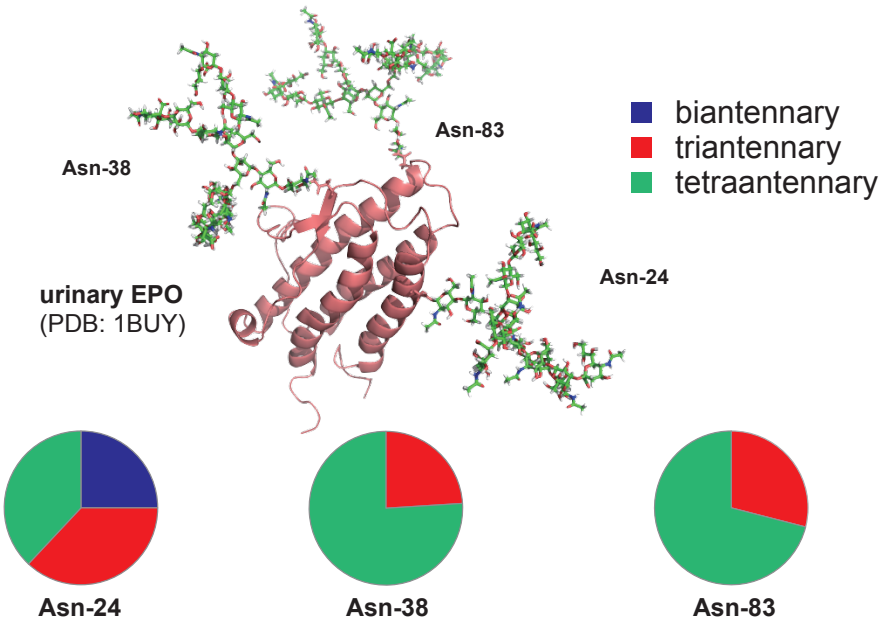

Figure 1-15. Structure of erythropoietin (EPO) and its oligosaccharide ratio at three different positions.[20]

One of the major methods to study interactions between enzymes and oligosaccharides is microarray employing a library of oligosaccharides fixed on a surface of an array, and in the same manner a glycopeptide library offers information of the relationship between glycan part and peptide part toward enzymes (Figure 1-16). In a glycan microarray, each oligosaccharide is immobilized on a surface of an array to make a library of carbohydrates, which are used to investigate the interaction with enzymes/lectins.[98-100]

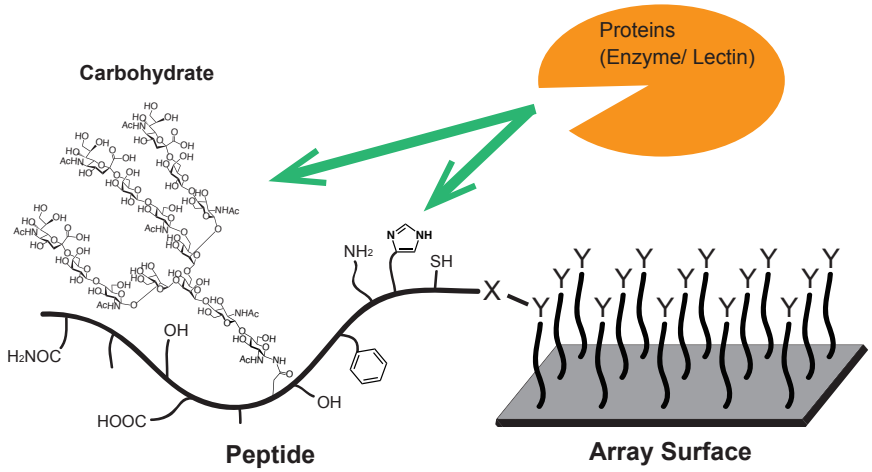

Figure 1-16. Concept of glycopeptide microarray to investigate the interaction with proteins such as enzymes and lectins.

In the case of glycoproteins such as cytokines, the oligosaccharide part often changes the physical properties and biological activities of proteins, therefore research on glycoproteins with homogeneous oligosaccharides can provide an insight into oligosaccharide functions. Okamoto *et al.* reported that glycosylation of CCL1, a chemokine protein secreted by activated T-cells, negatively changed chemotaxis properties,[101] however, on the other hand, Murakami *et al.* revealed that biological activity of EPO increases depending on the positions and the number of sialyloligosaccharides.[102] Thus, the investigation of homogeneous oligosaccharides with protein parts is critical to study function and necessities of oligosaccharides.

1-9. Synthesis of glycoprotein

While expression of glycoproteins by biological technologies suffers from heterogeneity of oligosaccharide structures, chemical synthesis offers glycoproteins with homogeneous oligosaccharide structures. The chemical synthesis of (glyco-)proteins consist of solid-phase peptide synthesis (SPPS), chemical ligation of peptides, and folding experiment steps as shown in Figure 1-17.

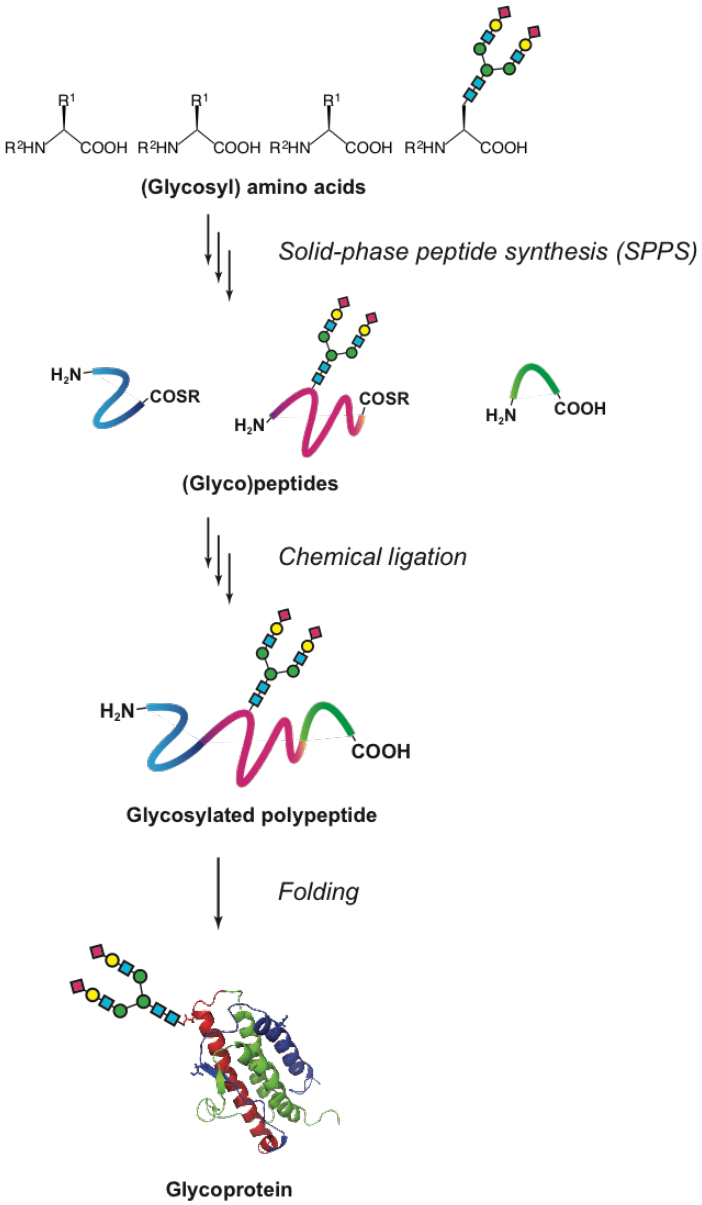

Figure 1-17. Outline of chemical glycoprotein synthesis

1-9-1. Solid-phase peptide synthesis (SPPS)

Peptide synthesis is usually enabled by two types of SPPS: *tert*-butyloxycarbonyl (Boc) SPPS and 9-fluorenylmethyloxycarbonyl (Fmoc) SPPS. In 1963, Merrifield reported solid phase peptide synthesis of tetrapeptide,[103] and he won the Novel Prize in Chemistry in 1984. Nowadays, Fmoc and Boc SPPS greatly contribute to peptide synthesis.[104] In Fmoc SPPS, the Fmoc group covers α-amino group, and *tert*-butyl and trityl groups are commonly used for side chain protecting groups.[105-107] During peptide elongation steps, Fmoc groups are removed by piperidine, while protecting groups for side chains are stable. On the completion of the coupling steps, treatment with TFA deprotects protecting groups on side chains. In the case of Boc SPPS, α-amino group is protected with *tert*-butyloxycarbonyl (Boc) group, and side chains are usually protected with benzyl groups.[108] Trifluoroacetic acid (TFA) removes Boc group, and HF or trifluoromethanesulfonic acid (TfOH) deprotects protecting groups on side chains. Synthesis of peptides sometimes encounters a difficulty in elongating peptides, however, in such cases, introduction of pseudoprolines and isoacyl dipeptides are known to improve the purity of the peptides.[109-110] Furthermore, microwave-assisted SPPS and flow SPPS have been developed to perform efficient and rapid SPPS.[111-112]

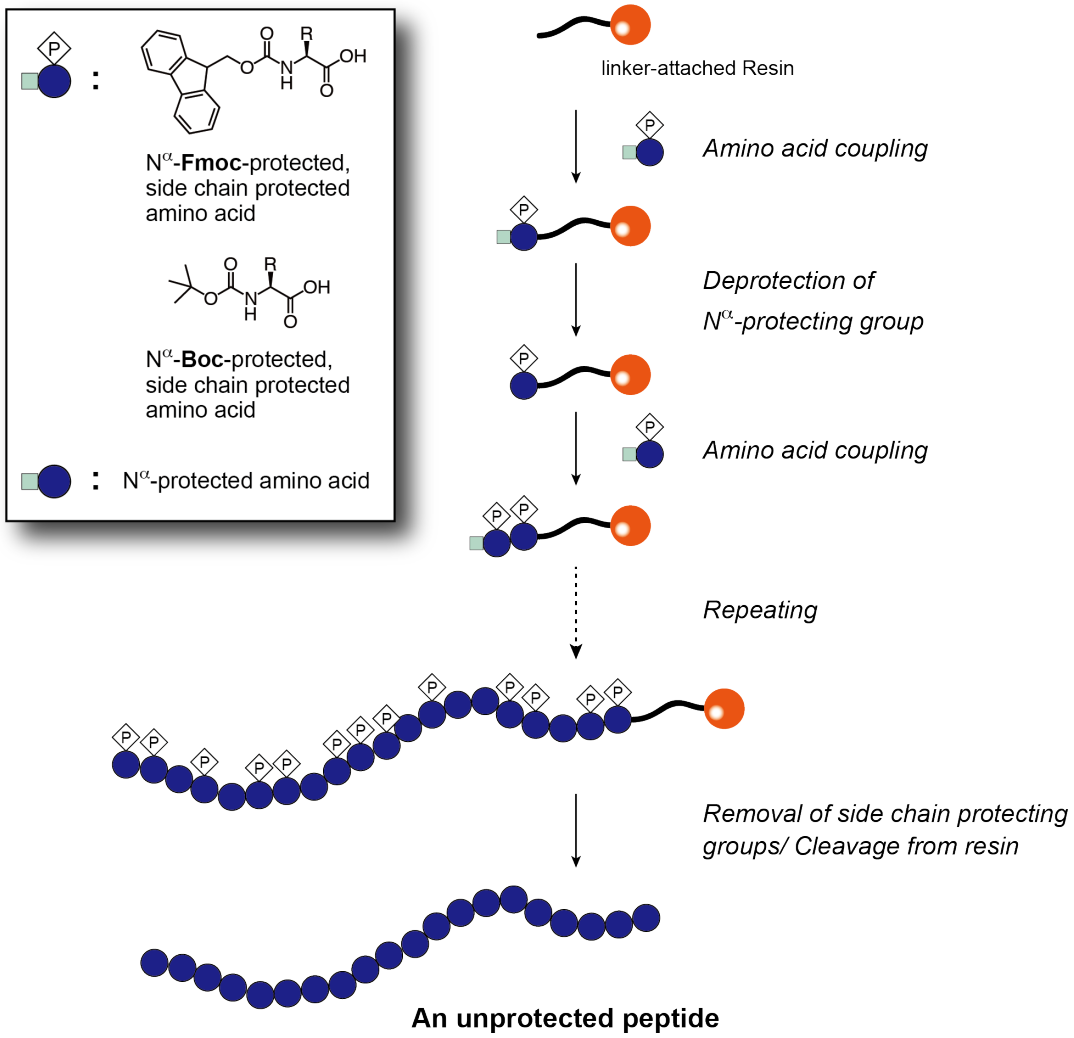

Figure 1-18. Outline of solid-phase peptide synthesis (SPPS). More details are mentioned in *Chapter 4 and 5*.

1-9-2. Chemical ligation of peptides

Once peptides are prepared by solid-phase peptide synthesis or biological expression, coupling of peptides to elongate polypeptides can be carried out by well-established peptide ligation chemistry: native chemical ligation (NCL). Dawson *et al.* developed native chemical ligation (NCL), which chemically ligate unprotected peptide-thioester and Cys-peptide to give a native peptide ligation product (Figure 1-19a).[113] This reaction works cleanly in buffer solution. The first step is thioester exchange reaction. A peptide thioester, which can be easily prepared by Boc SPPS,[114-115] reacts with a thiol additive (4-mercaptophenylacetic acid: MPAA) reversibly. Then, the thiol of Cys-peptide attacks the thiocarbonyl carbon of the formed peptide thioester to make a new thioester product, followed by S-to-N acyl shift by the nucleophilic attack of α-amine group to make a polypeptide with native amino acid sequence.[116] This versatile reaction has enabled chemical syntheses of proteins.[117-118]

While the NCL reaction couples peptides from C-to-N direction, kinetically controlled ligation (KCL) and hydrazide ligation enable peptide ligation from N-to-C direction. NCL reaction has limitation of i) requirement of Cys residues at ligation sites, and ii) sequential ligation in C-to-N direction. To expand the ligation sites, desulfurization chemistry in addition to syntheses of some β-mercapto amino acid derivatives have been developed.[119-125] Furthermore, to expand feasibility to ligate peptides in N-to-C directions, KCL reaction, *N*-acetylguanidine chemistry, and hydrazide ligation were established (Figure 1-19b).[126-129]

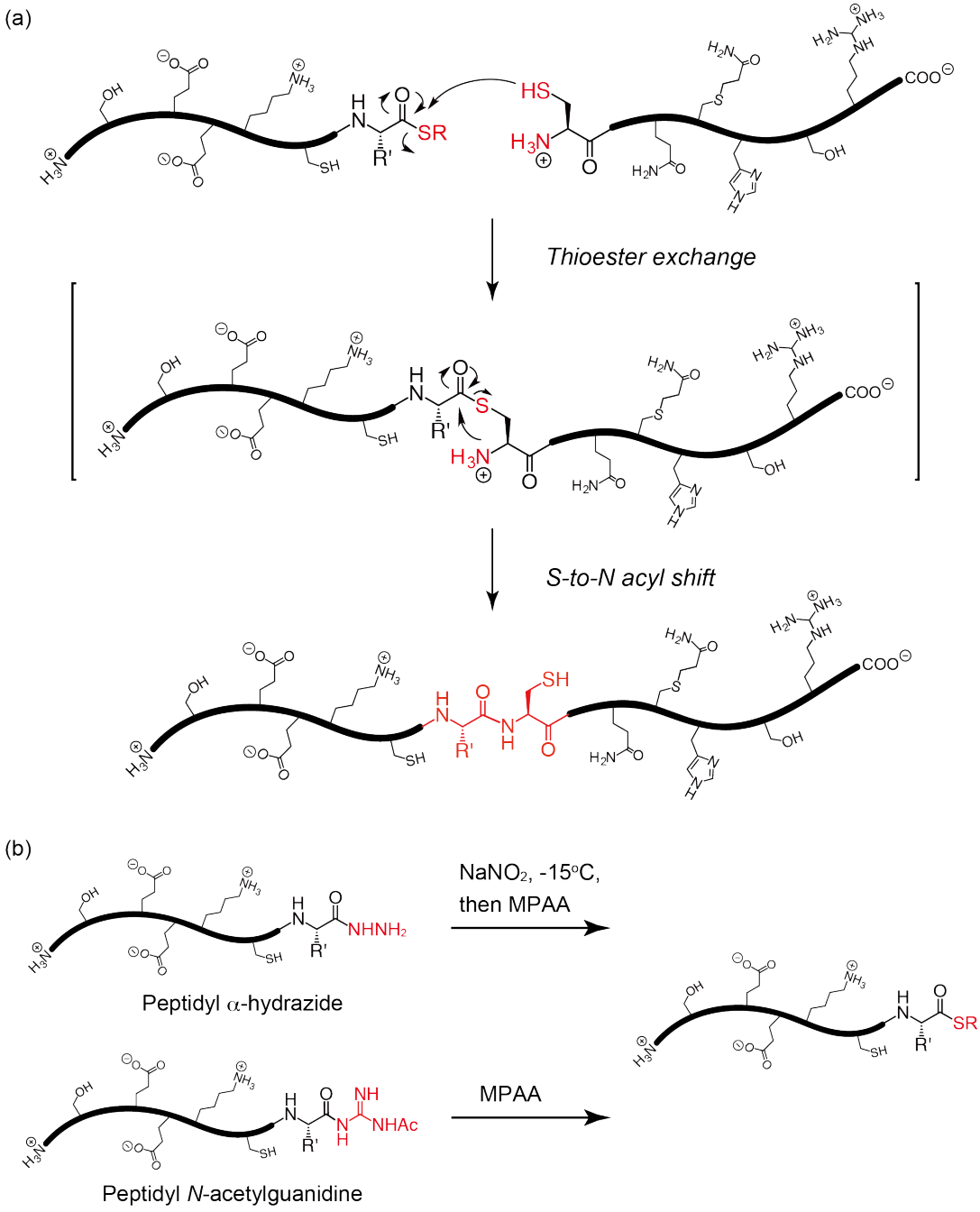

Figure 1-19. (a) Reaction mechanism of NCL. (b) Conversion of peptide-α-hydrazide and peptidyl *N*-acetylguanidine into peptide-α-thioester. These surrogates are inactive during usual NCL condition, thus allowing peptide ligation in the N-to-C direction.

1-9-3. Glycopeptide synthesis

The Kajihara group isolated an oligosaccharyl asparagine from hen egg yolk and examined coupling it to a peptide on resin by use of standard Boc or Fmoc SPPS to give a desired glycopeptide.[32, 130] Coupling of Fmoc-Asn(Oligosaccharide)-OH sometimes causes undesired aspartimide formation, and DEPBT was found to be a suitable coupling reagent in addition to PyBOP.[131-133] Unprotected hydroxy groups of oligosaccharides have a risk of undesired esterification during SPPS, however peptide elongation without any protecting groups found to be possible under diluted coupling conditions.[131] In terms of acid labile sialyl-oligosaccharide, they demonstrated that phenacyl ester renders sialyl linkage highly stable even under a TFA-TfOH deprotection condition in Boc SPPS.[132] Thus, sialic acid, which is highly important for biological activity of glycoproteins, can be easily introduced on glycoproteins (Figure 1-20).

In other way, a glycosyl amine can react with an activated ester of an aspartic acid residue in a peptide to make a native glycopeptide as reported by Lansbury.[134-135] β-glycosyl amine is usually prepared by reduction of the corresponding glycosyl azide or Kochetkov reaction, where treatment of a reducing oligosaccharide with saturated aqueous ammonium bicarbonate yields the glycosyl amine.[136] The resulting amine is then coupled with free Asp-containing protected peptide to yield a desired glycopeptide. But, this aspartylation reaction shows aspartimide formation.

While Lansbury aspartylation method shows troublesome aspartimide formation as a side product, the Unverzagt and Danishefsky groups developed convergent synthesis of *N*-glycopeptides facilitated by pseudoprolines at consensus sequence Ser/Thr residues. They found that a pseudoproline (ψPro) at Ser/Thr residue in the consensus sequence suppresses the formation of aspartimide (Figure 1-21a).[137-138] Their isolated yields were in the range of 14 – 32% with respect to oligosaccharylamine.

In addition to chemical synthesis of glycopeptide, an endoglycosidase has ability to catalyze a transfer of an oligosaccharide to a *N*-acetylglucosaminyl asparagine unit. Yamamoto *et al.* found that endo-β-*N*-acetylglucosaminidase (*Mucor hiemalis*) has transglycosylation activity,[139] and the Inazu group synthesized a glycopeptide using the enzyme and complex-type disialyloligosaccharide.[140] Nowadays, several types of enzymes were found, and use of glycosyl

oxazolines as donors improved efficiency of glycosylation reactions, which enables syntheses of glyco-peptides/proteins.[141-145] However, these reactions require excess of oligosaccharides with respect to peptides/proteins,[145] and, in addition, they have a potential risk of undesired glycosylation reactions.[146-147]

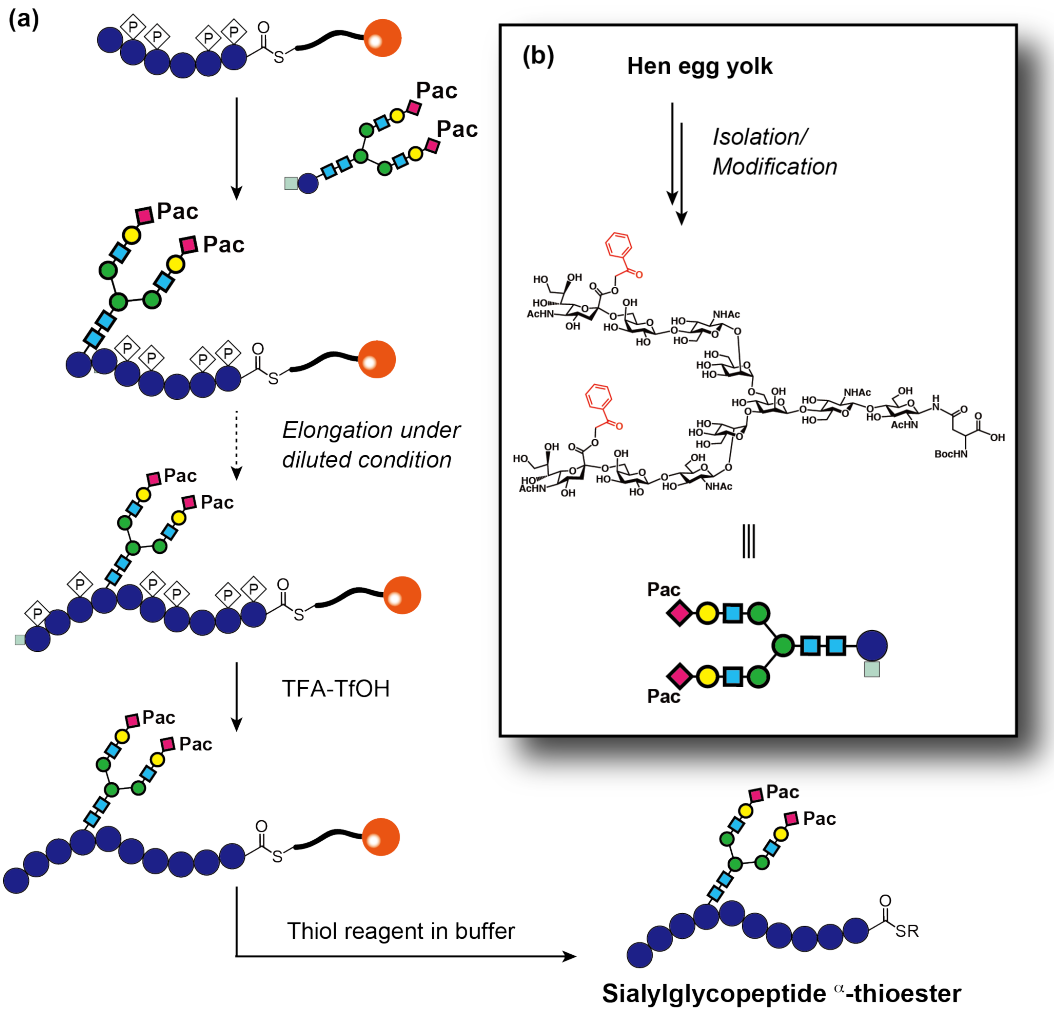

Figure 1-20. (a) Scheme for preparation of sialylglycopeptide thioester. (b) Phenacyl ester protection of sialic acid.

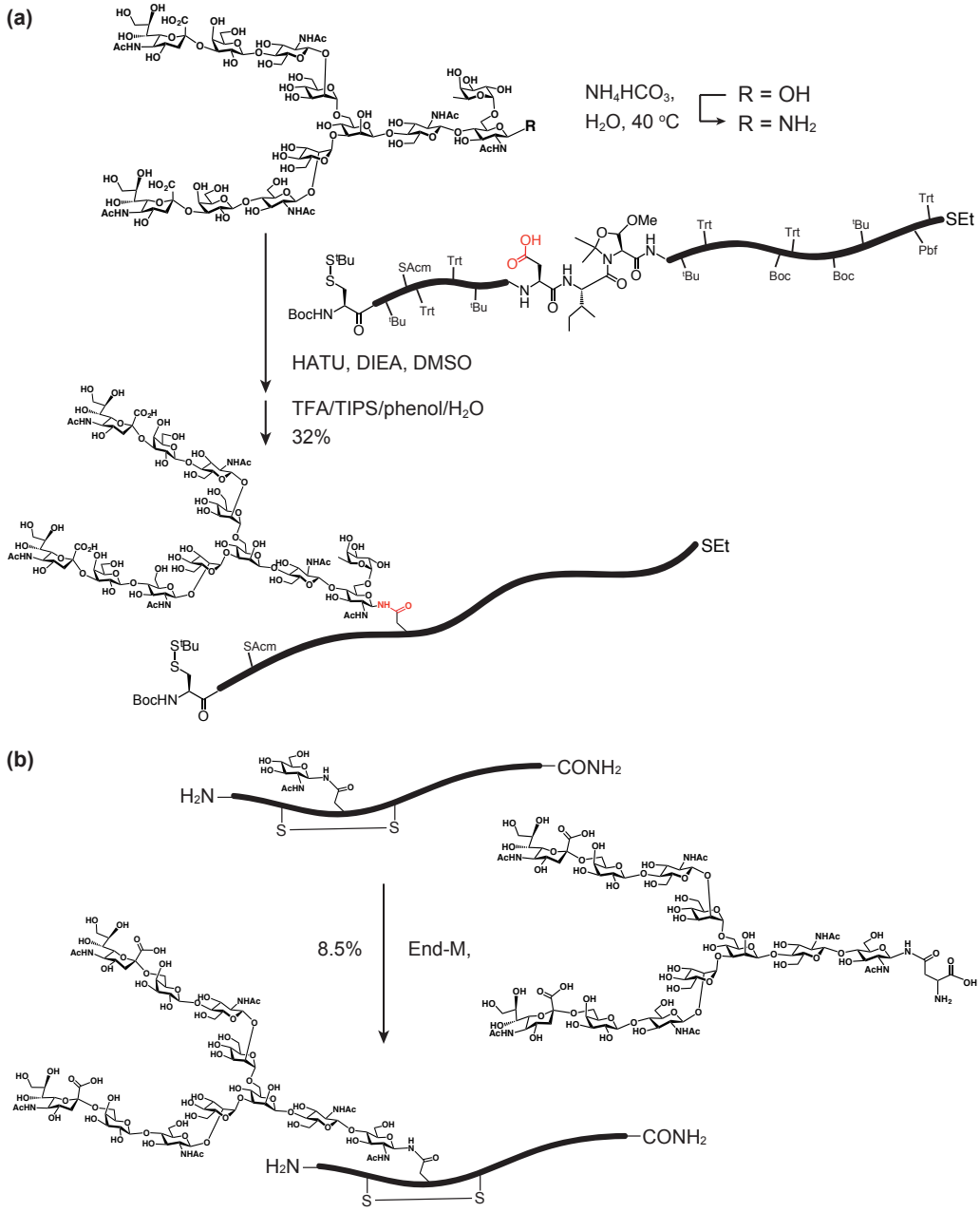

Figure 1-21. (a) Convergent aspartylation reaction enabled by the pseudoproline motif at the (n+2) position by the Danishefsky group.[138] (b) Transglycosylation using endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (End-M) by the Inazu group.[140]

1-9-4. Problems in glycopeptide synthesis

Considering glycopeptide synthesis using a glycosyl asparagine, isolated yield is generally moderate because of crude glycopeptide purity, amino acid couplings in diluted condition, and the efficiency of cleaving a glycopeptide from the resin. In general, isolated yield of glycopeptides is around 5-10% based on the oligosaccharyl asparagine in Boc SPPS, where 1.5-2.0 equiv of the oligosaccharyl asparagine residues was used.[102, 148] Low crude purity decreases the isolated yield, and the length of glycopeptide is limited even though suitable ligation sites are needed for NCL. In terms of preparation of glycopeptide-α-thioester, glycopeptides are cleaved from the resin by thioester exchange using sodium 2-mercaptoethanesulfonate or hydrazine monohydrate. The cleavage efficiency depends on peptide sequence, thus affecting the yield. Moreover, coupling of an oligosaccharyl asparagine and a peptide on resin is performed in heterogeneous mixture, and generally slight excess of oligosaccharyl asparagine is required.

1-10. An objective of this doctoral thesis

My doctoral thesis research focused on i) establishment of an unprecedented strategy to synthesize complex-type triantennary oligosaccharides and ii) development of a new strategy for synthesis of a glycopeptide having a synthesized triantennary oligosaccharide toward the synthesis of a glycoprotein. A detail including novelty and difficulty is mentioned as blow.

In order to elucidate the effect of branching degree of oligosaccharides on proteins, chemical synthesis can provide homogenous oligosaccharides and glycoproteins; above all however, synthesis of highly branched *N*-glycans is a very difficult task as previously described. Chemical synthesis of one triantennary oligosaccharide required 71 synthetic steps and amount of the final compound is a tiny amount, [92] which is a typical problem in general oligosaccharide synthesis. The repetitive chemical reactions and purification steps disturb the large amount preparation. In order to advance not only glycochemistry but also glycobiology, a new strategy for the synthesis of branched oligosaccharides is demanded and crucial.

Under these circumstances, I developed a semisynthetic strategy using an asialononasaccharide for the synthesis of two types of complex-type triantennary oligosaccharides (Figure 1-22). The starting material, asialononasaccharide, can be easily prepared from a natural source in over gram scale, [32] thus allowing chemical conversion of the biantennary asialononasaccharide into triantennary oligosaccharides through selective protections and additional glycosylation with a LacNAc disaccharide unit. This new synthetic concept could considerably reduce the number of synthetic steps in comparison to traditional oligosaccharide syntheses using sequential and repetitive glycosylations of monosaccharides.

The biantennary oligosaccharide has 24 hydroxy groups in total, and consequently a regioselective protection/deprotection strategy is newly essential. In general, protecting groups can be introduced to mono- or di-saccharides to obtain pure and desired product under easy control. A large number of hydroxy groups in the biantennary oligosaccharide may cause undesired and unexpected side reactions, and structural similarities between the desired and undesired products makes purification steps extremely troublesome. Therefore, I needed to establish a novel protection protocols toward the biantennary asialononasaccharide to overcome the regioselectivity among 24 hydroxy groups.

The synthesis of natural products and drug compound have sometimes employed the concept of semisynthesis, [149-150] however, large molecules such as oligosaccharides having many hydroxy groups as well as other functional groups have never been used as substrates in semisynthesis.[151-155] With respect to sugar derivatives, protection of mono- and di-saccharide units has been performed for semisynthetic purpose. But, highly complicated molecules such as *N*-linked oligosaccharides have never used in semisynthesis.

In addition to synthetic difficulties, unambiguous NMR assignment was required to determine the chemical structures of the intermediates. Not only 1D NMR but also rigorous 2D NMR experiments overcame the complicated molecular structures and sustained the successful synthesis with uncovering protected, deprotected, and glycosylated positions.

During the course of semisynthesis of triantennary oligosaccharides, I found an unprecedented selectivity in benzylidene acetal deprotection between Man and Gal residues. This new finding enabled me to develop the new semisynthetic method for the triantennary oligosaccharides. I would also like to discuss some mechanistic studies of the selectivity with thermodynamic parameters.

Since a new efficient glycopeptide synthesis was necessary to synthesize a glycoprotein bearing the triantennary oligosaccharide, I also established a new strategy for the efficient chemical synthesis of glycopeptides by using segment coupling reactions in liquid phase rather than standard solid phase glycopeptide synthesis. The synthesized triantennary oligosaccharide is very precious, and typical glycopeptide synthesis employs an excess of oligosaccharide for SPPS. I examined the segment coupling reactions with protected peptides in liquid phase to yield sufficient amount of glycopeptide toward chemical synthesis of EPO.

In order to evaluate the effect of branching structures on proteins, chemical synthesis of EPO having a triantennary oligosaccharide and two biantennary oligosaccharides was examined. I established a new synthetic strategy of EPO to convert a glycopeptide having a triantennary oligosaccharide into a full length of glycosylated EPO.

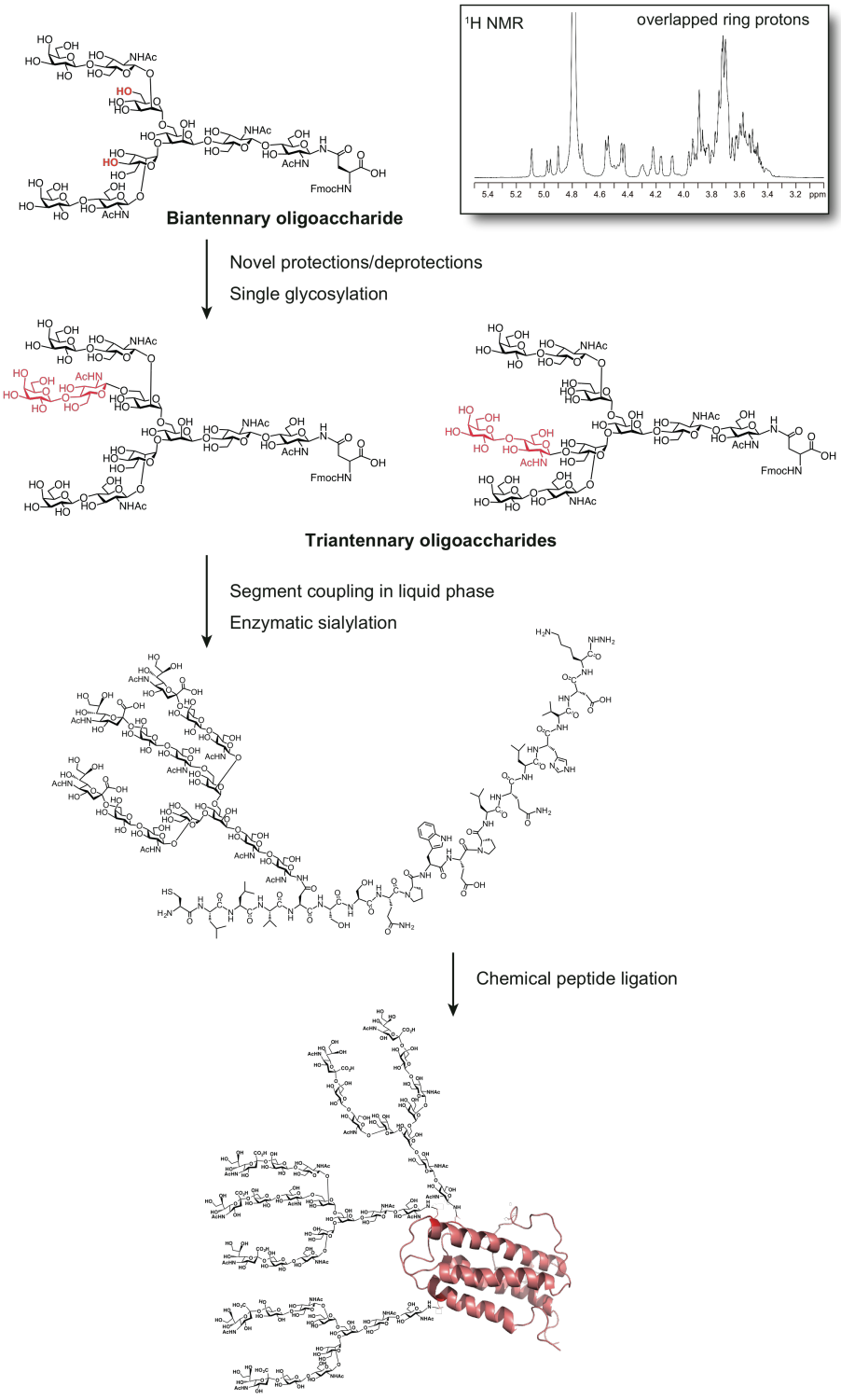

Figure 1-22. Outline of the synthesis in this doctoral thesis.

1-11. Contents of the thesis

As I mentioned, traditional oligosaccharide synthesis suffers from repetitive synthetic steps and low total yield. Therefore, establishment of a novel strategy for the synthesis of oligosaccharide is essential for the practical synthesis. In *Chapter 2*, I will discuss the detail of semisyntheses of β1,6- and β1,4-branched triantennary oligosaccharides. A finding of selective deprotection of benzylidene acetal enabled me to synthesize triantennary oligosaccharides. Therefore, the mechanistic studies of benzylidene acetal hydrolysis of monosaccharides will be discussed in *Chapter 3*. To make a glycoprotein, efficient and practical synthesis of glycopeptides still needs to be investigated. In *Chapter 4*, I will discuss the new segment coupling strategy for the synthesis glycopeptide. In the end, in *Chapter 5*, I would mention a successful synthesis of EPO having a tri- and two bi-antennary oligosaccharides.

Chapter 2 will describe the chemical syntheses of Galβ1→4GlcNAcβ1→2Manβ1→3[Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manβ1→6]Manβ1→4GlcNAcβ1→4GlcNAc→Asn and Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→4)Manβ1→3(Galβ1→4GlcNAcβ1→2Manβ1→6)Manβ1→4GlcNAcβ1→4GlcNAc→Asn.

Chapter 3 will discuss a mechanistic study on the benzylidene hydrolysis of monosaccharides.

Chapter 4 will describe a new segment coupling strategy to synthesize a sialylglycopeptide having a triantennary oligosaccharide.

Chapter 5 will describe chemical synthesis of an EPO glycoform having a triantennary oligosaccharide and two biantennary oligosaccharides.

1-12. References

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Chapter 2

Semisynthesis of Intact Complex-Type Triantennary Oligosaccharides from a Biantennary Oligosaccharide Isolated from a Natural Source

2-1. Introduction of *Chapter 2*

To synthesize complex-type triantennary oligosaccharides, I envisioned that biantennary asialononasaccharide could be used as a starting material. As described in the introduction of this thesis, efficient chemical synthetic methods are required, especially when the target oligosaccharide consists of over 10 monosaccharides. Practical and prompt preparation of complex-type triantennary oligosaccharides without repetitive glycosylation reactions was designed by an unprecedented semisynthetic strategy using a nonasaccharide as a scaffold.

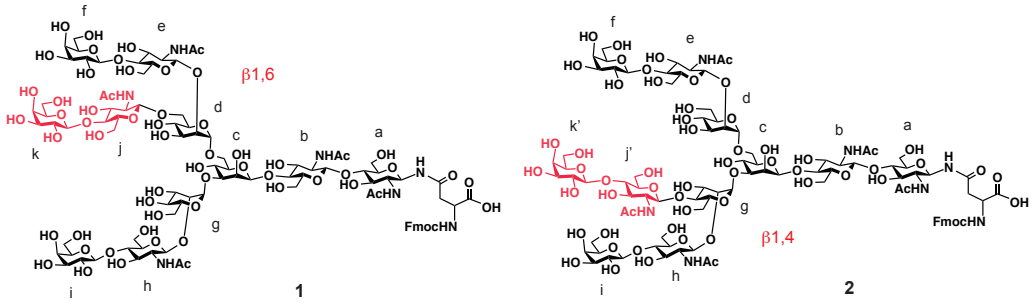


Figure 2-1. Structures of the desired complex-type triantennary oligosaccharides

2-2. Protection of Asn-linked biantennary asialononasaccharide

The synthesis began with the sequential selective protection of 24 hydroxy groups of asialononasaccharide **3**, which was prepared from hen egg yolk as previously reported (experimental section).[1] A specific hydroxy group needs to be retained by selectively protecting other hydroxy groups, thus allowing the conversion of oligosaccharide **3** into suitable glycosyl acceptors. Such acceptors can then be used for a glycosylation reaction in order to yield a triantennary oligosaccharide.

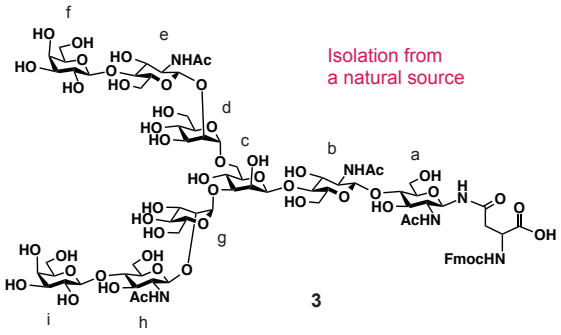


Figure 2-2. Structure of an isolated complex-type biantennary asialononasaccharide linked with Fmoc-protected asparagine

First, I examined the selective protection of primary hydroxy groups, but these attempts were not successful because of the low solubility of **3** in organic solvents and the similar reactivities of the many hydroxy groups of the molecule. Trityl, *tert*-butyldimethylsilyl, and *tert*-butyldiphenylsilyl groups were tested for 6-OH protection (two examples are shown in Figures 2-3 and -4). When these reactions were conducted with a few equivalents of reagents to primary alcohols, the conditions did not yield suitable results. Although the most of the substrate remained as a precipitate, a small amount of the substrate dissolving into the solvent reacted with reagents and resulted in multiple products. Diluting the solvent did not improve the conditions. Furthermore, using an excess of reagents caused overprotection of the substrate dissolved in the solvent. As a result, I could not regulate any selective protection of primary and secondary hydroxy groups among the 24 hydroxy groups.

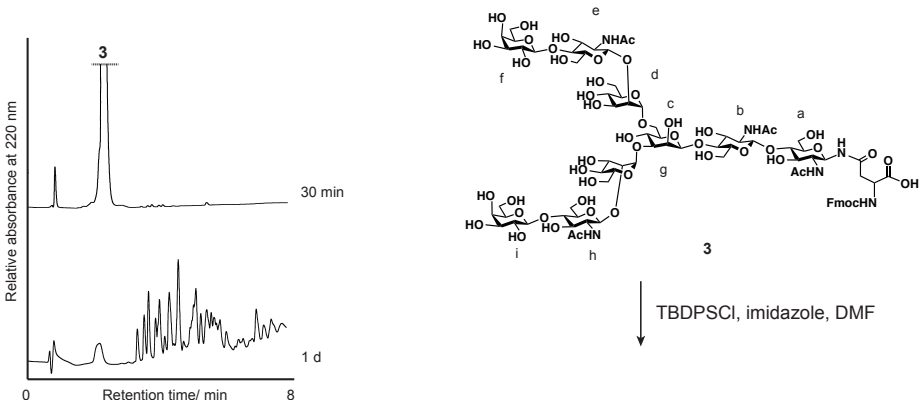


Figure 2-3. Monitoring of silylation of **3**. To a solution of Asn-linked biantennary asialononasaccharide **3** (9.8 mg, 5 μmol) in DMF (1.0 mL) were added imidazole (20 μmol) and TBDPSCl (25 mmol). The reaction mixture was stirred at rt. However, reaction did not proceed. After 12 hours, TBDPSCl (25 μmol) was added again to the mixture, followed by further addition of TBDPSCl (193 μmol). This reaction found to give multiple products as shown in HPLC chromatograms.

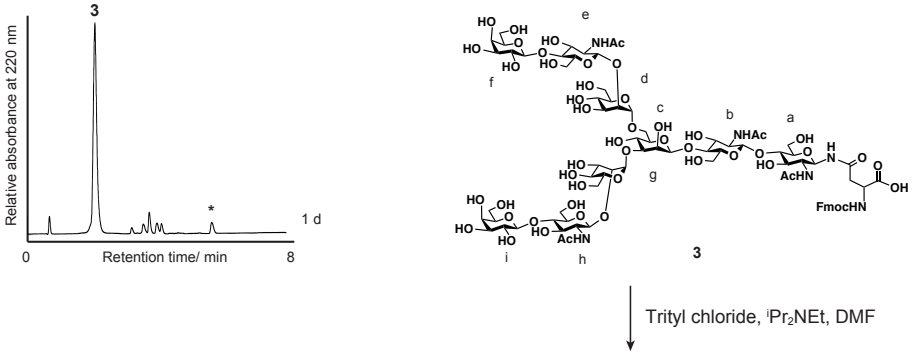
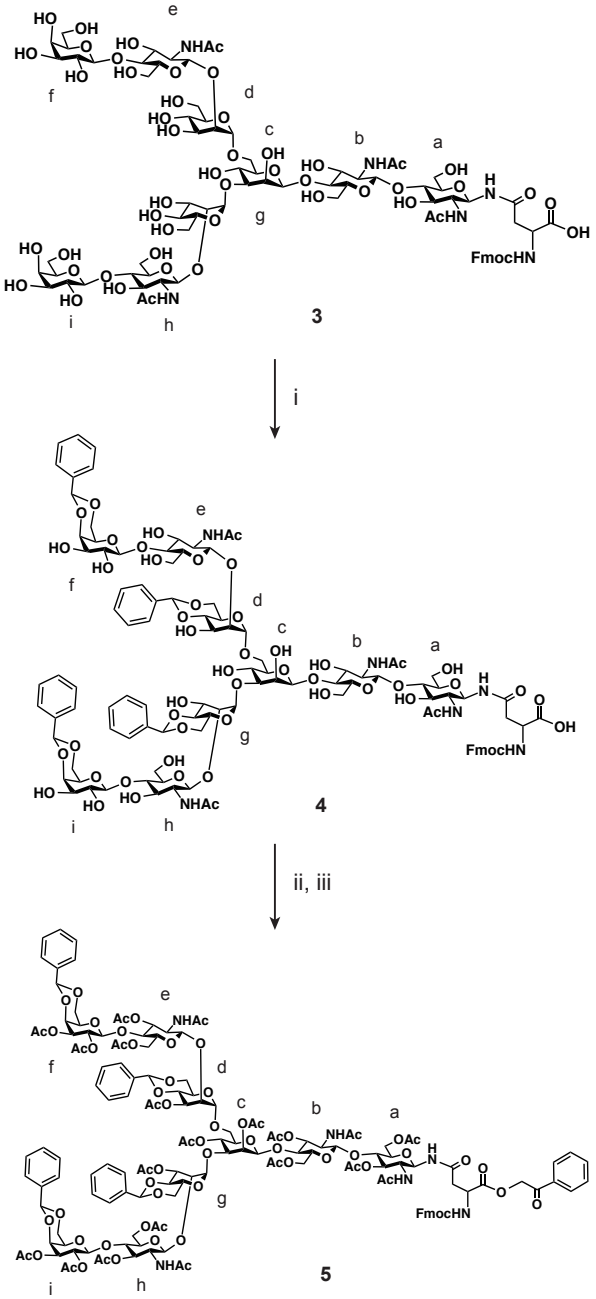


Figure 2-4. Monitoring of tritylation of **3**. To a solution of Asn-linked biantennary asialononasaccharide **3** (10 mg, 5 μmol) in DMF (630 μL) were added $^{i}Pr_2NEt$ (25 μmol) and TrCl (25 μmol). The reaction mixture was stirred at rt. This reaction yielded over 5 derivatives as shown in the chromatogram, and therefore I did not use this protection for the synthesis of triantennary oligosaccharides. *This peak is not an oligosaccharide peak.

Next, I examined selective protections with benzylidene acetal toward the primary 6-OH and secondary 4-OH groups of both $Man_{d,g}$ and $Gal_{f,i}$ of oligosaccharide **3**. After extensive optimization of the reaction conditions, the desired tetrabenzylidene derivative **4** was successfully obtained. The hydroxy groups of Man_d-6-OH and Man_g-4-OH were found to be protected by benzylidene acetals as shown in Scheme 2-1 and Figures 2-5 and -8. Thus, the resulting product could be used for future glycosylation at the Man_d-6-OH and Man_g-4-OH. The benzylidenation reaction usually promotes an equilibrium state, giving a thermodynamically stable product with the protection of a specific 1,3-diol among multiple hydroxy groups. Even benzylidenation of a monosaccharide sometimes causes (S)- or (R)-stereoisomers at a benzyl positions and regioisomers such as protection of 1,2-diol system. In terms of benzylidenation of oligosaccharide **3**, some isomers that show same *m/z* to the desired product were found in LC-MS analysis. These compounds are thought to be regio- or/and stereoisomers. Then, I employed the resultant product for the next reaction just after ether precipitation.

Scheme 2-1



Reagents and conditions: (i) $PhCH(OMe)_2$, CSA, DMF, rt, 17 h. (ii) 2-bromoacetophenone, $^{i}Pr_2NEt$, DMF, rt, 3 h. (iii) Ac_2O/piridine (1:1), DMAP, rt, 3 h, 18% (over three steps).

Subsequent phenacyl esterification of the carboxylic acid in the asparagine moiety followed by peracetylation of residual hydroxy groups afforded fully protected oligosaccharide **5** (*Scheme 2-1*) in 18% yield over the three steps. In order to determine the positions of the benzylidene acetals, two-dimensional (2D) NMR experiments were performed, and the heteronuclear single-quantum coherence (HSQC) signals were successfully assigned as shown in Figure 2-6b. In addition, HSQC and the heteronuclear multiple-bond correlation (HMBC) spectra of oligosaccharide **5** are shown in Figure 2-6c. HMBC correlation signals between the benzyl protons of the benzylidene acetals and C-4 and C-6 of $Gal_{f,i}$ were clearly observed. Similar correlation signals were found in $Man_{d,g}$ as well. These correlation patterns indicated that the benzylidene acetals were exclusively introduced at the 4- and 6-OH of $Gal_{f,i}$ and $Man_{d,g}$.

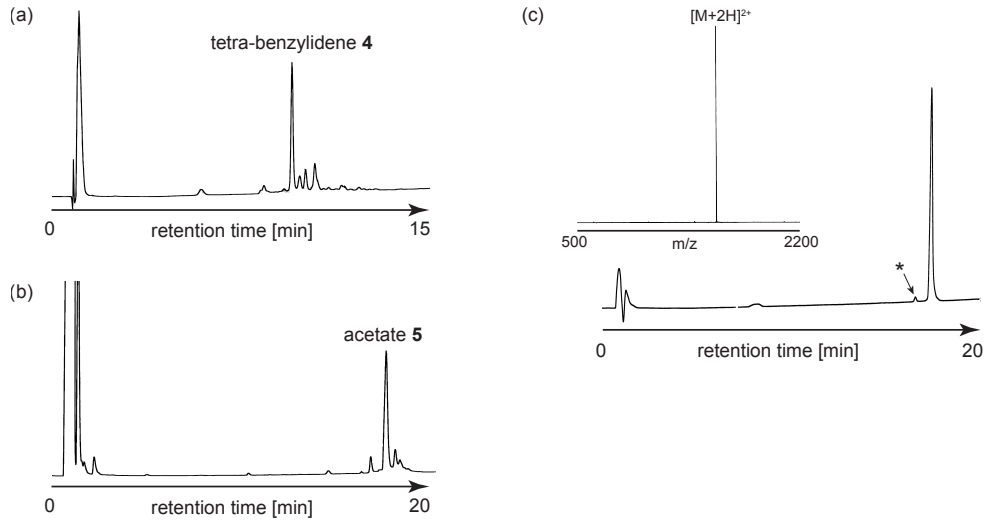


Figure 2-5. (a) RP-HPLC profiles of crude tetra-benzylidene derivative **4** after completion of the benzylidene acetalation. (b) RP-HPLC profile of crude acetate **5** after acetylation of **4**. (c) RP-HPLC profile and ESI-MS spectrum of purified acetate **5**. *Aspartimide byproduct was observed as 1.6% area on HPLC analysis. This aspartimide formation occurred either right after HPLC purification or during HPLC analysis. Adapted with permission from *J. Am. Chem. Soc.* **2016**, *138*, 3461. Copyright 2017 American Chemical Society.

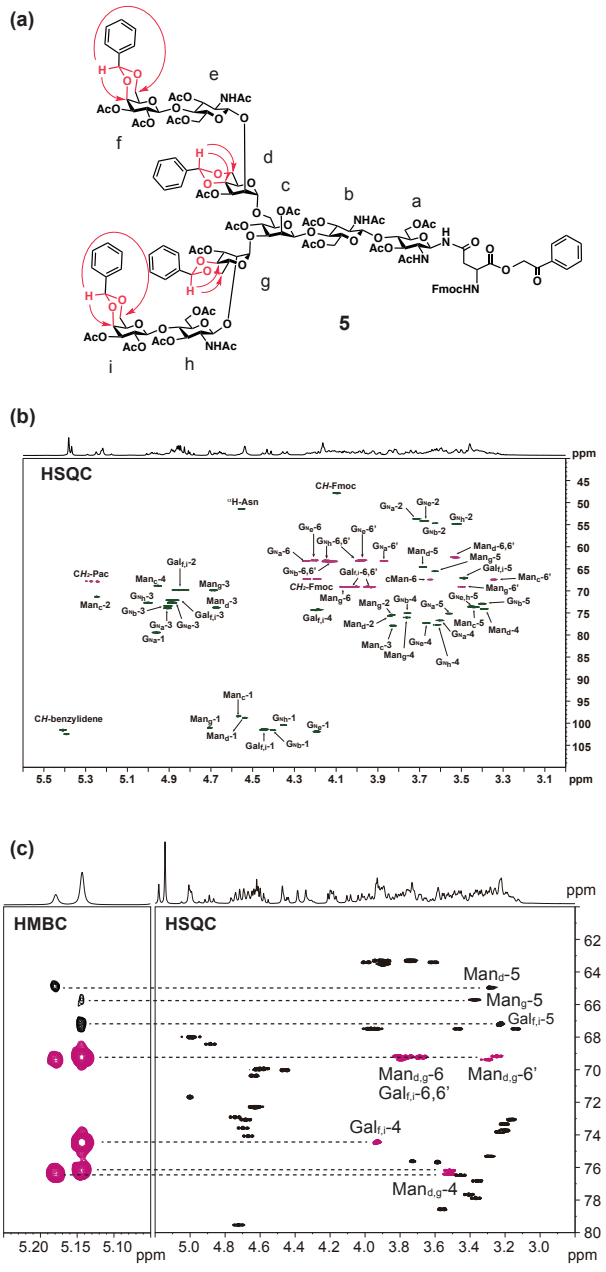


Figure 2-6. (a) Structure of fully protected oligosaccharide **5**. The observed HMBC correlation signals are shown by red arrows. (b) The assigned HSQC spectrum of **5**. (c) Selected regions of the HMBC (left square) and HSQC (right square) spectra of **5**. HMBC correlation signals between the benzyl protons of the benzylidene acetals and C-4 and C-6 were observed in $Man_{d,g}$, and similar correlation signals were also confirmed in $Gal_{f,i}$.

In terms of the phenacyl ester of asparagine, this ester was found to form a cyclic product (aspartimide) via nucleophilic attack of the *N*-glycosylated side-chain nitrogen under basic conditions and upon purification with ammonium acetate buffer (Figure 2-5c and -7). This aspartimide formation was also observed during glycopeptide syntheses using oligosaccharyl asparagine derivatives.[2] To prevent this side reaction, the fractions of purified oligosaccharide **5** were kept on ice during the HPLC purification step and were then lyophilized as soon as possible. Moreover, I took care to keep the reaction from strongly basic conditions in order to prevent undesired aspartimide formation.

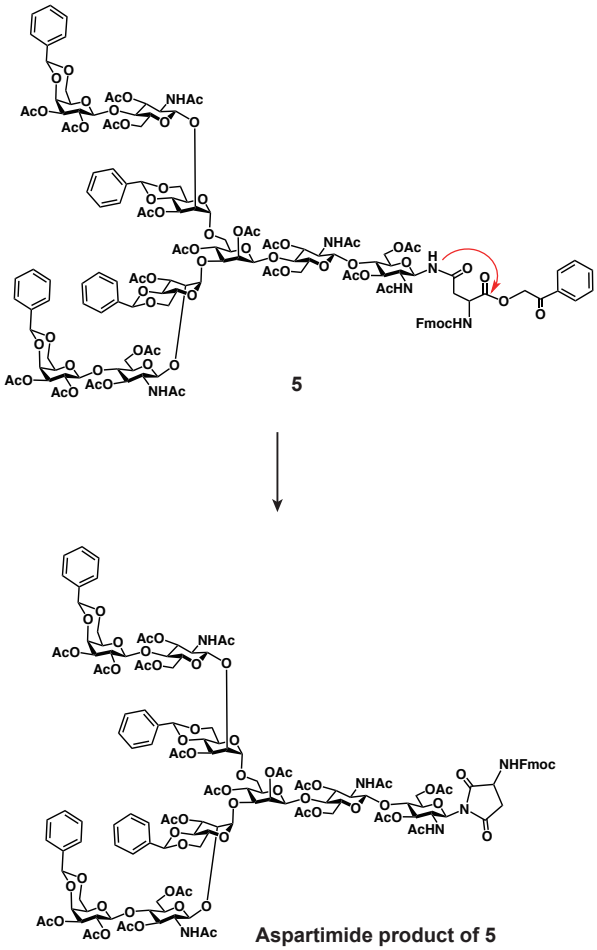


Figure 2-7. Nucleophilic attack of the nitrogen in *N*-glycoside to give an undesired aspartimide product.

In addition to the protection protocol yielding oligosaccharide **5**, in which $Gal_{f,i}$ and $Man_{d,g}$ were protected with benzylidene acetals, I also examined the selective protection of only $Man_{d,g}$ with benzylidene acetals in order to yield suitable glycosyl acceptors. I conducted this step because the third Gal-β-1,4-GlcNAc antenna links with Man_d-6-OH or Man_g-4-OH in the target triantennary oligosaccharides **1** and **2**, but I could not find any selective protection protocol toward Man_d and Man_g residues. Multiple products were formed during benzylidenation of **3** (Figure 2-8).

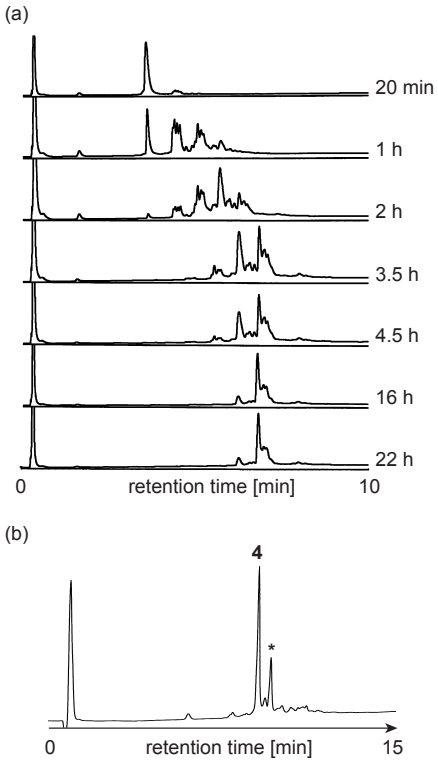


Figure 2-8. Kinetic benzylidenation of oligosaccharide **3**. (a) Time-dependent RP-UltraFast-HPLC chromatograms (low resolution) monitoring the benzylidenation reaction. This reaction was conducted with CSA (5 equiv) and benzaldehyde dimethyl acetal (20 equiv) at 37 ^{o}C. (b) A typical HPLC profile of benzylidenation reaction with high resolution. The conversion yield of desired tetrabenzylidene derivative **4** is 50-70% based on the peak areas of the HPLC chromatogram. *MS analysis suggested that this side-product might be regio- or stereoisomers of benzylidene derivatives, but the structures were not determined furthermore. Adapted with permission from *J. Am. Chem. Soc.* **2016**, *138*, 3461. Copyright 2017 American Chemical Society.

2-3. Selective deprotection of fully protected oligosaccharide **5**

Therefore, I examined the selective deprotection of benzylidene acetals of fully protected oligosaccharide **5** in order to convert it into suitable acceptors for the syntheses of triantennary oligosaccharides. During the extensive investigation of the selective removal of benzylidene groups, I found that the two benzylidene acetals of the $Man_{d,g}$ residues are prone to deprotection in comparison with those of the $Gal_{f,i}$ residues under mild acidic conditions. This selectivity will be further discussed in *Chapter 3*. This unexpected finding enabled me to obtain biantennary oligosaccharyl acceptors **6** and **7** that were suitable for the semisyntheses of triantennary oligosaccharides **1** and **2**, respectively (Figure 2-9). As shown in Figure 2-9b, this deprotection of the benzylidene groups in the mannosides gradually proceeded under the optimized conditions using 60% aqueous acetic acid. The increase of two tribenzylidene derivatives (peaks B and C) and concomitant decrease of tetrabenzylidene derivative **5** (peak A) were observed by HPLC/mass spectrometry. When reaction time was extended, all four benzylidene acetals were removed. The maximum conversion yield of a mixture of two tribenzylidene derivatives was found to be 46% based on the peak areas of the HPLC chromatogram, and individual isomers were isolated in yields ranging from 9 to 17% for **6** (peak B, 12% on average), and from 13 to 23% for **7** (peak C, 17% on average) by reversed-phase HPLC (RP- HPLC).

In order to confirm which hydroxy groups were made free under acidic removal of a benzylidene acetal, the structures of these two isomers (peaks B and C) were individually determined by 2D NMR experiments (Figures 2-10 and -11). According to these NMR assignments, I concluded that the structures corresponding to peaks B and C were tribenzylidene derivatives **6** and **7**, respectively (Figure 2-9, -10, and -11). I also checked for the possibility of acetyl migration to the newly formed hydroxy groups under these acidic conditions, because the migration is sometimes observed even in the case of monosaccharides. On the basis of NMR and HPLC analyses, I concluded that acetyl rearrangement did not occur.

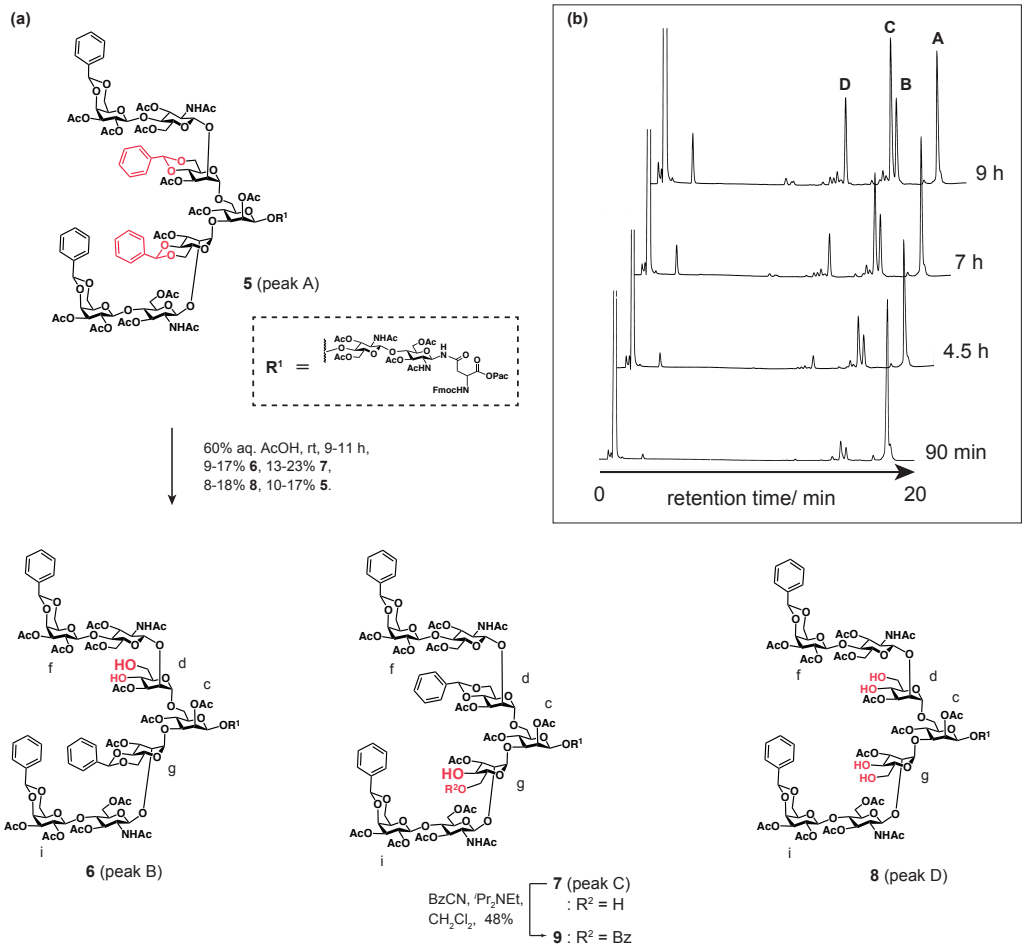


Figure 2-9. (a) Removal of benzylidene acetals of **5** to give partially debenzylidenated products **6**, **7**, and **8**. Man_g-6-OH of diol **7** was further protected with BzCN in CH_2Cl_2 to afford glycosyl acceptor **9** (48%). (b) Typical time-dependent HPLC chromatograms to monitor the selective debenzylidenation reaction.

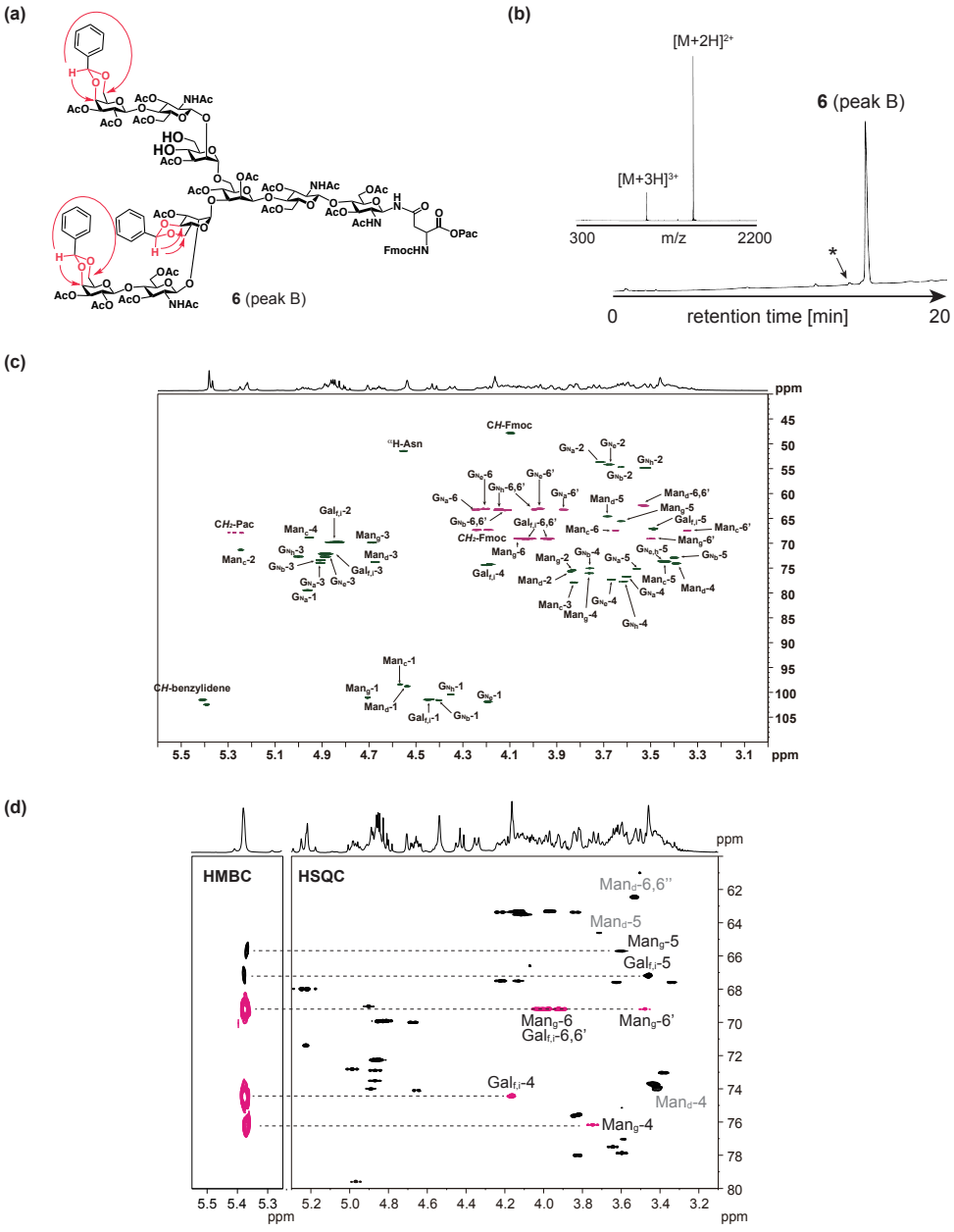


Figure 2-10. (a) The structure of acceptor **6** (peak B). The observed HMBC correlation signals are shown by red arrows. (b) HPLC profile and ESI-MS spectrum of purified **6**. *Aspartimide byproduct was observed as 1.4% area on HPLC analysis. This aspartimide occurred either right after HPLC purification or during HPLC analysis. (c) Assigned HSQC spectrum of **6**. (d) Selected regions of HSQC (right square) and HMBC (left square) spectra of **6**.

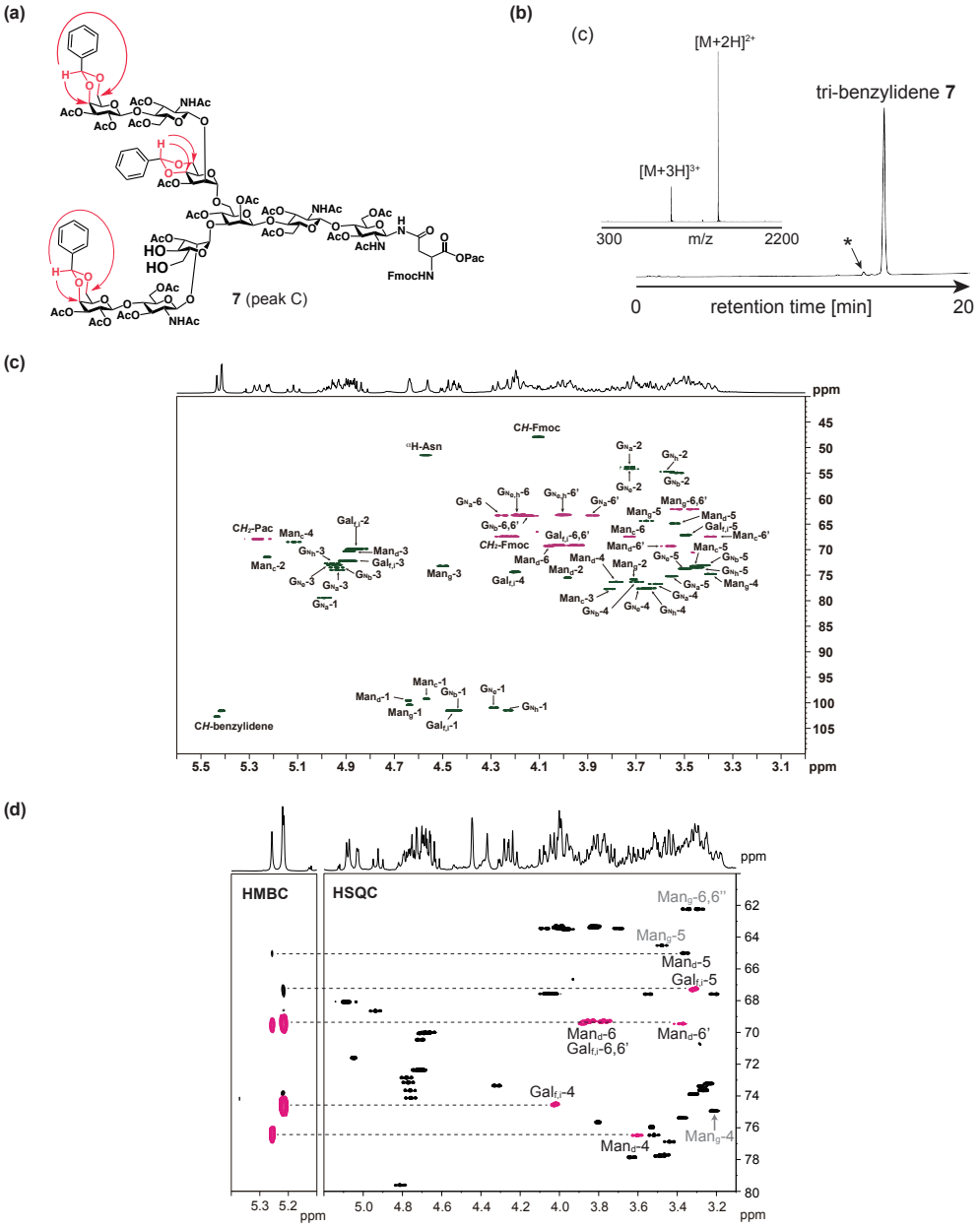


Figure 2-11. (a) The structure of tribenzylidene **7** (peak C). The observed HMBC correlation signals are shown by red arrows. (b) HPLC profile and ESI-MS spectrum of purified **7**. *Aspartimide byproduct was observed as 2.0% area on HPLC analysis. This aspartimide occurred either right after HPLC purification or during HPLC analysis. (c) Assigned HSQC spectrum of **7**. (d) Selected regions of HSQC (right square) and HMBC (left square) spectra of **7**.

For the synthesis of triantennary oligosaccharide **1**, I decided to use diol **6** as a glycosyl acceptor to examine selective glycosylation toward primary Man_d-6-OH, which is more reactive than Man_d-4-OH, but then, for the synthesis of triantennary oligosaccharide **2**, primary Man_g-6-OH in diol **7** was selectively benzoylated by treatment with BzCN to afford a 48% yield of glycosyl acceptor **9**, which has a free Man_g-4-OH hydroxy group. This structure was also confirmed by NMR experiments, in which I observed that the chemical shifts of H-6 and C-6 of Man_g changed due to the electron-withdrawing group, and HMBC spectrum shows a correlation signal between carbonyl carbon of Bz and H-6 of Man_g (Figure 2-12).

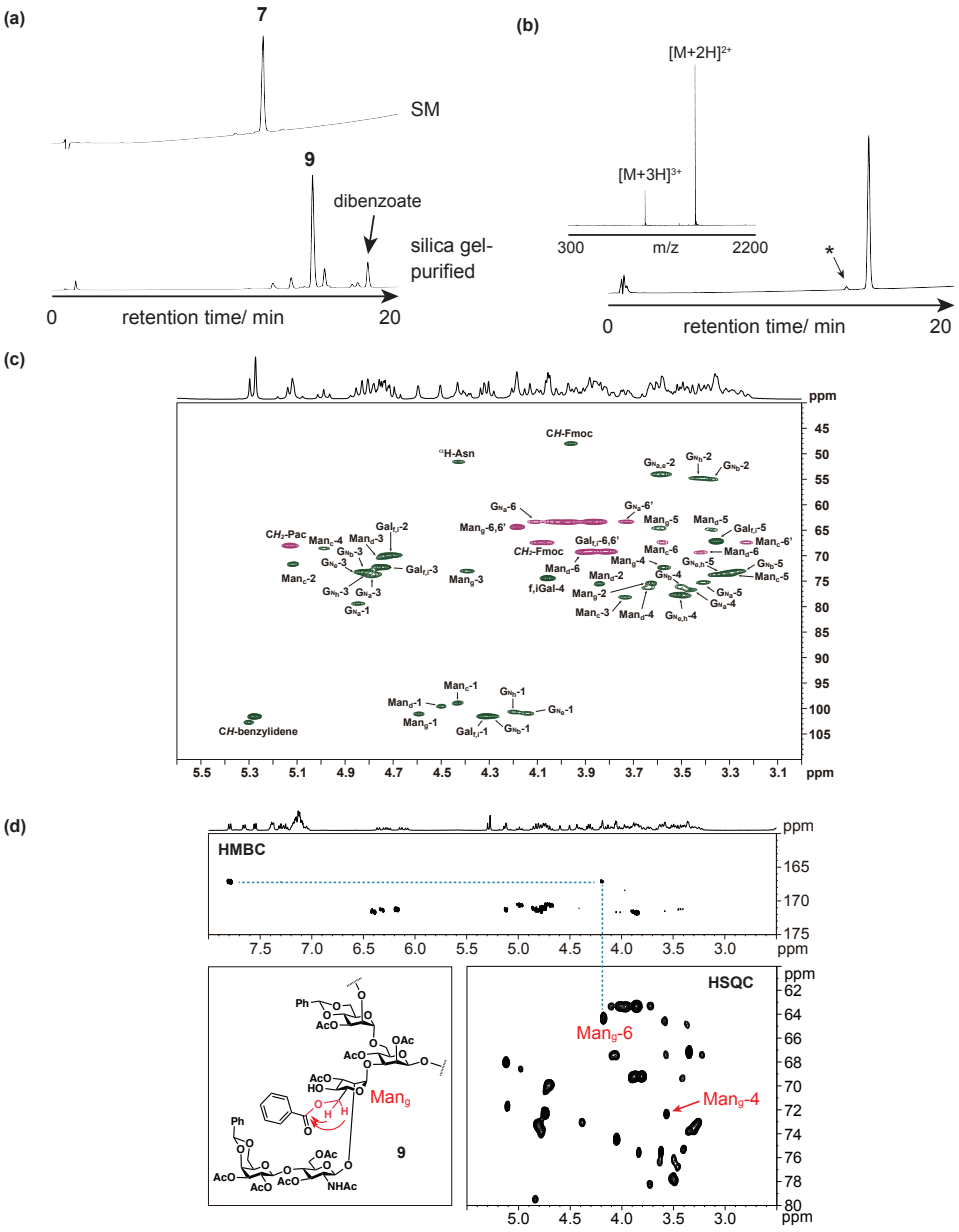


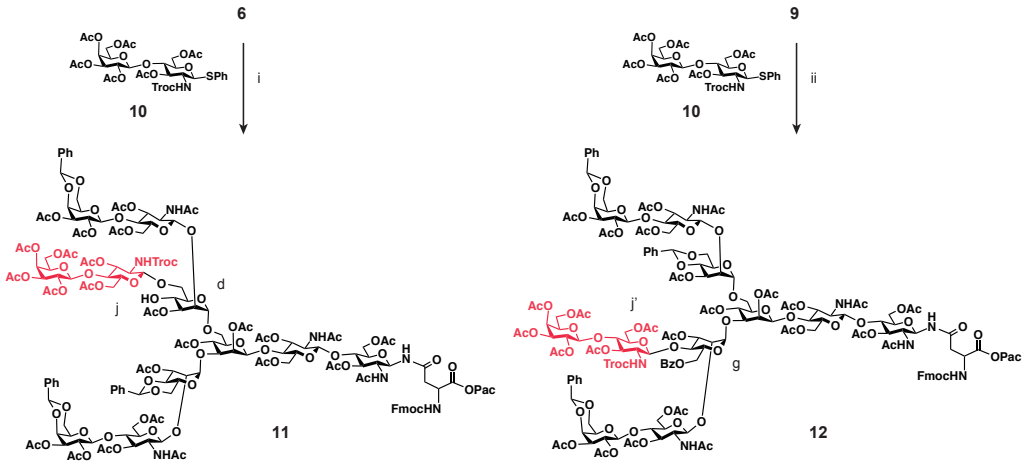
Figure 2-12. (a) RP-HPLC profiles of the benzoylation reaction of **7**. (b) HPLC profile and ESI-MS spectrum of HPLC-purified benzoate **9**. *Aspartimide byproduct was observed as 1.8% area on HPLC analysis. This aspartimide formation occurred either right after HPLC purification or during HPLC analysis. (c) Assigned HSQC spectrum of **9**. (d) Selected regions of HMBC and HSQC spectra. Chemical shift of carbonyl carbon of benzoyl group was assigned at 167 ppm because this carbon shows correlations with aromatic *o*-protons. A HMBC correlation signal from carbonyl carbon of benzoyl group to H-6 of Mang was observed. HSQC signal of Man_g-6 shows down field shift in terms of ^{1}H and ^{13}C after benzoylation reaction.

2-4. Glycosylation reactions of the obtained acceptors

I then examined the glycosylation of suitably protected biantennary oligosaccharyl acceptors **6** and **9** with thioglycoside **10** (Scheme 2), which was prepared from lactose in eight steps via an azidonitration reaction (Scheme 3 and experimental section).[3-6] The reactivity of the oligosaccharyl acceptor is thought to be low due to multiple electron-withdrawing groups, and reactivity of a glycosyl donor and an acceptor needs to be adjusted to make an efficient reaction. Thus, thioglycoside was considered to be the best donor compared with highly reactive glycosyl imidate donor. Donor **10** was prepared as shown in Scheme 3 and the detail of the synthesis is described in experimental section. For the synthesis of triantennary oligosaccharide **11**, diol **6** was coupled with glycosyl donor **10** by TfOH/NIS activation. I found the best conditions to be acceptor **6** (5 mM in CH_2Cl_2) and excesses of donor **10** (10 equiv) and *N*-iodosuccinimide (NIS) (10 equiv) with a reaction time of 1 h at 0 °C. These conditions yielded the desired triantennary oligosaccharide **11** in 47% yield, while small amounts of byproducts, such as the diglycosylated product, were also observed. The formation of the diglycosylated product was accelerated when the amount of NIS was increased. In addition, this glycosylation reaction was prone to form the aspartimide derivative through intramolecular cyclization during the long reaction time. For example, using donor (2 equiv with respect to acceptor) and NIS (2 equiv with respect to donor) at −20 °C for 3.5 h did not complete the reaction and gave aspartimide (Figure 2-14). Moreover, in this reaction, I also observed another byproduct exhibiting the same mass as the desired product **11** by HPLC/MS analysis. This byproduct was thought to possibly be an α-linked isomer or a regioisomer, but because of its negligible amount, I could not isolate and assign this byproduct.

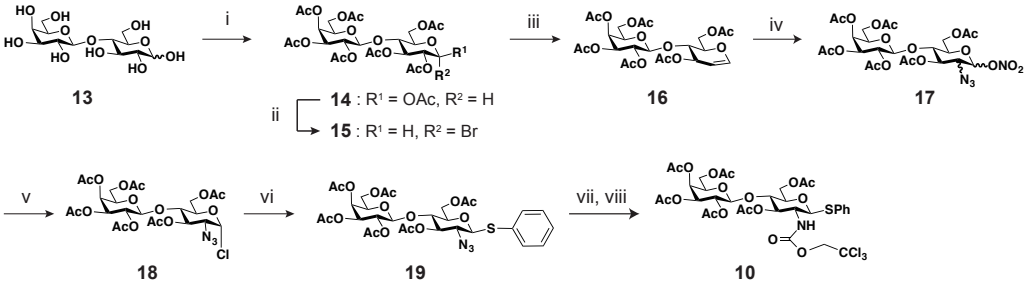
Contrary to the glycosylation of Man_d-6-OH for the synthesis of triantennary oligosaccharide **11**, the glycosylation of 4-OH of Man_g for the synthesis of triantennary oligosaccharide **12** was slow (Scheme 2). Thioglycoside donor **10** was also used in this glycosylation reaction with TfOH/NIS activation. Although the desired triantennary oligosaccharide **12** was obtained, acceptor **9** (5 mM in CH_2Cl_2) still remained even when an excess of donor **10** (20 equiv) was employed and the reaction temperature was elevated to room temperature (Figure 2-13). Unreacted acceptor **9** was recovered during the purification step and used for the glycosylation reaction repeatedly. After optimization of this reaction, the desired triantennary oligosaccharide **12** was obtained in 44% yield after HPLC purification.

Scheme 2



Reagents and conditions: (i) **10**, NIS, TfOH, CH_2Cl_2, 0 °C, 4A molecular sieves, 1 h, 47%. (ii) **10**, NIS, TfOH, CH_2Cl_2, 4A molecular sieves, 0 °C to rt, 2 h, 44%.

Scheme 3



Reagents and conditions. (i) Ac_2O, NaOAc, 140 ^oC, 2 h, 78 %. (ii) HBr/AcOH, 0 ^oC, 5 h. (iii) 50% AcOH, $CuSO_4$, Zn, 0-5 ^oC, 3 h, 92% (over two steps). (iv) CAN, NaN_3, CH_3CN, -15 ^oC, 15 h, 52% (α-gluco: β-gluco: α-manno = 1:2:1). (v) Et_4NCl, CH_3CN, rt, 15 h, 39%. (vi) Bu_4NHSO_4, PhSH, EtOAc/1 M Na_2CO_3, 40 ^oC, 2 h, 25%. (vii) AcOH, Zn, THF, rt, 30 m. (vii) triethylamine, TrocCl, THF, 0 ^oC to rt, 5 h, 35% (over two steps).

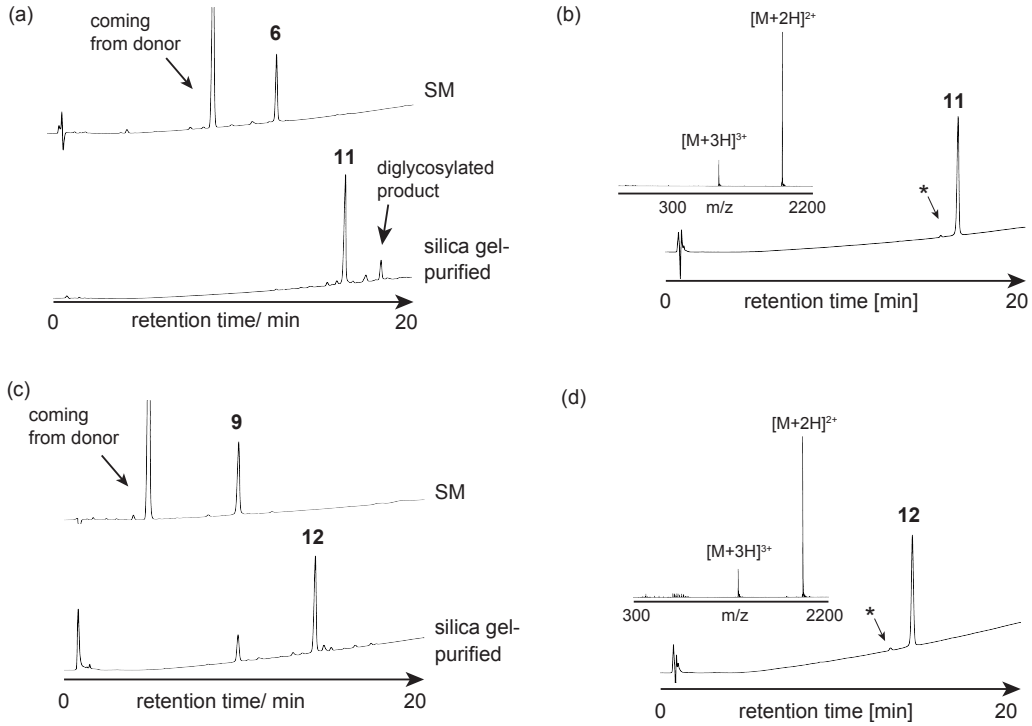


Figure 2-13. (a) RP-HPLC profiles of the glycosylation reaction of **6**, starting materials (upper) and silica gel-purified material (bottom). (b) HPLC profile and ESI-MS spectrum of HPLC-purified triantennary oligosaccharide **11**. *Aspartimide byproduct was observed as 1.4% area on HPLC analysis. This aspartimide formation occurred either right after HPLC purification or during HPLC analysis. (c) RP-HPLC profiles of the glycosylation reaction of **9**; starting materials (upper) and silica gel-purified materials (bottom). (d) HPLC profile and ESI-MS spectrum of HPLC-purified triantennary oligosaccharide **12**. *Aspartimide byproduct was observed as 2.3% area on HPLC analysis. This aspartimide formation occurred either right after HPLC purification or during HPLC analysis.

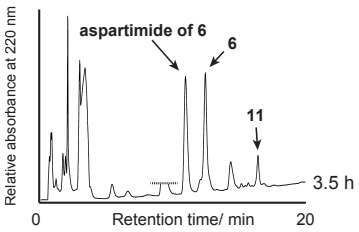


Figure 2-14. Monitoring a glycosylation reaction of **6** under another reaction condition. A mixture of acceptor **6** (1.7 mg, 0.6 μmol) and donor **10** (1.0 mg, 1.2 μmol, 2 equiv) was azeotropically dried with toluene and kept *in vacuo* for 2 hours, after which the mixture was dissolved in CH_2Cl_2 (100 μL) under an atmosphere of Ar, followed by addition of freshly activated 4 A molecular sieves (13 mg). The reaction mixture was cooled to -20 ^oC, and NIS (4.4 μmol) was added. Then, TfOH (0.44 μmol, with dilution by CH_2Cl_2) was added to the mixture, which was stirred at the same temperature for 3.5 h. This reaction afforded undesired by product, aspartimide rather than desired triantennary oligosaccharide **11.** Because donor was not enough, the reaction did not finish within 2 h and the condition of longer reaction time yielded undesired aspartimide derivative. Adapted with permission from *J. Am. Chem. Soc.* **2016**, *138*, 3461. Copyright 2017 American Chemical Society.

The structures of triantennary oligosaccharides **11** and **12** were confirmed by 2D NMR experiments. An HMBC correlation between the anomeric proton of $GlcN_j$ and C-6 of Man_d was observed in **11** (Figure 2-15c, dotted line). Similarly, an HMBC correlation between the anomeric proton of $GlcN_{j'}$ and C-4 of Man_g was also observed in **12** (Figure 2-16c, dotted line). Furthermore, newly formed glycosyl linkages showed $J_{1,2}$ 8.5 Hz and 8.3 Hz in **11** and **12**, respectively, that indicate the desired β-linkages. These NMR data prove that I indeed obtained the protected triantennary oligosaccharides **11** and **12** by the semisynthetic methods.

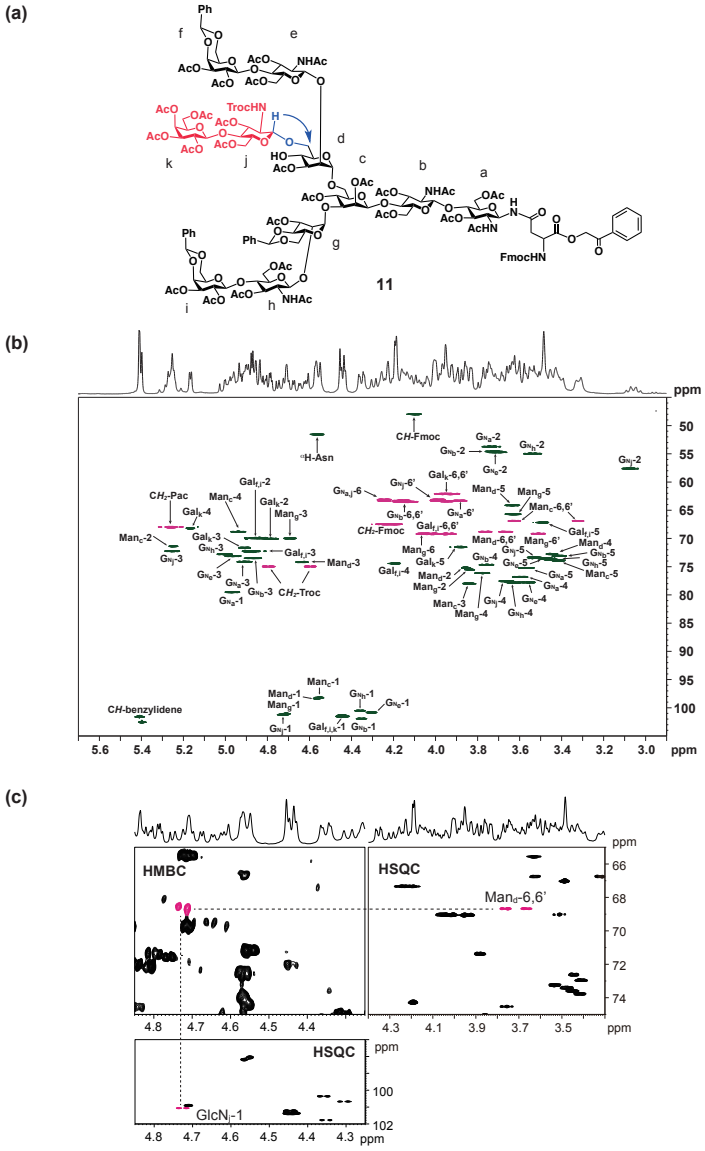


Figure 2-15. (a) The structure of **11**. An observed HMBC correlation signal is shown by a blue arrow. (b) Assigned HSQC spectrum of **11**. (c) Selected regions of HSQC and HMBC spectra of **11**. The HMBC correlation signal between the C-6 of Man_d and the H-1 of $GlcN_j$ (dotted line) was observed. The newly synthesized β-linkage was determined by the coupling constant observed for H-1 and H-2 in $GlcN_j$ (8.5 Hz).

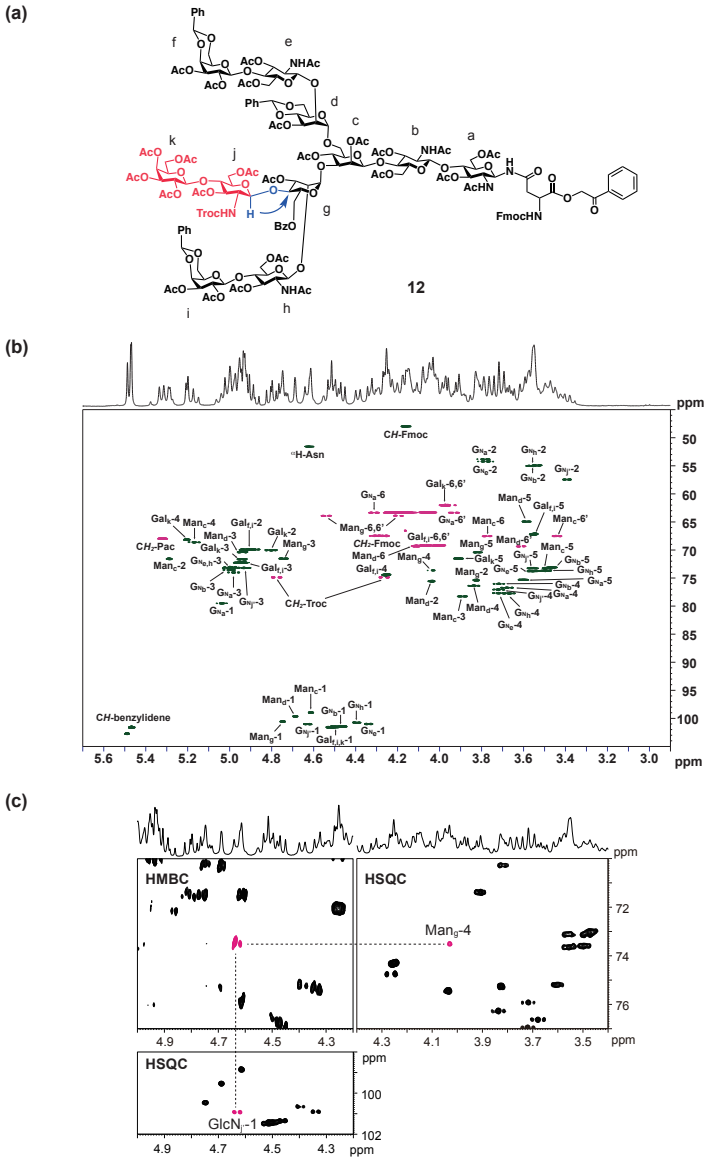
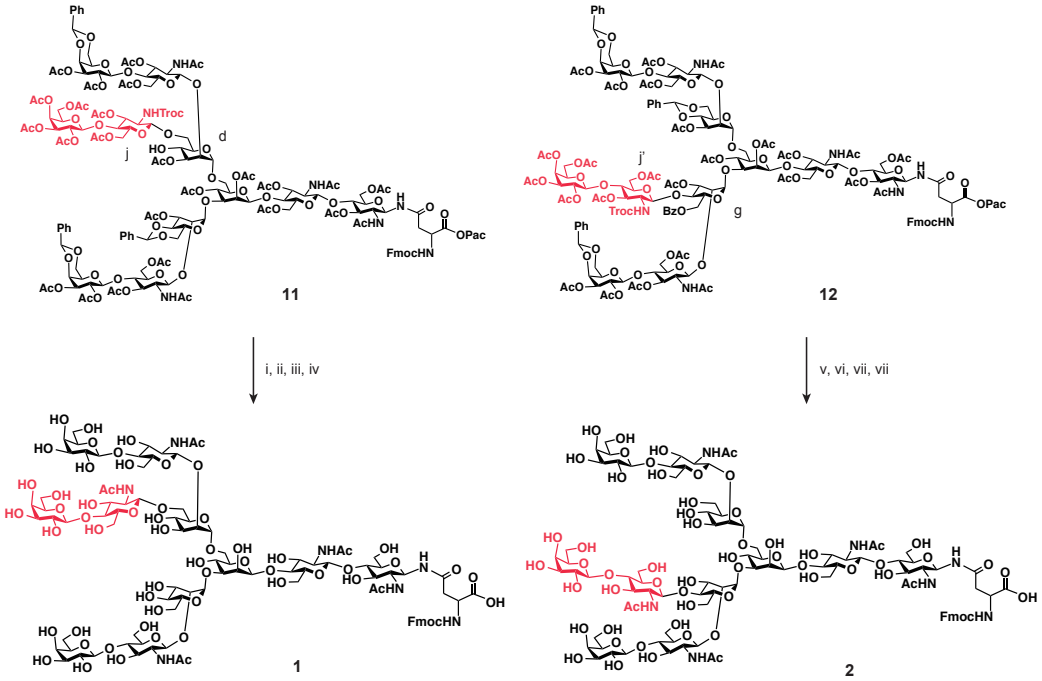


Figure 2-16. (a) The structure of **12**. An observed HMBC correlation signal is shown by a blue arrow. (b) Assigned HSQC spectrum of **12**. (c) Selected regions of HSQC and HMBC spectra of **12**. The HMBC correlation signal between the C-4 of Man_g and the H-1 of $GlcN_j$, (dotted line) was observed. The newly synthesized β-linkage was determined by the coupling constant observed for H-1 and H-2 in $GlcN_j$, (8.3 Hz).

2-5. Deprotection steps of the obtained triantennary oligosaccharides

Finally, I examined deprotection reactions of **11** and **12** in order to obtain the desired intact triantennary asialoundecasaccharides **1** and **2**, respectively (Scheme 4). First, the Troc group was removed by treatment with Zn powder, after which acetylation yielded the acetamide derivatives. The phenacyl group was also removed during this zinc treatment. Subsequently, all of the acyl groups and the Fmoc group were removed by treatment with NaOH in MeOH, and Fmoc groups were reintroduced to the asparagine moieties. This Fmoc groups enabled me to analyze reaction by RP-HPLC and can be used for glycopeptide synthesis after the completion of the synthesis. The removal of the remaining benzylidene acetals was carefully conducted by means of a brief treatment of 90% aqueous trifluoroacetic acid (TFA) at 0 °C, and the desired triantennary oligosaccharides **1** and **2** were obtained in 63% and 44% yield, respectively. Several analyses including high-resolution mass spectrometry and 2D NMR analysis were conducted (Figure 2-17 and experimental section). Based on these data, I established an unprecedented protocol of the synthesis of homogeneous triantennary oligosaccharides.

Scheme 4



Reagents and conditions: (i) Zn, THF/AcOH/Ac_2O (3:2:1), 0 °C to rt, 16 h. (ii) MeOH/aq. NaOH, 0 °C to rt, 2.5 h. (iii) FmocOSu, aq. $NaHCO_3$, acetone, 0 °C to rt, 3.5 h. (iv) aq. TFA, 0 °C, 10 min, 63% (over four steps). (v) Zn, THF/AcOH/Ac_2O (3:2:1), 0 °C to rt, 18 h. (vi) MeOH/aq. NaOH, 0 °C to rt, 2 h. (vii) FmocOSu, aq. $NaHCO_3$, acetone, 0 °C to rt 3 h. (viii) aq. TFA, 0 °C, 10 min, 44% (over four steps).

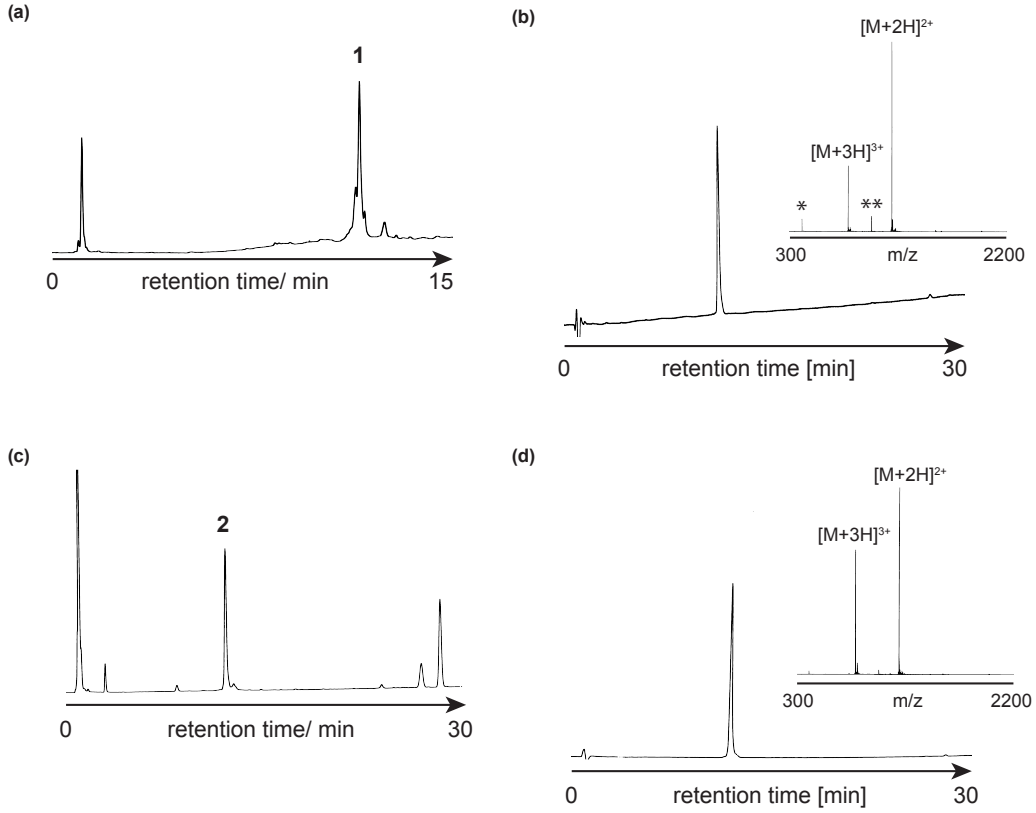


Figure 2-17. (a) RP-HPLC profile of crude β-1,6-branched triantennary oligosaccharide **1** after completion of the deprotection reactions. (b) HPLC profile and ESI-MS spectrum of purified Asn-linked β-1,6-branched triantennary oligosaccharide **1**. Asterisk *and ** indicate fragmentation peaks during mass analysis and correspond to LacNAc and biantennary asialooligosaccharide, respectively. (c) RP-HPLC profile of crude β-1,4-branched triantennary oligosaccharide **2** after the deprotection reactions. (b) HPLC profile and ESI-MS spectrum of purified Asn-linked β-1,4-branched triantennary complex-type oligosaccharide **2**.

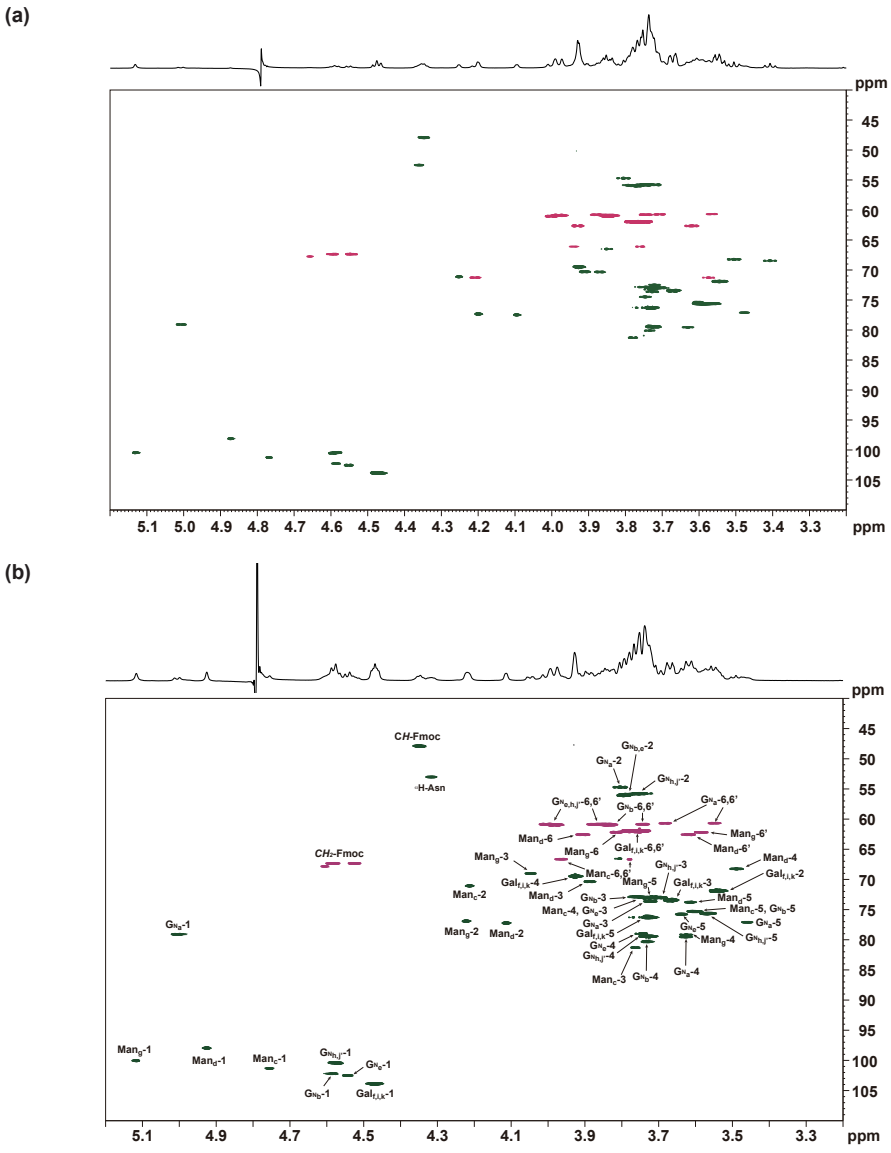


Figure 2-18. (a) The HSQC spectrum of triantennary oligosaccharide **1**. These signals prove the purity of the synthetic oligosaccharide. The signals are not assigned due to the small amount of the material and heavily overlapped signals. NMR assignment of oligosaccharides without protecting groups is especially difficult as previously described.[7] (b) The assigned HSQC spectrum of **2**. These signals were assigned based on the previous assignment of the triantennary structure.[8]

2-6. Enzymatic sialylation of a triantennary oligosaccharide

In addition, I examined enzymatic sialylation toward triantennary oligosaccharide **1** (Figure 2-19) because mature complex-type oligosaccharides have sialic acid residues at the non-reducing termini and sialic acid is known to be involved in many biological events. Enzymatic sialylation usually proceeds in a stereo- and regioselective manner in a single step.[9] Because α-2,3 and α-2,6 sialyl linkages with the terminal galactoside are found in the structures of natural oligosaccharides, enzymatic sialylation by specific α-2,3 or α-2,6 sialyltransferase seems to be effective in making the desired sialyl oligosaccharides. Wong and co-workers reported a combined enzymatic and chemical strategy for the syntheses of sialylated multiantennary complex-type oligosaccharides with their regeneration system of sugar nucleotides.[10] In this application, sialic acid residues were installed on **1** using α-2,6 sialyltransferase (*Photobacterium damsela*, [EC 2.4.99.1])[11] in the presence of CMP-Neu5Ac. This sialylation reaction proceeded in 78% conversion yield. The structure of triantennary sialyl oligosaccharide **20** was also confirmed by NMR experiments and mass spectrometry.

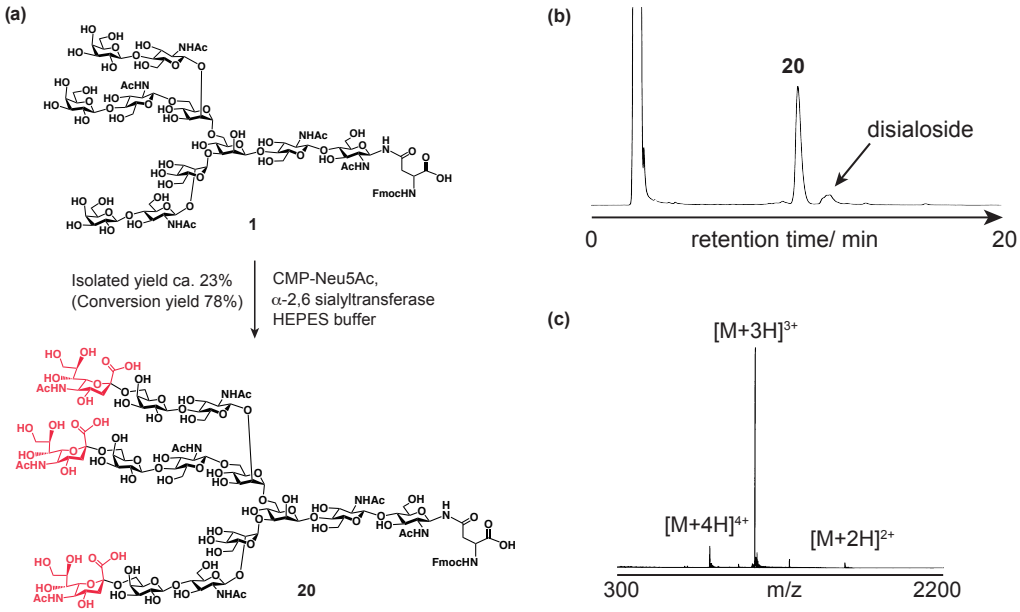


Figure 2-19. (a) Enzymatic sialylation of triantennary oligosaccharide **1**. (b) RP-HPLC profile of crude trisialyloligosaccharide **20**. (b) ESI-MS spectrum of purified triantennary sialyltetradecasaccharide **20**.

2-7. Summery of *Chapter 2*

I have established a strategy for the semisynthesis of complex-type triantennary oligosaccharides from a biantennary oligosaccharide isolated from a natural source. The sequential protection and deprotection of 24 hydroxy groups of this biantennary oligosaccharide successfully yielded two glycosyl acceptors bearing free hydroxy groups at the desired positions, which are essential for the semisyntheses of two types of natural triantennary oligosaccharides. Because the selective introduction of a protecting group is sometimes difficult for a complex molecule, the idea to regulate the reactivity during the deprotection step was critical.

This new strategy significantly reduced the number of synthetic steps and overcame limitations of traditional oligosaccharide syntheses that rely on repetitive protection/ deprotection and multiple glycosylation steps. In terms of the synthesis of triantennary oligosaccharide **1** having three Gal-β1,4-GlcNAc antennae through a β-1,6 and two β-1,2 linkages with the terminal mannosides of the core, more than 50 synthetic steps were eliminated compared with the previous conventional chemical oligosaccharide synthesis.[12]

Because the biantennary oligosaccharide can be isolated on multigram scales and the synthetic routes of the triantennary oligosaccharides have been established once on the basis of rigorous structural analyses, the synthesis of triantennary oligosaccharides can be repeatedly performed to promptly provide sufficient amounts, such as dozens of milligrams. My current experiments demonstrated that biantennary nonasaccharide **3** (ca. 600 mg) could be converted into triantennary undecasaccharides **1** (4 mg) and **2** (2 mg). Isolation of biantennary nonasaccharide **3** from egg yolk can be easily performed on over a 20 g scale, and I consider there to be no limitation on the synthesis of triantennary undecasaccharides.

2-8. References

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Chapter 3

Mechanistic Study on Deprotection of Benzylidene Acetals of Monosaccharides

3-1. Introduction of *Chapter 3*

I found that benzylidene acetals of mannosides of an oligosaccharide derivative are more easily removed than that of galactosides in aqueous acetic acid. This was an unexpected finding and enabled me to synthesize two types of triantennary oligosaccharides. To deeply understand what causes the selectivity, benzylidene removal reactions were investigated by use of monosaccharides: methyl α-D-glucopyranosides, methyl α-D-mannopyranosides, and methyl α-D-galactopyranosides.

3-2. Preparation of benzylidenated monosaccharides

Six types of benzylidenated monosaccharides were synthesized according to the previously reported procedures as shown in Figure 3-1.[1-3] In brief, **methyl α-D glucopyranoside (21)**, **methyl α-D-mannopyranoside (22)**, and **methyl α-D-galactopyranoside (23)** were purchased. In terms of glucoside derivatives, glucoside **21** was dissolved in acetonitrile, to which (±)-10-camphorsulfonic acid (CSA, 0.3 equiv) and benzaldehyde dimethyl acetal (1.5 equiv) were added. The resulting mixture was then stirred at room temperature under an atmosphere of Ar, and then the reaction mixture was neutralized by the addition of triethylamine (2 equiv). After evaporation of the reaction mixture, the resulting residue was purified by silica gel column chromatography to give **methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (24)** in 42%. Using same procedure, **methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (26)** was obtained in 74%. In the case of mannoside, **22** was dissolved in DMF, and *p*-toluenesulfonic acid (*p*-TsOH, 0.3 equiv) and benzaldehyde dimethyl acetal (1.0 equiv) were added to the mixture. The mixture was mixed at 60 °C under reduced pressure (200 hPa). After 3 h, EtOAc was added to the mixture, and then the mixture was washed by $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated. Silica gel column chromatography of the resulting residue afforded **methyl 4,6-*O*-benzylidene-α-D-mannopyranoside** (**25**) in 31%.

Acetylation reactions of the obtained benzylidene derivatives were performed by pyridine and acetic anhydride (Ac_2O). Benzylidene derivatives were dissolved in pyridine/Ac_2O (1:1), and the reaction mixtures were stirred at rt under an atmosphere of Ar. On the completion of the reaction, MeOH was added to the mixture on an ice bath. The resulting mixture was evaporated, azeotropically dried with toluene, which was then purified by silica gel column chromatography and/ or recrystallization to give the desired products (**24**: 40%, **25**: 94%, **26**: 82%).

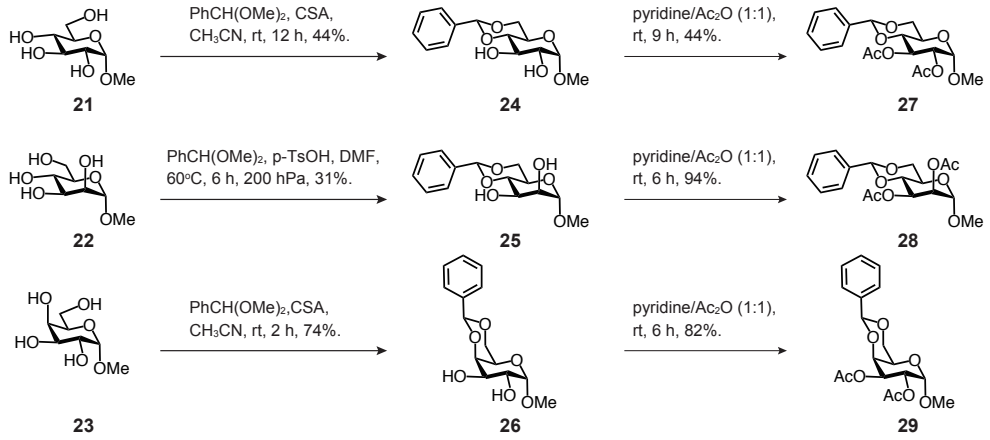


Figure 3-1. Synthesis of benzylidene derivatives **24-29**.

3-3. Hydrolysis rate of benzylidene derivatives at room temperature

To confirm the dependence of the benzylidene group on the sugar stereochemistry for selective deprotection, I first investigated the deprotection velocity of benzylidene groups of monosaccharides without Ac groups (**24**, **25**, and **26**). Individual substrate was dissolved in 60% d-AcOH in D_2O. Concentration of the monosaccharides was adjusted to 10 mM, which is as same as the case of oligosaccharide deprotection (5 mM oligosaccharide containing two benzylidenated galactosides and two benzylidenated mannosides). Ratio of starting material versus product was measured by proton NMR at 23 ^oC at times. Plotting data of the result is shown in Figure. 3-2, and benzylidene acetal of Gal was obviously hydrolyzed slower compared with Glc and Man (Figure 3-2a). Furthermore, I examined a competition experiment by use of three substrates in same NMR sample tube, which also clarified slow deprotection of benzylidene of Gal (Figure 3-2b). These results clearly showed that stereochemistry at the 4-position was critical for the observed selectivity.

Next, I employed monosaccharides with two acetyl groups (**27**, **28**, and **29**) to compare the deprotection velocity of benzylidene acetal, and this experiment also showed the same selectivity (Figure 3-3). Acidic hydrolysis of three substrates in 60% d-AcOH at 23^oC was measured by ^1H proton NMR as described above, respectively (Figure 3-3a). These Gal substrates showed slower deprotection of benzylidene groups compared with non-acetylated substrates due to electron-withdrawing property. I also tested a competition experiment employing three substrates in same reaction mixture (Figure 3-3b and d). All results indicated that deprotection velocity of benzylidene acetal Gal was slower than that of Glc and Man because of the difference of stereochemistry at 4-position.

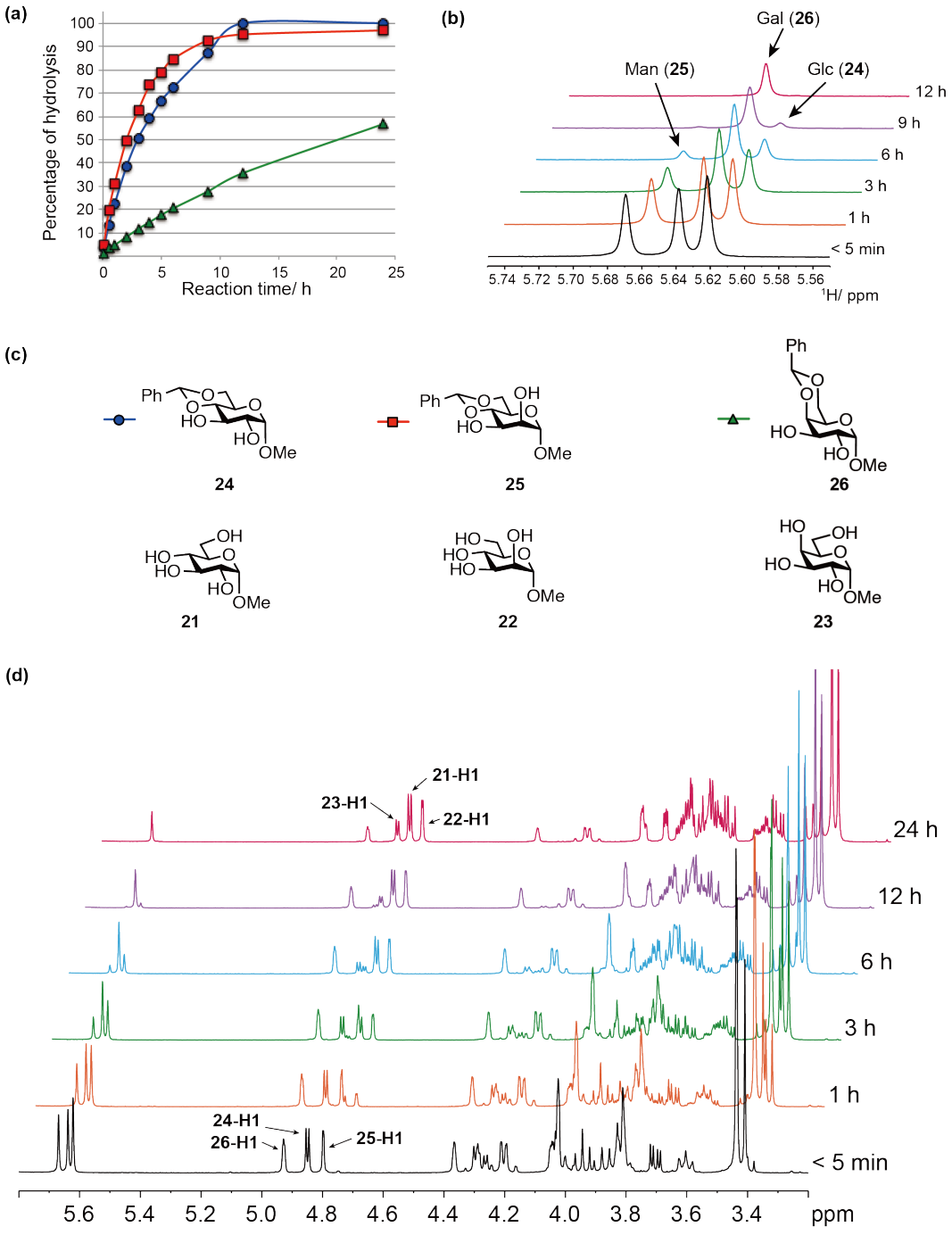


Figure 3-2. Comparison of benzylidene hydrolysis using non-acetylated monosaccharides **24**, **25**, and **26**. (a) Plotting data for percentage of acetal hydrolysis. (b) Monitoring of the competitive experiment by ^{1}H NMR. Selected regions for benzyl proton of benzylidene are shown. (c) Structures of monosaccharides. (d) Monitoring of the competitive experiment by ^{1}H NMR. All regions are shown.

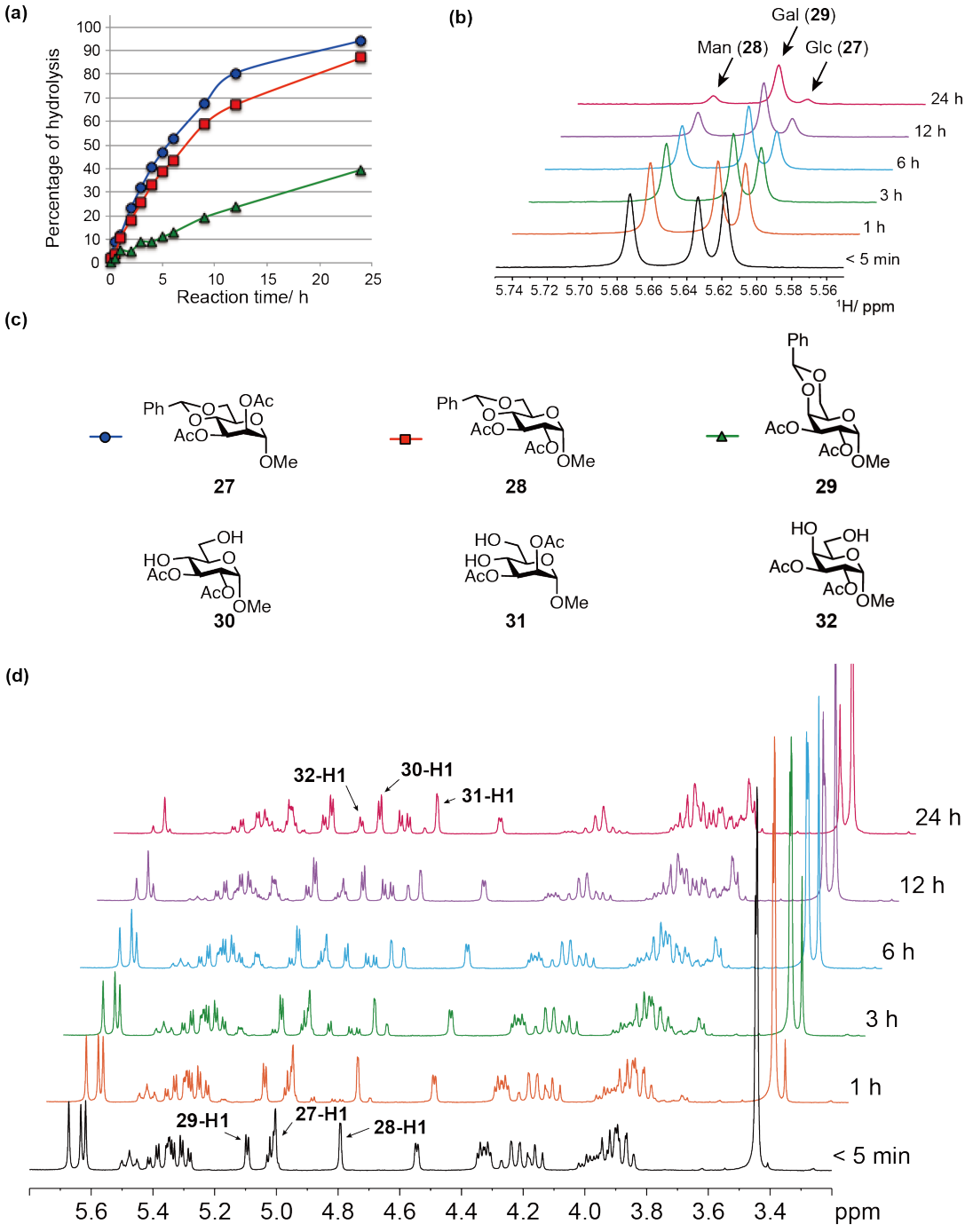


Figure 3-3. Comparison of benzylidene hydrolysis using non-acetylated monosaccharides **27**, **28**, and **29**. (a) Plotting data for percentage of acetal hydrolysis. (b) Monitoring of the competitive experiment by ^{1}H NMR. Selected regions for benzyl proton of benzylidene are shown. (c) Structures of monosaccharides. (d) Monitoring of the competitive experiment by ^{1}H NMR. All regions are shown.

3-4. Thermodynamic parameters derived by the Arrhenius and Eyring plotting

In general, thermodynamic parameters provide us deeper information about chemical reactions, and thus benzylidene deprotection reactions were furthermore investigated with varied reaction temperature to obtain thermodynamic parameters by the Arrhenius and Eyring equations. Six monosaccharide substrates were dissolved in 60% d-AcOH (10 mM), and the reactions were monitored by NMR as described above, however they were performed at 4 ^{o}C (277 K) and 33 ^{o}C (306 K), along with 23 ^{o}C (296 K), in this experiment to see the temperature dependency of reaction rates. The obtained ratio of [starting material]/ [hydrolyzed product] was used to calculate a reaction velocity using Eq. 3.5, where $[A]_0$ is the initial concentration of benzylidenated monosaccharide, [P] is the concentration of the product, k is the reaction rate, and t is the reaction time [sec]. This equation can be applicable for pseudo-first order reaction such as hydrolysis of benzylidene acetal.

$$A \rightarrow P \qquad \text{Eq. 3.1}$$

$$\frac{d[P]}{dt} = -\frac{d[A]}{dt} = k[A] \qquad \text{Eq. 3.2}$$

$$\ln[A] = \ln[A]_0 - kt \qquad \text{Eq. 3.3}$$

$$[A] = [A]_0 \times \exp(-kt) \qquad \text{Eq. 3.4}$$

$$\ln\left(\frac{[A]_0 - [P]}{[A]_0}\right) = kt \qquad \text{Eq. 3.5}$$

Plotting data and calculated reaction rates [s^{-1}] are summarized in Figure 3-4.

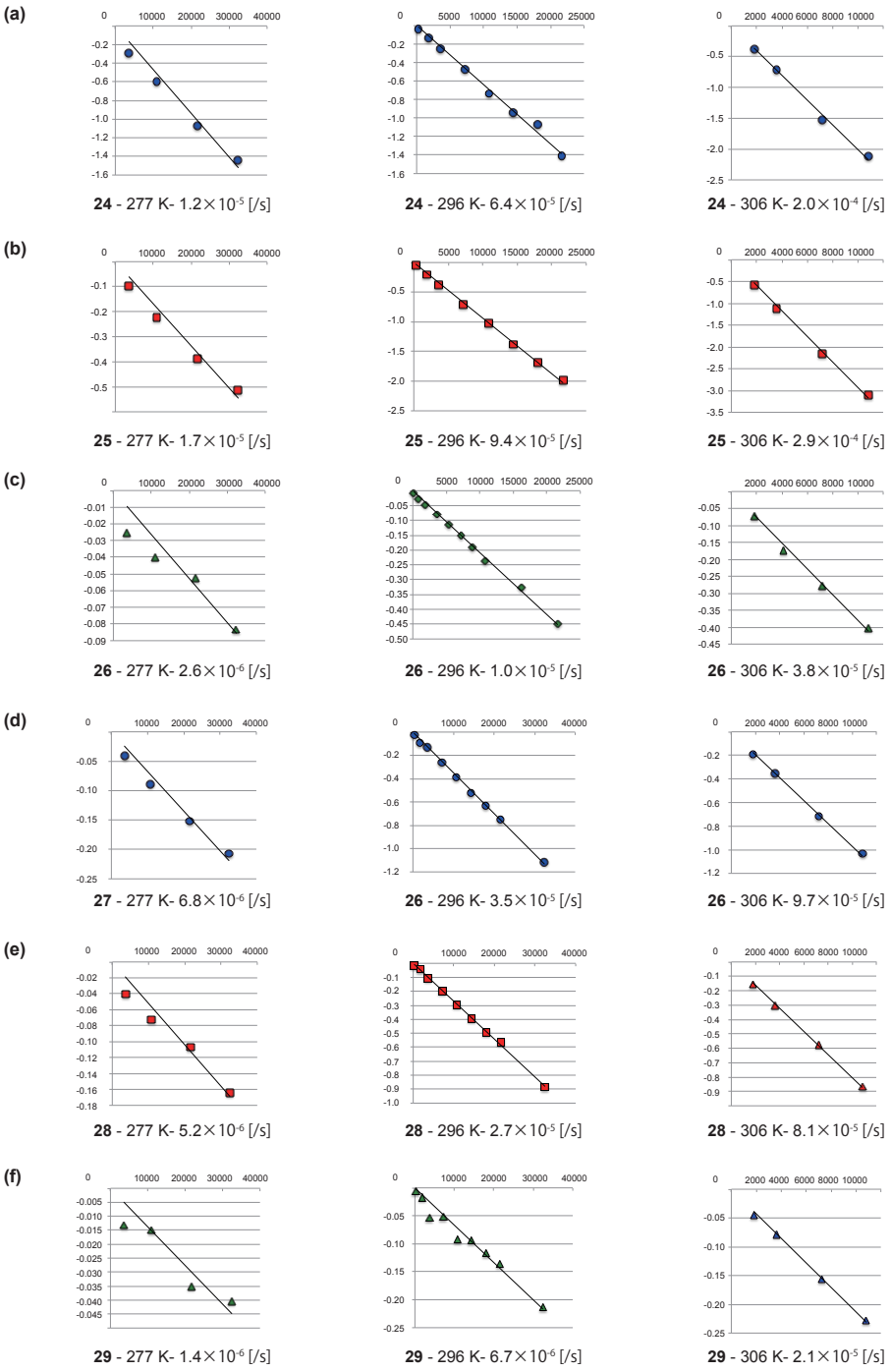


Figure 3-4. Plotting data for individual experiments. In all data, Y axis is $\ln\{([A]_0-[P])/[A]_0\}$ and X axis is reaction time [sec]. Reaction velocities [sec^{-1}] were calculated based on their slopes.

Based on the Arrhenius equation, plotting ln(k) as a function of reciprocal of temperature, in other words ln(k) versus 1/T, give a straight line revealing an activation energy (E_a), thus allowing determination of E_a values for six substrates. The Arrhenius rate law (Eq. 3.7) arises from empirical observations of the macroscopic rate constants for a particular conversion. For examples, A goes to B by various paths. This analysis determines energies for the reaction barrier. The Boltzmann distribution of molecules varies depending on reaction temperatures, thus giving different reaction kinetics. Plotting data using the Arrhenius equation (Eq. 3.7) are shown in Figure 3-5. Activation energy (E_a) and pre-exponential factor (A) were determined based on Eq. 3.8 and Eq. 3.9. Regarding both of non-acetylated and acetylated substrates, E_a values are almost same between Glc/Man, and Gal, however, on the other hand, the pre-exponential factor (A) indicate a non-negligible difference between Glc/Man, and Gal.

$$k = A \times \exp\left(\frac{-E_a}{RT}\right) \qquad \text{Eq. 3.6}$$

$$\ln(k) = \ln(A) - \frac{E_a}{RT} \qquad \text{Eq. 3.7}$$

$$slope = -\frac{E_a}{R} \qquad \text{Eq. 3.8}$$

$$intercept = \ln(A) \qquad \text{Eq. 3.9}$$

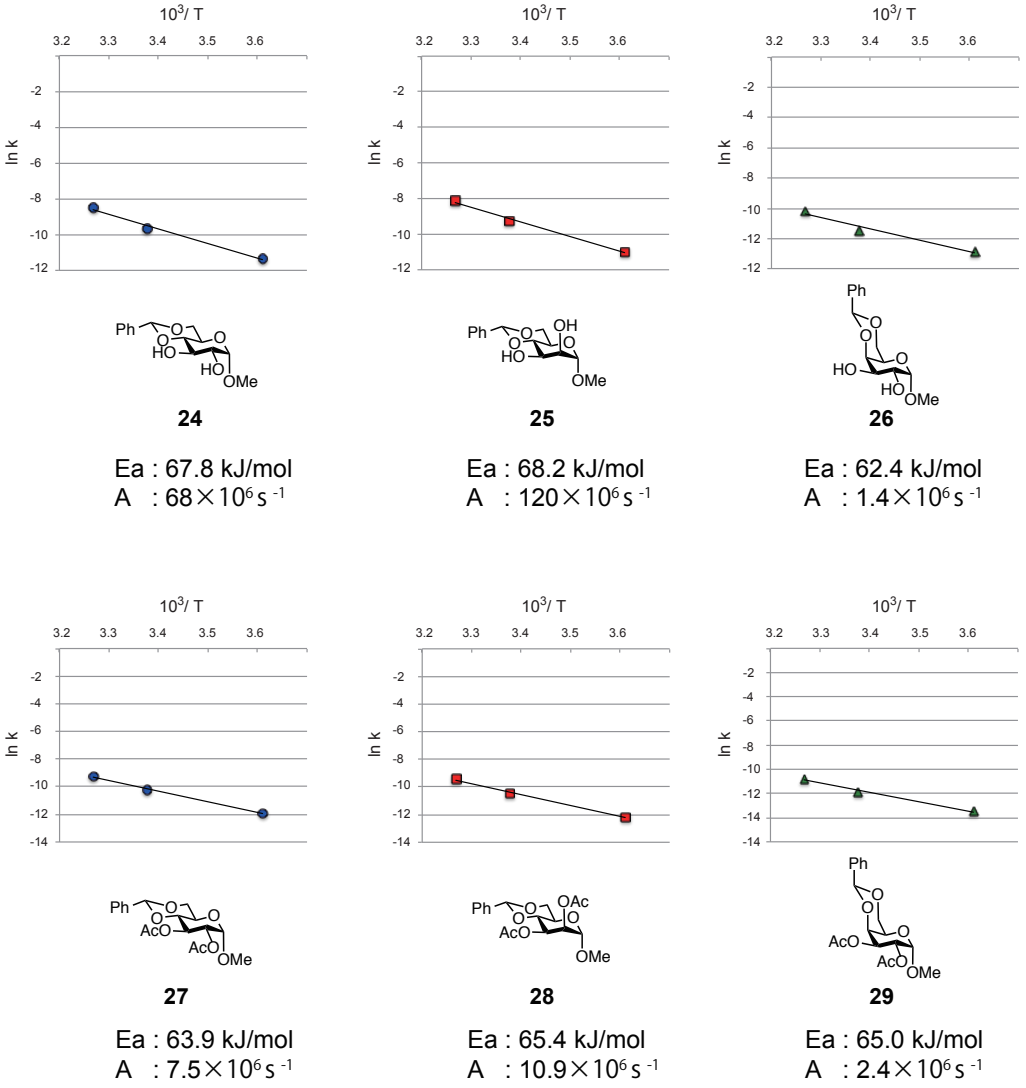


Figure 3-5. The Arrhenius plotting data of six substrates and their activation energy (E_a) and pre-exponential factor (A).

The Eyring plot produces information about the enthalpy of activation ($\Delta H^{\ddagger}$) and the entropy of activation ($\Delta S^{\ddagger}$), and the plotting data indicated that Glc/Man and Gal have entropic difference. The Eyring equation (Eq. 3.10) is derived from transition state theory (TST), where it is assumed that the reactants and activated complex are in equilibrium (Figure 3-6a). Activation parameters ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$) can be given on the basis of TST. Eq. 3.10 can be transformed in Eq. 3.11, where k_B is Boltzmann's constant, h is Planck constant, and κ is transmission coefficient. Plotting ln(k/T) versus 1/T gives a straight line, whose slope is $-\Delta H^{\ddagger}/R$ and intercept is $\Delta S^{\ddagger}/R + \ln(\kappa k_B/h)$. This Eyring plot is often used for experimental analysis. In TST, vibration in the activated complex has relationship with the rate constant for the activated complex converting to products. But, not every oscillation associated with the frequency of vibration will convert the activated complex into product. This is due to improper arrangement of other atoms in the molecule for conversion of the activated complex into products or because rotational state of the molecule interferes with the transition. Transmission coefficient (κ) is used to take these factors into account, and in my study, κ values are uniformly assumed to be 1.0 to compare the monosaccharides. I investigated removal of benzylidene acetals using same reaction condition, and structurally similar substrates were employed. Thus, difference of κ is not thought to be considerable. The plotting data, the obtained activation energy ($\Delta H^{\ddagger}$), activation entropy ($\Delta S^{\ddagger}$), and activation Gibbs free energy ($\Delta G^{\ddagger}$) are summarized in Figure 3-7.

$$k = \kappa\left(\frac{k_B T}{h}\right)\exp\left(\frac{\Delta S^{\ddagger}}{R}\right)\exp\left(\frac{-\Delta H^{\ddagger}}{RT}\right) \qquad \text{Eq. 3.10}$$

$$\ln\left(\frac{k}{T}\right) = -\frac{\Delta H^{\ddagger}}{RT} + \ln\left(\frac{\kappa k_B}{h}\right) + \frac{\Delta S^{\ddagger}}{R} \qquad \text{Eq. 3.11}$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \qquad \text{Eq. 3.12}$$

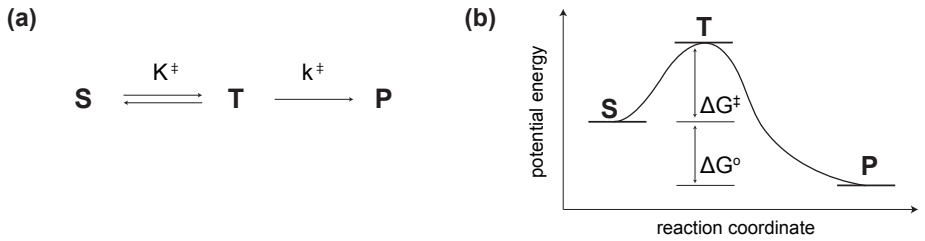
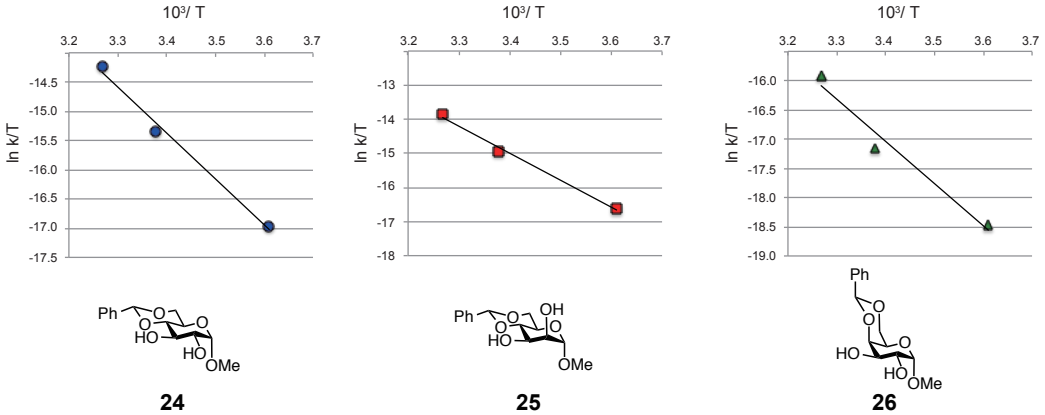


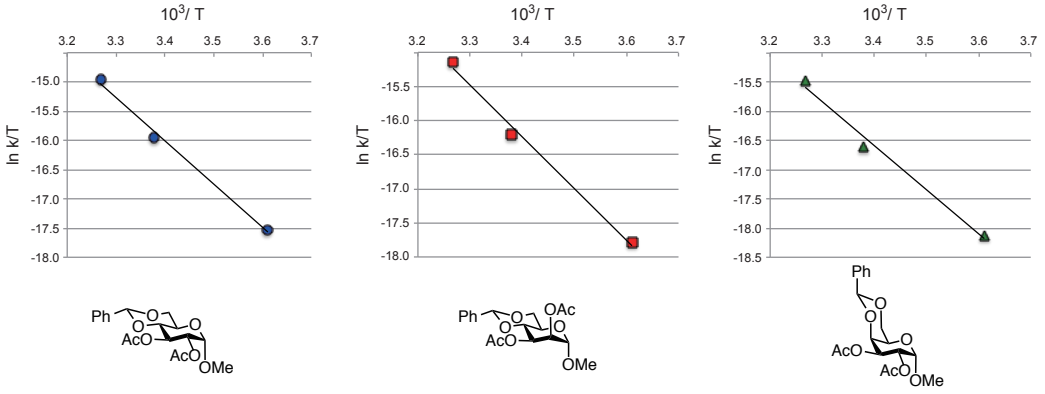
Figure 3-6. (a) Reaction scheme including transition state. (b) Diagram showing energy level of starting material, transition state, and product. ΔG^{o} is standard Gibbs free energy.



ΔH‡ : 65.3 kJ mol^{-1}
ΔS‡ : -103 J mol^{-1} K^{-1}
ΔG‡ : 96.0 kJ mol^{-1}

ΔH‡ : 65.8 kJ mol^{-1}
ΔS‡ : -98 J mol^{-1} K^{-1}
ΔG‡ : 95.1 kJ mol^{-1}

ΔH‡ : 60.0 kJ mol^{-1}
ΔS‡ : -135 J mol^{-1} K^{-1}
ΔG‡ : 100 kJ mol^{-1}



ΔH‡ : 61.5 kJ mol^{-1}
ΔS‡ : -121 J mol^{-1} K^{-1}
ΔG‡ : 97.7 kJ mol^{-1}

ΔH‡ : 63.0 kJ mol^{-1}
ΔS‡ : -118 J mol^{-1} K^{-1}
ΔG‡ : 98.2 kJ mol^{-1}

ΔH‡ : 62.6 kJ mol^{-1}
ΔS‡ : -131 J mol^{-1} K^{-1}
ΔG‡ : 102 kJ mol^{-1}

Figure 3-7. The Eyring plot data of six substrates and their activation enthalpy ($\Delta H^{‡}$) and activation entropy ($\Delta S^{‡}$). κ values are assumed to be 1.0 to give $\Delta S^{‡}$ and $\Delta G^{‡}$ values.

3-5. Interpretation and discussion

Arrhenius plot showed the difference in terms of pre-exponential factor (A), and following Eyring plot proved the difference in $\Delta S^{\ddagger}$ rather than $\Delta H^{\ddagger}$, which suggests that stereochemical difference at 4-position have influence on the reaction rate of benzylidene removing entropically. Considering the stereochemistry at 4-position, Glc and Man have equatorial hydroxy groups while Gal has an axial hydroxy group. In general, equatorial hydroxy group is more reactive than axial hydroxy group due to its less sterically hindered environment. In the case of Gal, axial stereochemistry disturbs protonation of oxygen atoms in benzylidene acetal and/or the nucleophilic attack of H_2O to oxocarbenium cation (Figure 3-8b). These steric difference around 4-OH may cause the entropic difference in the transition state of hydrolysis. In the view of *trans*- and *cis*-decalin structures, *cis*-decalin has an opened "convex" face and a hindered "concave" face, and organic synthesis sometimes employs the reactivity difference of them. Eq. 3.10 shows that reaction rate (k) is proportional to $\exp(\Delta S^{\ddagger})$, and thus it is considered that entropic 10~40 $Jmol^{-1}K^{-1}$ difference between Glc/Man and Gal (Figure 3-7) distinguishes faster and slower benzylidene hydrolysis. For example, at 296 K, hydrolysis velocities of **24** and **25** were 6.4×10^{-5} [s^{-1}] and 9.4×10^{-5} [s^{-1}], while the velocity of **26** was 1.0×10^{-5} [s^{-1}].

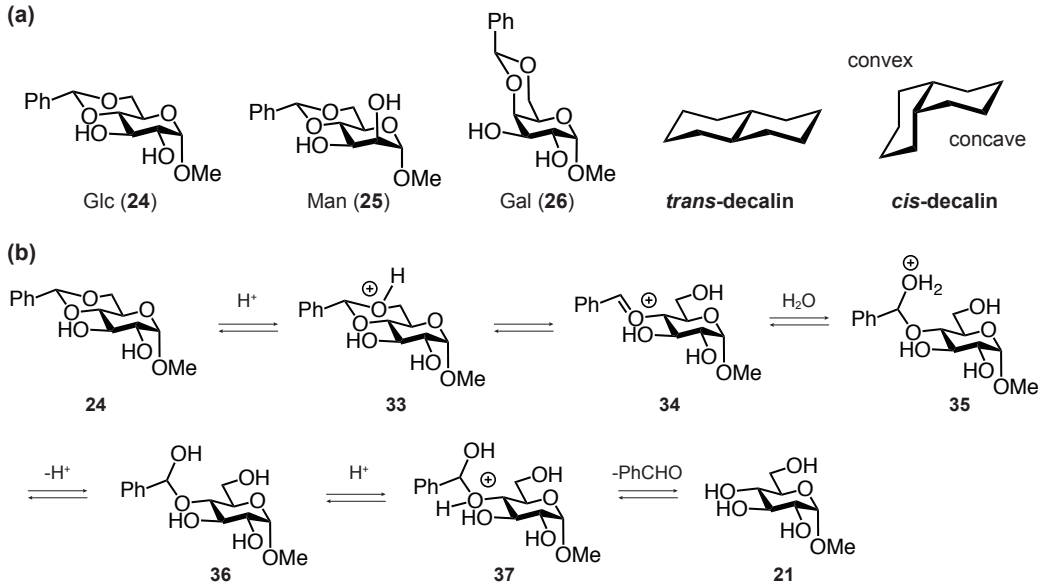
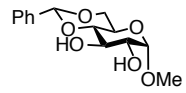


Figure 3-8. (a) Structures of benzylidenated monosaccharides and *trans*- and *cis*-decalin. Convex face is sterically less hindered face, while concave face is sterically hindered face. (b) An example of reaction mechanism of benzylidene acetal hydrolysis.

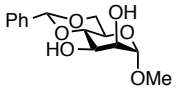
In principle, E_a derived from the Arrhenius plot and $\Delta H^{\ddagger}$ derived from the Eyring plot can be correlated by use of Eq. 3.13, and this comparison indicates the accuracy of the experiments and plotting as shown in Figure 3-9. While hydrolysis experiments were not conducted in duplicate or triplicate, discussion on thermodynamic parameters is meaningful and significant as Figure 3-9 shows that E_a values are in good accordance with E_a' values, which was calculated as $\Delta H^{\ddagger}$ +RT.

$$E_a = \Delta H^{\ddagger} + RT \qquad \text{Eq. 3.13}$$



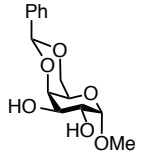
24

Ea : 67.8 kJ mol^{-1}
ΔH‡ : 65.3 kJ mol^{-1}
Ea' : 67.8 kJ mol^{-1}



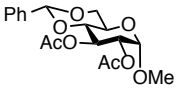
25

Ea : 68.2 kJ mol^{-1}
ΔH‡ : 65.8 kJ mol^{-1}
Ea' : 68.3 kJ mol^{-1}



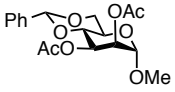
26

Ea : 62.4 kJ mol^{-1}
ΔH‡ : 60.0 kJ mol^{-1}
Ea' : 62.5 kJ mol^{-1}



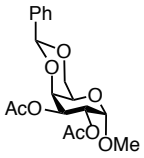
27

Ea : 63.9 kJ mol^{-1}
ΔH‡ : 61.5 kJ mol^{-1}
Ea' : 64.0 kJ mol^{-1}



28

Ea : 65.4 kJ mol^{-1}
ΔH‡ : 63.0 kJ mol^{-1}
Ea' : 65.5 kJ mol^{-1}



29

Ea : 65.0 kJ mol^{-1}
ΔH‡ : 62.6 kJ mol^{-1}
Ea' : 65.1 kJ mol^{-1}

Figure 3-9. Comparison of activation energy and activation enthalpy derived from the Arrhenius plot and Eyring plot, respectively (Ea' = $\Delta H^{\ddagger}$ +RT = $\Delta H^{\ddagger}$ [kJmol^{-1}] + 8.31 [JK^{-1}mol^{-1}] ×298 [K] / 1000).

Formation of carbonium cation is generally considered as a rate-determining step (RDS) in hydrolysis of acetal (Figure 3-10a, (i)),[4] however negative values of $\Delta S^{\ddagger}$ indicates a possibility that RDS is one of other steps. In principle, $\Delta S^{\ddagger}$ includes information about structure of transition state complex and often used to predict the structure of transition state. When two molecules react and make a unimolecular complex in transition state, $\Delta S^{\ddagger}$ will basically show a negative value, while a positive value will be observed in the case that one molecule produces two molecules by cleavage of a covalent bond. Thus, RDS in carbonium cation formation (cleavage of C-O bond in step [i] in Figure 3-10) would show a positive value. In my study, the Eyring plot produced negative $\Delta S^{\ddagger}$, so that formation of carbonium cation may not be RDS in deprotection of benzylidene acetal of monosaccharides. Some exceptions, where generation of oxocarbenium cation is not RDS, have been found, and they have RDS in a later step under some conditions such as hydration step (step [ii] in Figure 3-10).[5-10] Especially, Mcclelland *et al.* reported a change in RDS in the hydrolysis of cyclic ketals (Figure 3-10b).[11] Step [iv] is the RDS at pH >6, however at pH <5, the RDS is step [vi]. Thus, in the case of hydrolysis of benzylidene acetals in monosaccharides, cleavage of C-O bond to yield an oxocarbenium ion might not be RDS (Figure 3-8b), however other experiments such as kinetic isotope effect are required for deeper discussion on RDS in monosaccharides.

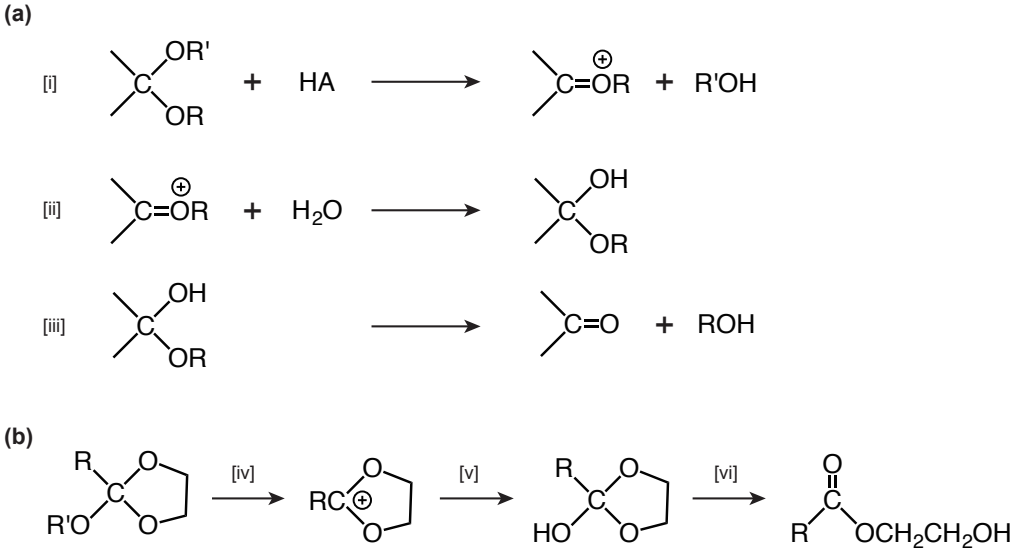


Figure 3-10. (a). Three-stage reaction mechanism in acetal hydrolysis. (b) Hydrolysis steps of cyclic ketals.

In summary, considering acetal hydrolysis of six monosaccharides, difference of stereochemistry at 4-position entropically influenced on the hydrolysis of benzylidene acetal of an oligosaccharide derivative. Thermodynamic parameters derived from six benzylidene derivatives and two types of plotting revealed the difference in $\Delta S^{\ddagger}$, which is considered to be due to stereochemistry at 4-position. It is assumed that axial and equatorial hydroxy groups make a difference where oxonium ion and/or H_2O molecules approach benzylidene acetal (protonation and hydration). Thus, sterically unfavorable galactose showed slower hydrolysis of benzylidene acetals in the case of not only monosaccharides but also the oligosaccharide (Figure 3-11). Moreover, in the case of oligosaccharide, Gal has two acetyl groups and Man has one acetyl group. As seen in Figure 3-4, acetyl group causes degreasing of hydrolysis rate when non-acetylated and acetylated monosaccharides are compared. The number of acetyl groups in Gal and Man might also affects the selective removal of benzylidene acetals of Man in the oligosaccharide.

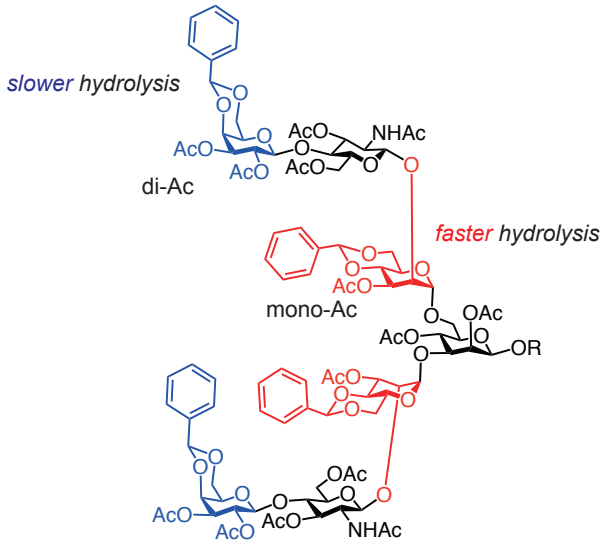


Figure 3-11. Partial structure of an oligosaccharide derivative that showed faster benzylidene removal in mannosides.

3-6. References

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In addition, for the basic theory of physical chemistry, two textbooks were greatly helpful.

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Chapter 4

Development of a New Strategy for Chemical Synthesis of a Glycopeptide Having a Complex-Type Triantennary Oligosaccharide

4-1. Introduction of *Chapter 4*

Glycopeptide synthesis is usually performed by Boc- or Fmoc-SPPS, while glycopeptide synthesis using SPPS is generally low yield and results in difficulties in obtaining a final target glycosylated polypeptide as described in *Chapter 1*. Thus, I established a new efficient strategy for the synthesis of glycopeptides by using segment coupling reactions in liquid phase. The detail of the concept and the results will be discussed in this chapter.

4-2. Outline of the strategy

I chose EPO segment [C^{79}-N^{83}(oligosaccharide)-K^{97}] as a model glycopeptide to perform the new glycopeptide synthesis. This glycopeptide will be used for further EPO synthesis, which will be described in *Chapter 5*. Retrosynthetic analysis is shown in Figure 4-1. I designed to perform enzymatic sialylation reaction after the synthesis of glycopeptide having a triantennary asialooligosaccharide **34**, [C^{79}-N^{83}(oligosaccharide)-K^{97}], to obtain sialylglycopeptide **33**. The key of the synthesis is to incorporate synthesized oligosaccharyl Asn **1** into glycopeptide **34** efficiently. I chose a segment coupling strategy as shown in Figure 4-1. To convert oligosaccharyl Asn **1** into glycopeptide **34**, carboxylic acid of the asparagine residue can be coupled with an amino group of *C*-terminal peptide **36**, of which side chains are protected. After deprotection of an Fmoc group of *N*-terminal Asn of the resulting peptide, the amino group is condensed with a carboxylic acid of *N*-terminal peptide **35**. After acidic removal of side-chain protecting groups of **34**, HPLC purification affords desired glycopeptide **34** whose *N*-terminus and *C*-terminus are functionalized with Cys and hydrazide for further reactions such as peptide ligation chemistry.[1-2]

In this strategy, protected peptide **35** and **36** are purified by RP-HPLC or gel permeation/silica gel column chromatography to yield pure materials, thus allowing efficient conversion of oligosaccharyl Asn **1**. Furthermore, coupling reaction in liquid phase is thought to perform more efficient incorporation of oligosaccharyl Asn **1** than that on solid phase, because reaction mixture is heterogeneous and small-scale reaction is difficult to be handled in solid phase synthesis.

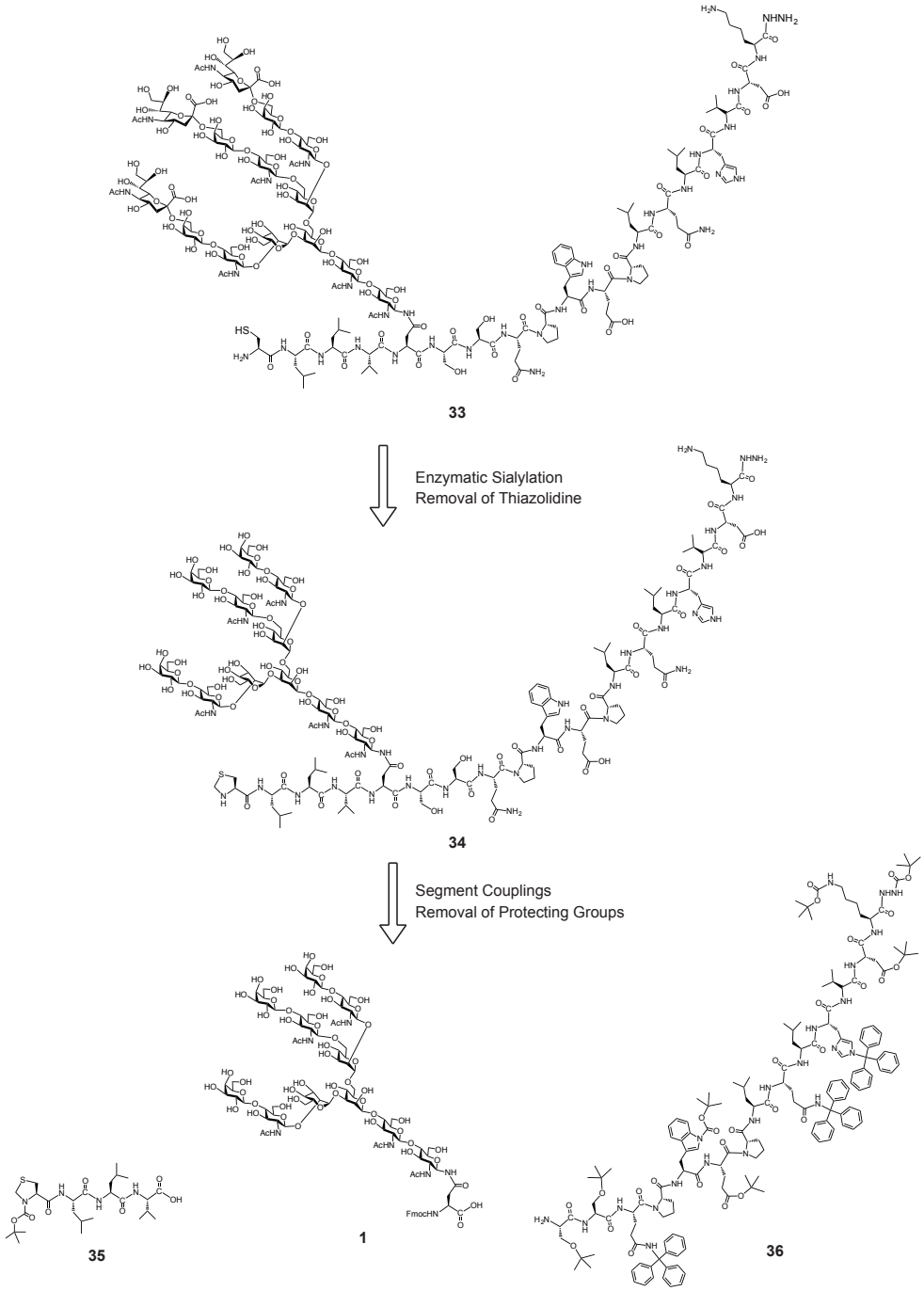

Figure 4-1. Retrosynthetic analysis of glycopeptide **33**. Sialylglycopeptide **33** can be synthesized by enzymatic α2,6-sialylation of glycopeptide **34**, which can be obtained by segment coupling reactions in liquid phase. Standard Fmoc-SPPS affords protected peptides **35** and **36**.

4-3. Preparation of *N*-terminal and *C*-terminal peptides

First, *C*-terminal peptide Boc-[Thz^{79}-Leu^{80}-Leu^{81}-Val^{82}]-OH **35** was synthesized by Fmoc SPPS, and the peptide was purified by RP-HPLC to give a pure material (Figure 4-2). The synthesis began with coupling of 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker to aminomethyl ChemMatrix resin using TBTU and *N*-ethylmorphorine. This resin is a widely used and 100% polyethylene glycol (PEG) based resin, and the HMPB linker enables generation of a side chain protected peptide with mild acidic treatment at the final step. After introduction of the HMPB linker to aminomethyl ChemMatrix resin, the first amino acid (Fmoc-Val-OH) was coupled by *N*-methylimidazole and 2,4,6-mesitylene-sulfonyl-3-nitro-1,2,4-triazolide (MNST). This coupling reagent facilitates the efficient esterification of HMPB linker-alcohol with suppressing epimerization as reported by Blankemeyer-Menge *et al.*[3] Following deprotection of an *N*-terminal Fmoc group was performed by treatment of 20% piperidine in DMF. Then, Fmoc-Leu-OH, Fmoc-Leu-OH, and Boc-Thz-OH were coupled by HBTU (3 equiv), HOBt (3 equiv), and DIEA (6 equiv) in DMF after deprotection of Fmoc group in each step. After the completion of all steps, the side chain protected peptide was detached from the resin by the treatment with AcOH/trifluoroethanol (TFE), followed by purification by RP-HPLC afforded desired tetrapeptide **35** as shown in Figure 4-2.

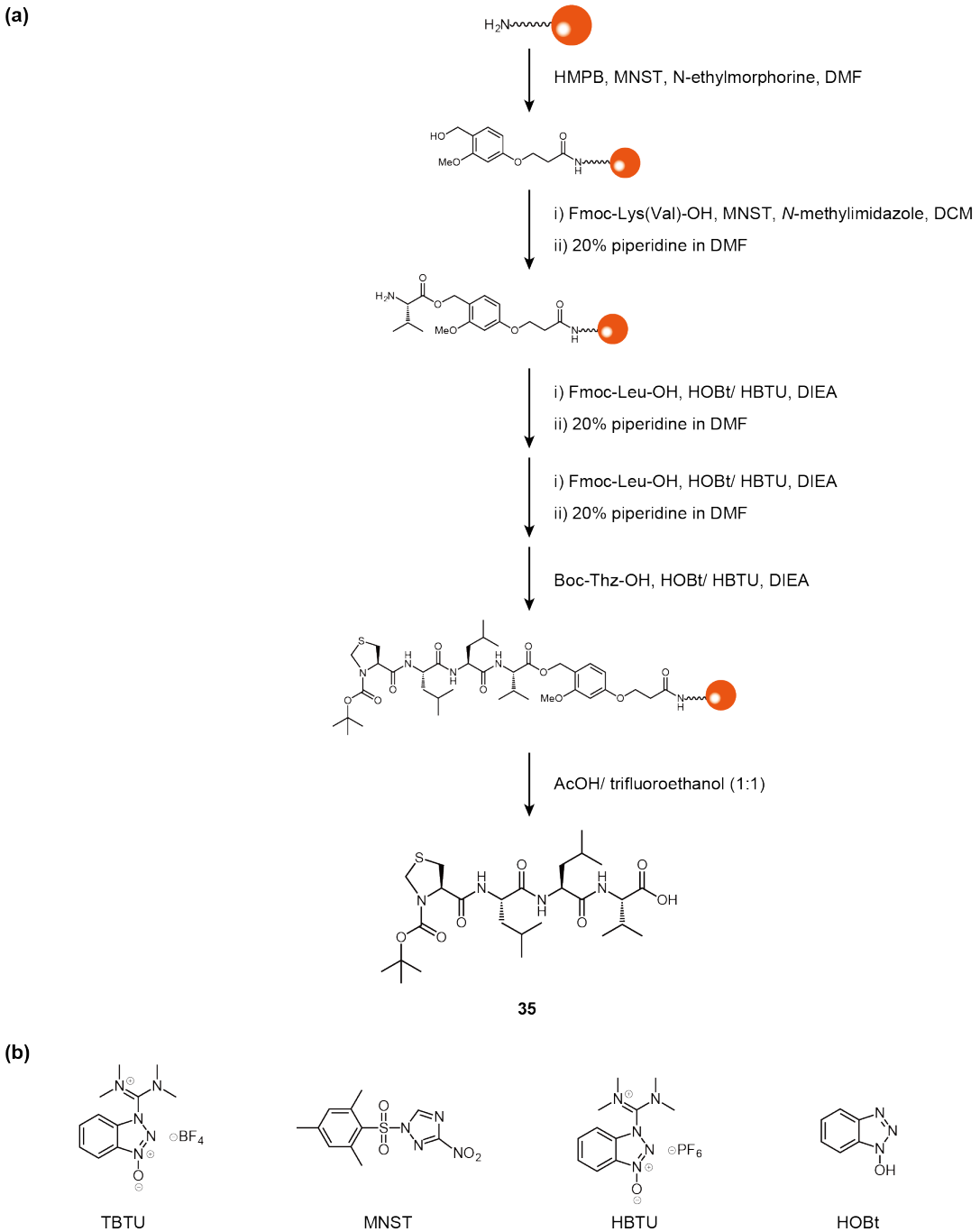

Figure 4-2. (a) Synthesis of tetrapeptide **35** by Fmoc SPPS. (b) Structures of used coupling reagents.

Next, *N*-terminal peptide of 83rd Asn position, H-[Ser(^{t}Bu)^{84}-Ser(^{t}Bu)-Gln(Trt)-Pro-Trp(Boc)-Glu(^{t}Bu)-Pro-Leu-Gln(Trt)-Leu-His(Trt)-Val-Asp(^{t}Bu)-Lys(Boc)^{97}]-OH **37**, was also synthesized by standard Fmoc-SPPS using HMPB linker as mentioned above (Figure 4-3). Fmoc-Lys(Boc)-OH was linked to HMPB-ChemMatrix resin using *N*-methylimidazole and MNST. After deprotection of Fmoc group, the peptide was elongated by use of Fmoc-AA-OH (4 equiv, 200 mM), HBTU (3 equiv), HOBt (3 equiv), and DIEA (6 equiv) in DMF. After the completion of elongation, the resulting peptide was cleaved from the resin by AcOH/ trifluoroethanol (TFE) with keeping protecting groups on side chains.

The obtained peptide mixture was purified by both gel permeation column eluted with MeOH and silica gel column chromatography eluted with chloroform/MeOH/AcOH to obtain pure protected peptide **37**. TLC analysis of the peptide showed single spot, and after removal of protecting groups of side chains, LC-MS analysis showed suitable single peak (Figure 4-4). During the peptide cleavage step, trityl group was partially deprotected because of the acidic treatment. This was confirmed by MS analysis of protected peptides after silica gel separation. The partially deprotected material was removed by silica gel column chromatography because it showed undesired side reactions during the coupling reactions in model experiments.

Then, the *C*-terminal carboxylic acid of **37** was coupled with *tert*-butyl carbazate by use of 1H-benzotriazol-1-yloxy-tri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) at -20 ^{o}C based on a modified strategy reported by Kajihara and co-workers previously.[4] They reported that immediate coupling with a large excess of nucleophiles at low temperature (-20 ^{o}C) is essential to suppress *C*-terminal racemization. This reaction allowed complete conversion of carboxylic acid into hydrazide on the *C*-terminus of **37**. After the completion of the reaction, the mixture was directly applied to gel permeation column to remove the solvent and reagents. This reaction was monitored by RP-HPLC after removal of protecting groups of side chains using TFA/TIPS/H_2O (95:2.5:2.5) as shown in Figure 4-4.

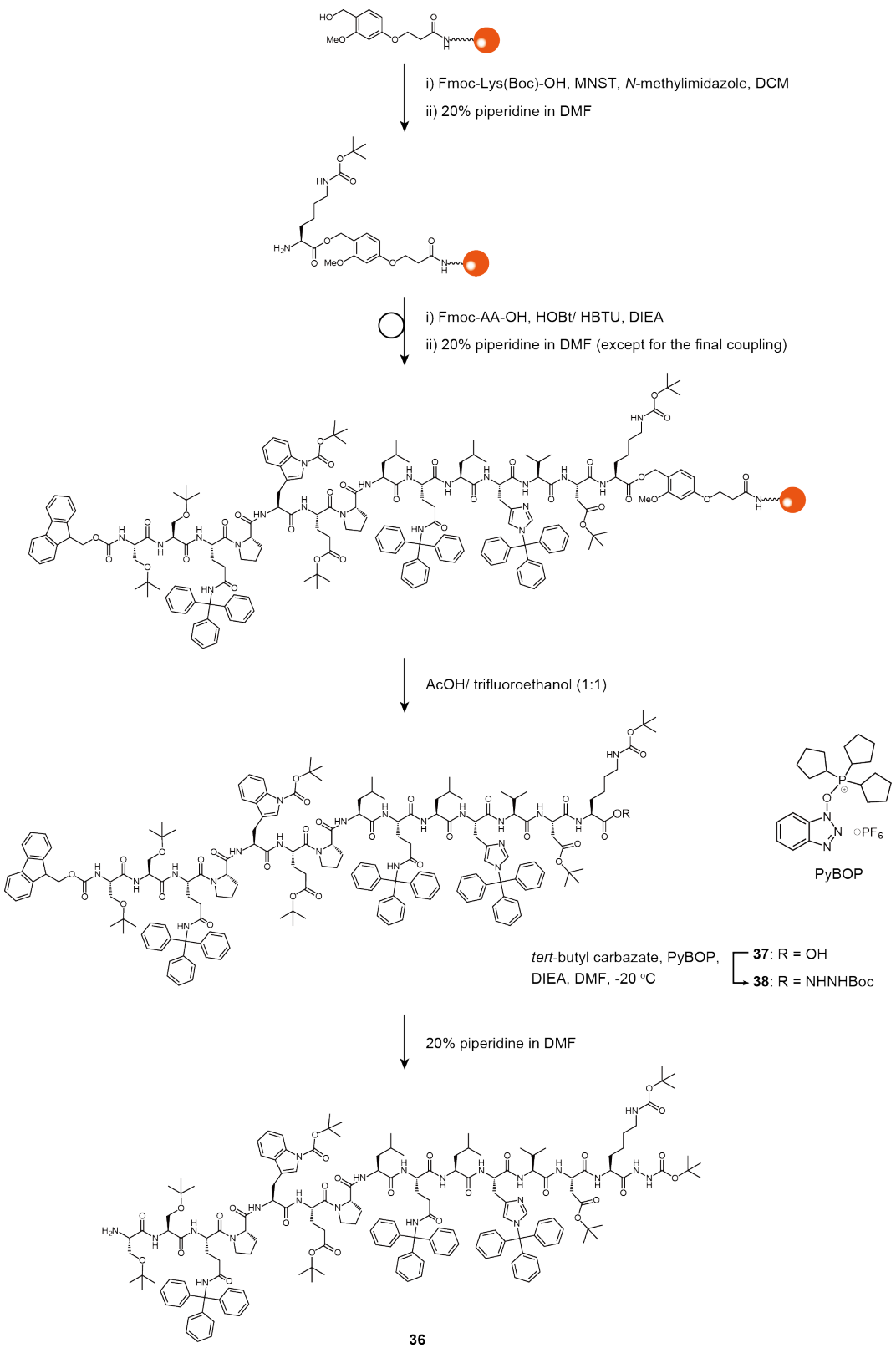

Figure 4-3. Synthesis of protected *C*-terminal peptide **36**.

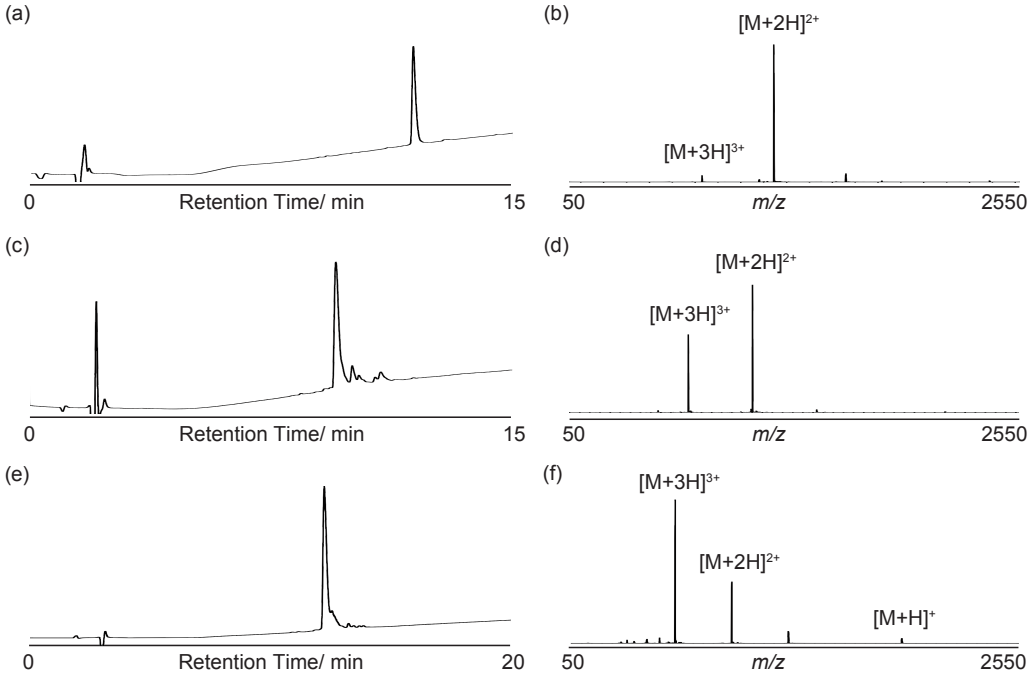

Figure 4-4. (a) LC-MS analysis of **37** after deprotection of side-chain protecting groups. (b) is the ESI-MS for the major peak in (a). (c) LC-MS analysis of **38** after deprotection of side-chain protecting groups. (d) is the ESI-MS for the major peak in (c). (e) LC-MS analysis of **36** after deprotection of side-chain protecting groups. (f) is the ESI-MS for the major peak in (e).

Boc protection of hydrazide was needed because hydrazide moiety showed reactivity in the model experiment (Figure 4-5). During this experiment, coupling of *N*-terminal peptide **35** was examined with glycopeptide having biantennary glycan **39**. This reaction produced considerable amount of **41** in addition to desired **40** when PyBOP, HOBt, and HODhbt were used as coupling reagents. Optimizing equivalents of reagents and diluting the reaction conditions were not successful to make selectivity between amine and hydrazide. This is thought to be because of steric hindrance of the amino group due to the close oligosaccharide, thus lowering the reactivity of the amine group. The double coupling of **35** was completely not observed when *C*-terminal hydrazide was protected with Boc group.

Then, the peptide **38** was treated with 20% piperidine to deprotect *N*-terminal Fmoc group for the next reaction (Figure 4-3). After the completion of the reaction, the reaction mixture was also directly applied to gel permeation column to remove the solvent and undesired products.

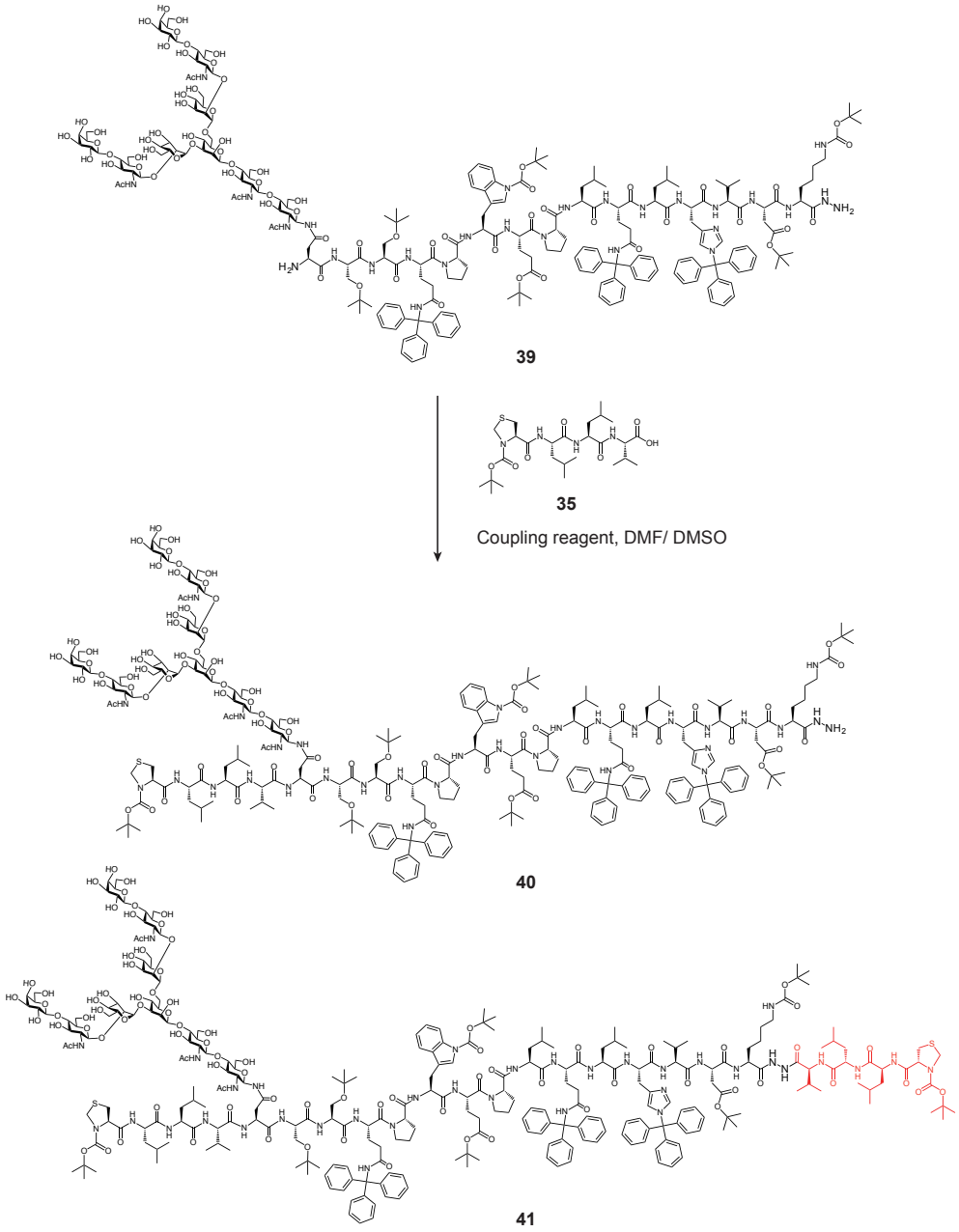

Figure 4-5. Undesired side reaction at the *N*-terminal hydrazide without Boc group.

4-4. Glycopeptide synthesis by segment coupling reactions

In order to introduce synthesized triantennary oligosaccharide **1**, the carboxylic acid of **1** was coupled with the *N*-terminus of the peptide in DMF/DMSO by using PyBOP (Figure 4-6). This reaction used 1.8 equivalent of protected peptide **36** to ensure full conversion of oligosaccharyl asparagine into desired product **42**. I selected PyBOP as a coupling reagent because it is usually used for coupling of oligosaccharyl asparagine on resin during standard glycopeptide synthesis.[5] In the case of triantennary oligosaccharyl asparagine, aspartimide formation was not observed in the optimized conditions. This reaction was monitored by TLC analysis directly and LC-MS analysis after removing side chain protections. After the completion of the reaction, the reaction was directly applied to gel permeation column to remove solvents and reagents. A mixture of glycopeptide **42** and remaining peptide **36** was used for the next reaction without further reaction due to a difficulty of separation.

Glycopeptide **42** was then treated with 20% piperidine to remove Fmoc group, and the resulting glycopeptide **43** was further coupled with *N*-terminal tetrapeptide **35** (Figure 4-6). Peptide-glycopeptide coupling of glycopeptide **43** and **36**, along with residual protected peptide **36**, was performed by HODhbt-DIC activation. This coupling was repeated twice using 20 equivalents of tetrapeptide **35** (with respect to **43**) to increase conversion into **44**, however a small amount of **43** remained (88% conversion from **43** into **44**). Reactivity of *N*-terminal amine of **43** was low possibly due to steric hindrance of triantennary oligosaccharide. After the reaction, the mixture was also applied to gel filtration.

After construction of the desired glycopeptide with protecting groups, it was treated with 2.5% TIPS and 2.5% H_2O in TFA to remove all protecting groups for side chains and Boc group of *C*- and *N*-termini. RP-HPLC purification afforded the desired glycopeptide **34** in 28% isolated yields (calculated based on oligosaccharyl asparagine **1**). This yield is higher compared with glycopeptide synthesis by standard Boc- or Fmoc-SPPS. Using purified segments and oligosaccharyl Asn coupling in liquid phase are expected to produce the higher yield.

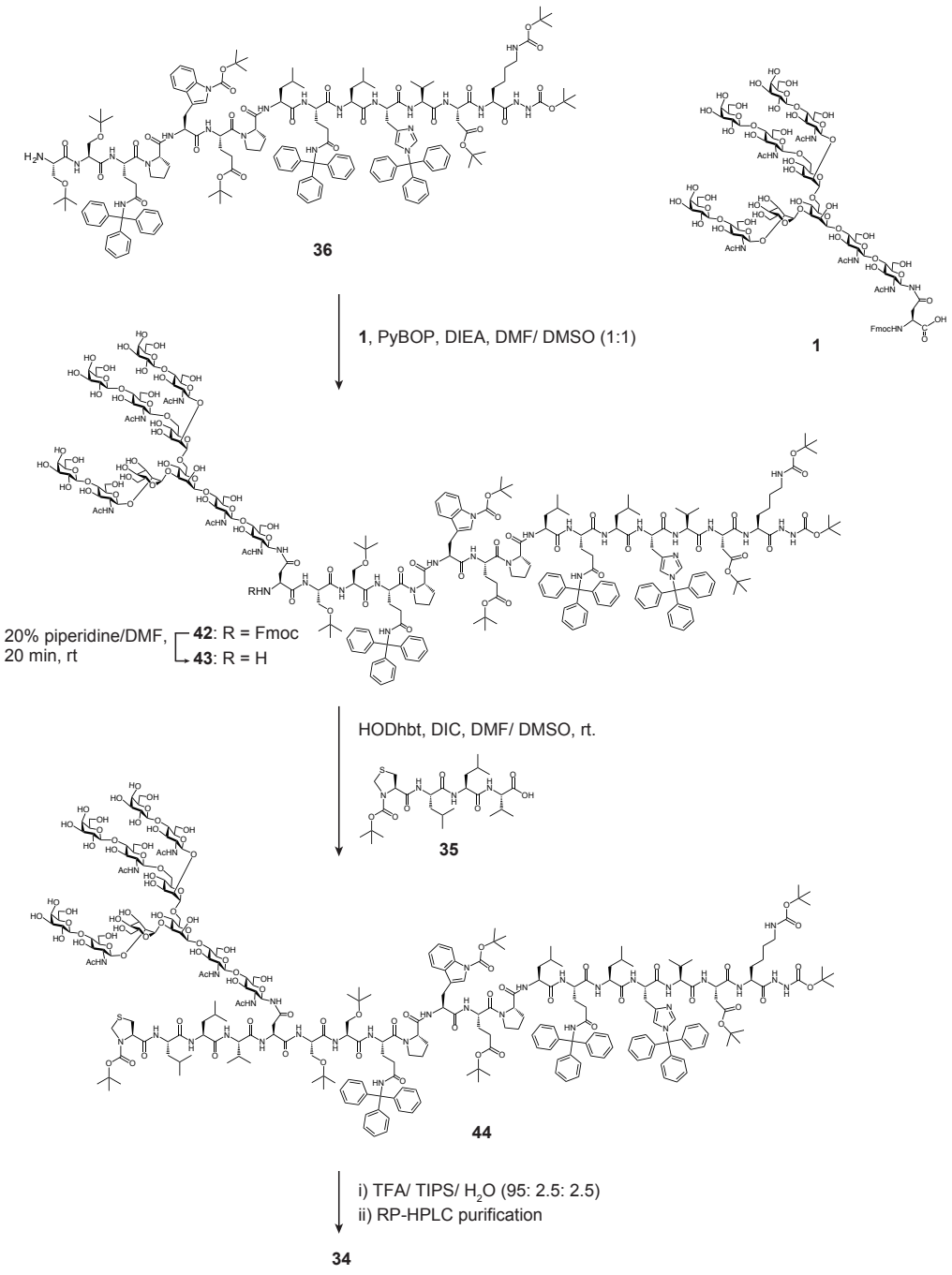

Figure 4-6. Synthesis of desired glycopeptide **34**.

For this peptide-glycopeptide segment coupling, three types of coupling reagents were investigated to suppress epimerization at Val by use of glycopeptide having biantennary oligosaccharide **39** and tetrapeptide **35** (The reaction scheme is shown in Figure 4-4). As standard, glycopeptide **44** (all L-amino acids) and **45** (including a D-Val) were prepared by Boc-SPPS. Briefly, glycopeptides were synthesized by HBTU activation on a $HSCH_2CH_2C(=O)NH$-PEGA resin (Protocols of glycopeptide synthesis is shown *Chapter 5*). Coupling reaction of **39** and **35** were tested by B) PyBOP-DIEA, C) HOBt-DIC, and D) HODhbt-DIC activation in DMF-DMSO. Six equivalents of individual reagents were used for the coupling reactions in addition to 6 equivalents of tetrapeptide **35** at rt. After the reactions, aliquots of the reaction mixtures were treated with 2.5% TIPS and 2.5% H_2O in TFA to remove protecting groups and analyzed by RP-HPLC as shown in Figure 4-7. LC chromatograms of standard **44** (A) and **45** (E), and a mixture of **44** and **45** (F) are also shown in Figure 4-7. As a result, it was found that HODhbt-DIC is the best condition to decrease the racemization (<2%) compared with PyBOP (28%) and HOBt (9%).

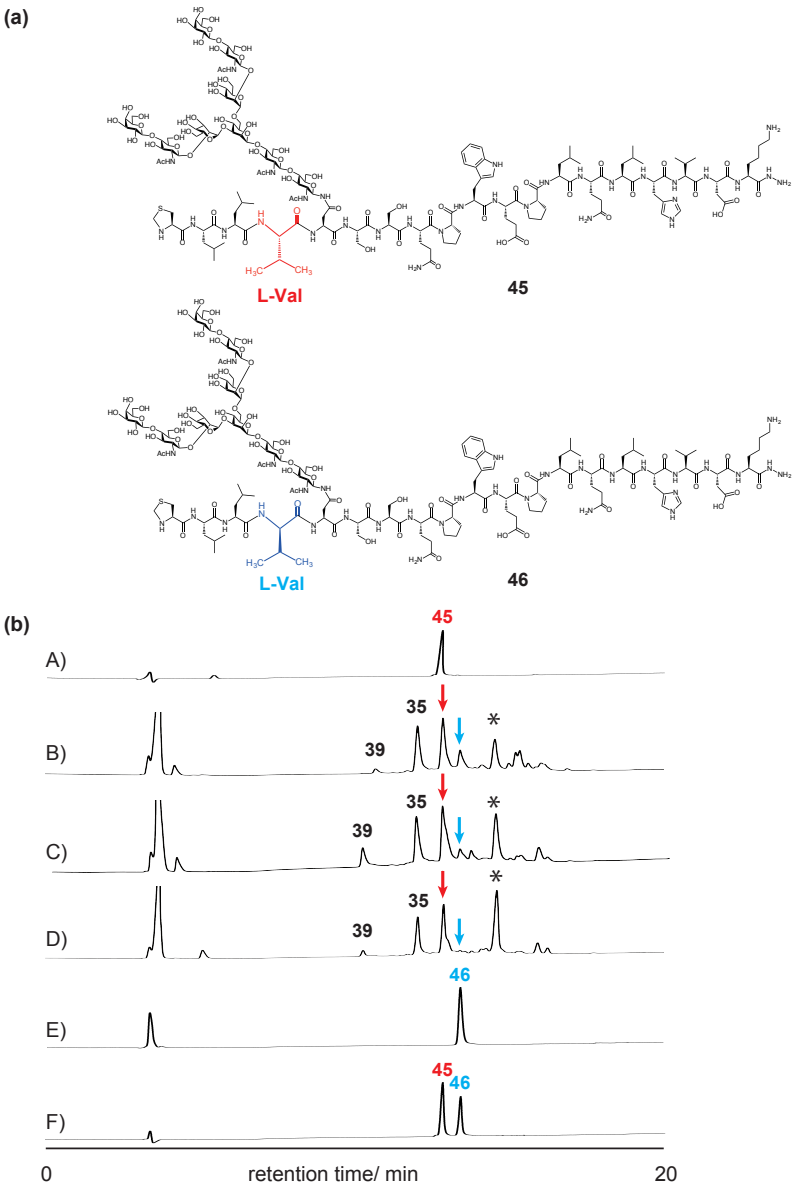

Figure 4-7. An epimerization study for the coupling of tetrapeptide **35** and model glycopeptide having a biantennary oligosaccharide **39**. (a) Structures of glycopeptide standards **45** and **46** prepared by Boc SPPS. (b) LC chromatograms of standards (A, E, and F) and coupling reactions (B, C, and D). * is a mixture of two compounds. One is coupling product of **36** and tetrapeptide **35**, which lacks oligosaccharyl asparagine. Another product is deprotection form of **41** (shown in Figure 4-5), which has additional tetrapeptide on the hydrazide moiety.

To confirm the structure of the glycopeptide having a triantennary oligosaccharide, MSMS analysis of **34** was performed by electron-capture dissociation (ECD) method.[6-7] In brief, in the ECD process, a low-energy electron (<0.2 eV) is captured by multiply protonated peptides, $[M+nH]^{n+}$, and that yields the $[M+nH]^{(n-1)+\cdot}$. By an energetic H transfer to the backbone carbonyl group, the species dissociate to form c- and z-type ions (Figure 4-8). This MS/MS method cleaves only peptide bonds (N-Cα), thus allowing the observation of oligosaccharyl asparagine residues. By using this method, all peptide bonds including Val-Asn(oligosaccharide) and Asn(oligosaccharide)-Ser bonds were identified, except for Glc-Pro and Glu-Pro bonds. AA-Pro bonds are, in principle, not observed because amine is tertiary. These data prove the success of a new segment coupling strategy.

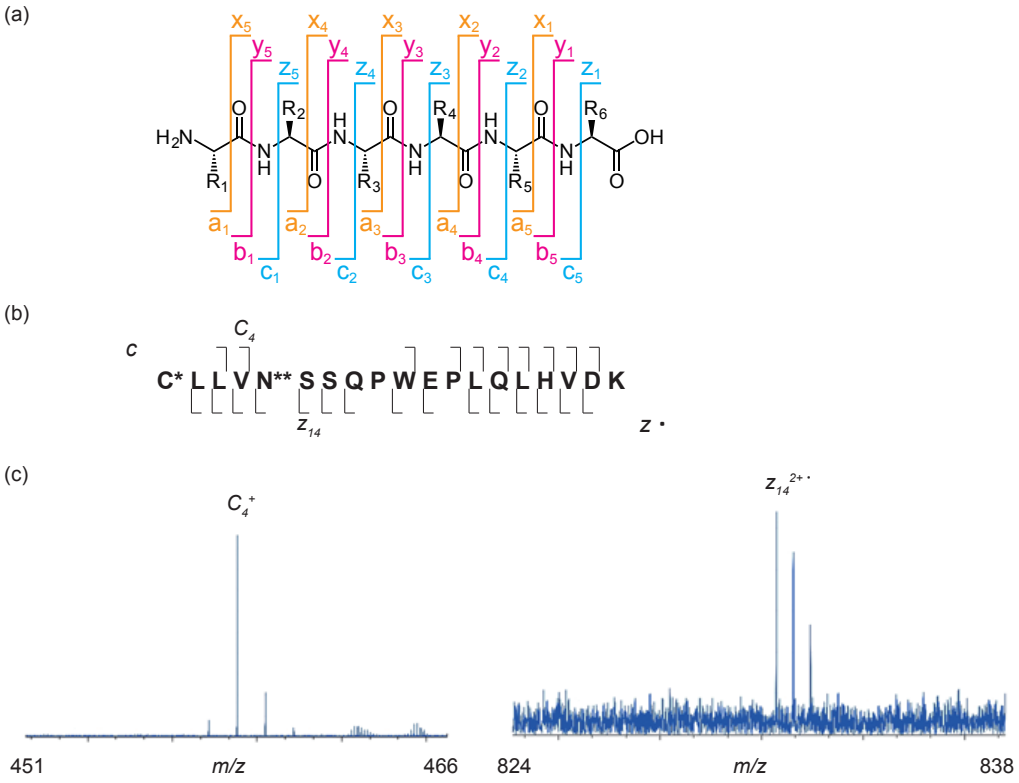

Figure 4-8. (a) Nomenclatures used for assignment of product ions in the ECD spectra of peptides and proteins in addition to CID and ETD spectra. (b) Observed fragmentation pattern of *c* and *z* ions produced from glycopeptide having a triantennary asialooligosaccharide **34**. (c) Selected regions of MS/MS spectrum to show the observed c_4+ and $z_{14}^{2+\cdot}$ ions, which indicates the peptide bonds between Val-Asn(oligosaccharide) and Asn(oligosaccharide)-Ser. * Cysteine position is thiazolidine in this glycopeptide. **Asn has a triantennary oligosaccharide.

4-5. Synthesis of sialylglycopeptide by enzymatic sialylation and deprotection of thiazolidine.

In order to use synthesized glycopeptide **34** for protein synthesis, glycopeptide was subjected to enzymatic α2,6-sialylation in addition to removal of thiazolidine. Bacterial enzyme (*Photobacterium damsela*, [EC 2.4.99.1]) was used for this glycosylation.[8] The reaction was performed using the enzyme, alkaline phosphatase (calf intestine), and CMP-Neu5Ac in HEPES buffer (pH 5.9) containing 0.5% triton X. To ensure the maximum conversion of glycopeptide **34** into sialyl-glycopeptide **33**, excess of CMP-Neu5Ac (30 equiv × 2) was employed. After installation of sialic acid moieties on terminal galactosides, *O*-methylhydroxylamine was added to the reaction mixture carefully and the pH value of the mixture was adjusted to 4.3, thus resulting in conversion of thiazolidine into cysteine residue. After purification by RP-HPLC, the desired sialyl-glycopeptide was obtained 55% isolated yield. *C*-terminus of sialylglycopeptide **33** is cysteine residue, which allows NCL reaction at the site. Moreover, N-terminus is α–hydrazide enabling the hydrazide ligation at the site.

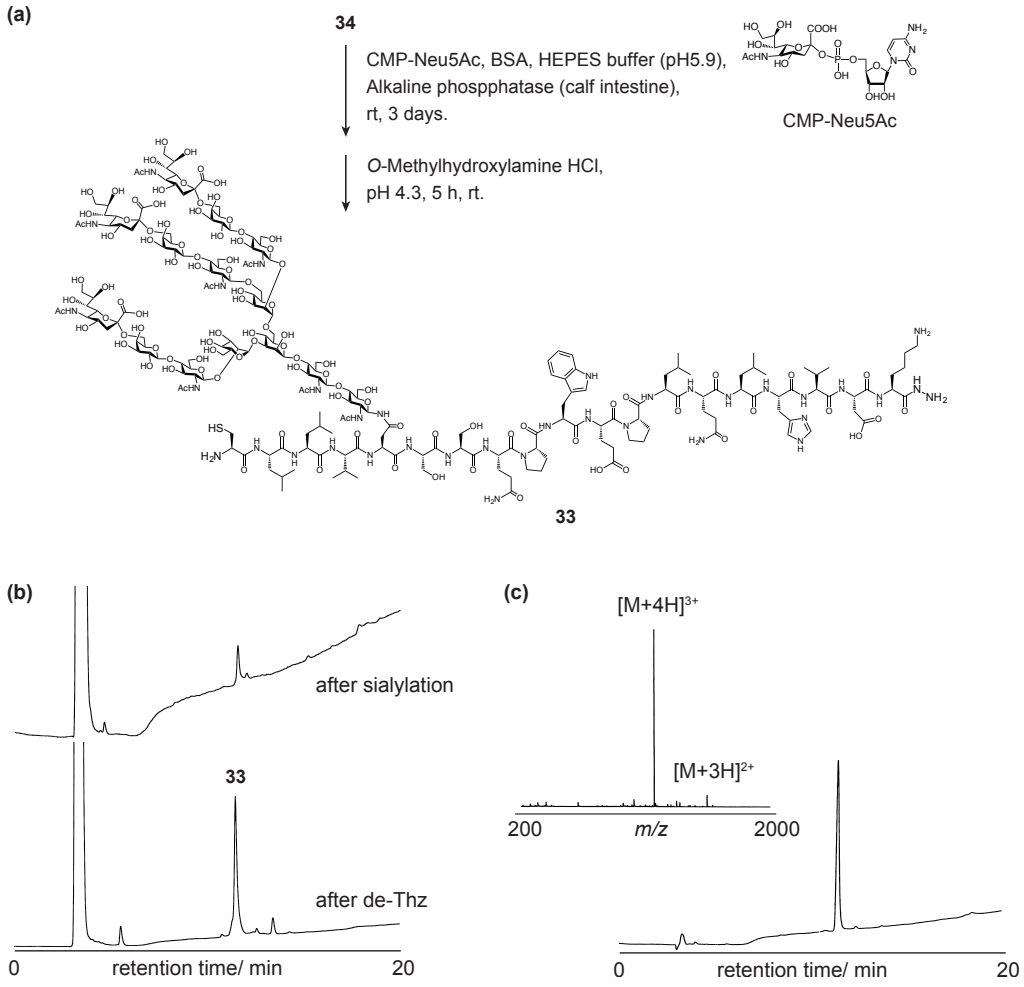

Figure 4-9. (a) Synthesis of sialylglycopeptide **33**. (b) HPLC chromatograms for sialylated product and thiazolidine-removed product. (c) HPLC chromatograms and MS spectrum of purified **33**.

4-6. Summery of *Chapter 4*

I established an efficient practical strategy for the synthesis of glycopeptide having a synthesized triantennary oligosaccharide **34**. Based on coupling reactions in liquid phase, oligosaccharyl asparagine was efficiently converted into glycopeptide in good yield (28%). Using purified peptides is also thought to increase the isolated yield. Furthermore, sialylation reaction successfully produced sialylglycopeptide **33** whose both of *C*- and *N*-terminus can be used for peptide ligation chemistry for synthesis of proteins/ glycoproteins.

This strategy is applicable for other glycopeptide syntheses, which are expected to show better isolated yield compared with standard Boc or Fmoc SPPS. Based on my synthesis, the key is to prepare highly pure materials and suppress epimerization. In my synthesis, tetrapeptide **35** can be purified by RP-HPLC and it was not difficult to obtain pure material. In terms of preparation of *C*-terminal protected peptide, standard Fmoc SPPS afforded carboxylic acid **37** with high purity even though a trityl group was partially removed during mild acidic cleavage. Combination of gel filtration and silica gel column chromatography in addition to efficient conversion into hydrazide was essential to prepare peptide **36**. With respect to epimerization of C-terminal Val of **35**, HODhbt activation can suppress the undesired reaction. This racemization is thought to depend on *C*-terminal amino acids and peptide sequence. I presume that side chain of Val caused 28% epimerization in the case of PyBOP due to its bulkiness.

Unverzagt has reported the synthesis of a glycopeptide by use of an oligosaccharyl asparagine, unprotected Fmoc-[Ser-Ser-Ser]-OH, and unprotected H-Tyr-OMe;[9] however, my strategy shows the feasibility of making 19-mer peptide and introducing ligation sites on both *N*- and *C*-termini. Thioester or equivalent hydrazide moieties of *C*-terminus of peptide, which can be easily incorporated in this strategy, is crucial in terms of protein synthesis and is not straightforward especially in Fmoc SPPS. Moreover, I chose thiazolidine to protect the thiol group on *N*-terminus. Thus, the first ligation of the peptide is limited on *N*-terminus because thiazolidine group is unstable during activation of hydrazide to thioester. However, this protecting group can be varied such as Acm group, which enables the peptide ligation reactions on both of *N*-and *C*-termini. In my synthesis, I avoided the use of Acm group because deprotection of Acm sometimes results in low yield, therefore I selected thiazolidine to synthesize glycopeptide and glycoprotein having the synthesized triantennary oligosaccharide

(Glycoprotein synthesis is described in *Chapter 5*).

4-7. References

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Chapter 5

Chemical Synthesis of an Erythropoietin Glycoform Bearing a Triantennary Sialyloligosaccharide and Two Biantennary Sialyloligosaccharides

5-1. Introduction of *Chapter 5*

Erythropoietin (EPO) is a glycoprotein hormone and the principal regulator of erythropoiethis, thus stimulating red blood cell production.[1] EPO consists of 166 amino acid residues and two disulfide linkages (Cys^{7}-Cys^{161} and Cys^{29}-Cys^{33}), and the syntheses of EPO glycoforms and analogs have been reported by some groups.[2-9] In the view of carbohydrate, EPO has three complex-type oligosaccharides at 24^{th}, 38^{th}, and 83^{rd} Asn, and an EPO glycoform having three biantennary oligosaccharides has been synthesized by Murakami *et al*.[9]

I have studied the synthetic methods of EPO having a triantennary oligosaccharide and two biantennary oligosaccharides, along with the findings of efficient synthetic methods of oligosaccharide and glycopeptide. Branching pattern of oligosaccharides of EPO is known to be critical for its biological activity *in vivo*.[10-12] Predominant structure of oligosaccharides at 38^{th} and 83^{rd} Asn are tri- and tetra-antennary forms.[13] Considering these properties, EPO is a suitable model glycoprotein to investigate how the oligosaccharide pattern alters chemical as well as biological property of a glycoprotein. Introduction of several types of branched oligosaccharides will reveal the effect of oligosaccharide part on EPO activity and physiological property, thus giving us insight into the relationship between oligosaccharide functions/diversity and glycoprotein activity. Although several groups have reported EPO syntheses, an efficient synthetic route is still required for the synthesis of EPO glycoforms having highly branched oligosaccharides. To the best of my knowledge, no group has reported the synthesis of EPO glycoform bearing fully sialylated highly branched oligosaccharides due to difficulties in not only oligosaccharide preparation but also tough chemical synthesis of the long polypeptide chain. Therefore, I examined chemical synthesis of EPO having a triantennary oligosaccharide at 83^{rd} Asn and two biantennary oligosaccharides at 24^{th} and 38^{th} Asn with improving the synthetic strategy in order to show the robustness of oligosaccharide/glycopeptide synthesis.

5-2. Synthetic strategy of EPO

In order to elongate polypeptides, 166 amino acid-sequence was divided into 6 segments as previously reported,[9] and NCL and hydrazide ligation couples them to give a full length of glycosylated polypeptide.[14-15] In general, *N*-terminus of peptides requires free Cys residue for the native chemical ligation and hydrazide ligation, and during ligation at *C*-terminus, acetamidomethyl (Acm) or thiazolidine (Thz) were designed to cap thiol of *N*-terminal Cys to make it inactive. In terms of the EPO synthesis, EPO has only four Cys residues, so that Ala$^{50, 79, 98, \text{and } 128}$ were substituted with Cys and used for ligation sites, which were reduced to native Ala using radical reaction in late stage.[16] Thus, individual ligation site is designed to be Cys or Ala residues in the native sequence. Gln^{78} was only mutated to Ala because of a difficulty and risk of side reaction such as epimerization during NCL. All (glyco-)peptides were prepared by the established Boc and Fmoc chemistry, except for segment 4 having a triantennary oligosaccharide (*Chapter 4*). To convert Seg4 into glycosylated EPO-full length, the ligation order was modified compared with that by Murakami *et al.,* where Seg 4 was first ligated with Seg 56, followed by ligation with Seg 23 to give Seg 23456. A detail of the synthesis is described later in this chapter.

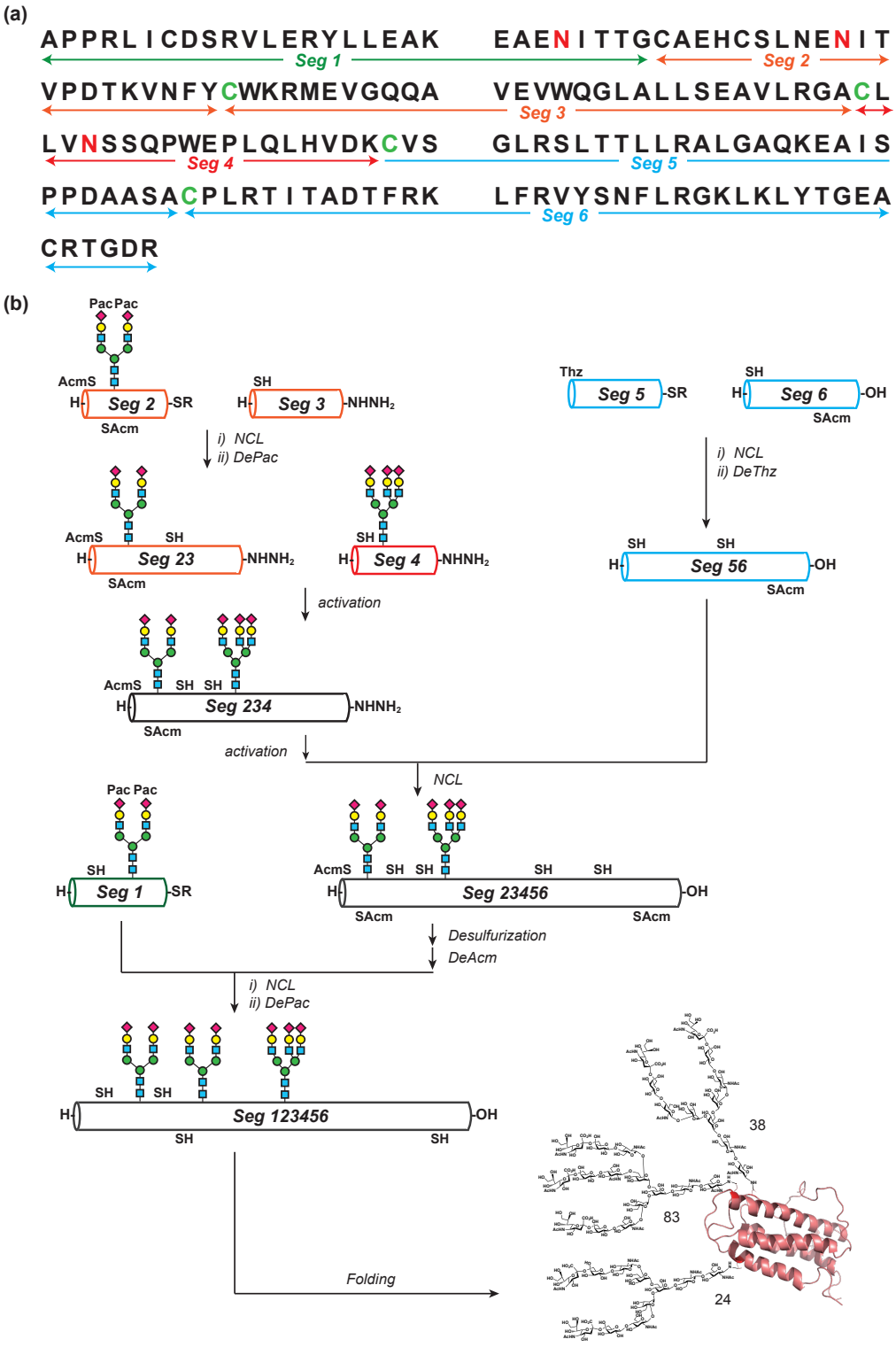


Figure 5-1. (a) Amino acid sequence of EPO, where some native Ala residues are drawn as Cys for NCL. (b) Ligation strategy for EPO.

5-3. Synthesis of phenacyl esterified biantennary sialylglycopeptide

Sialyl linkage is not compatible with deprotection condition in Boc SPPS; however esterification of carboxylic acid of sialic acid with phenacyl group renders sialoside stable even under TFA/TfOH deprotection in Boc SPPS.[6] First, Fmoc-Asn(Sialyloligosaccharide)-OH **47** was treated with Cs_2CO_3 followed by esterification by 2-bromoacetophenone (Phenacyl bromide) gave selectively esterified oligosaccharyl asparagine **48** in 59% according to Murakami's procedure. Following deprotection of Fmoc group and subsequent installation of Boc group by BocOSu gave the desired product (**49**), which is compatible with Boc SPPS.

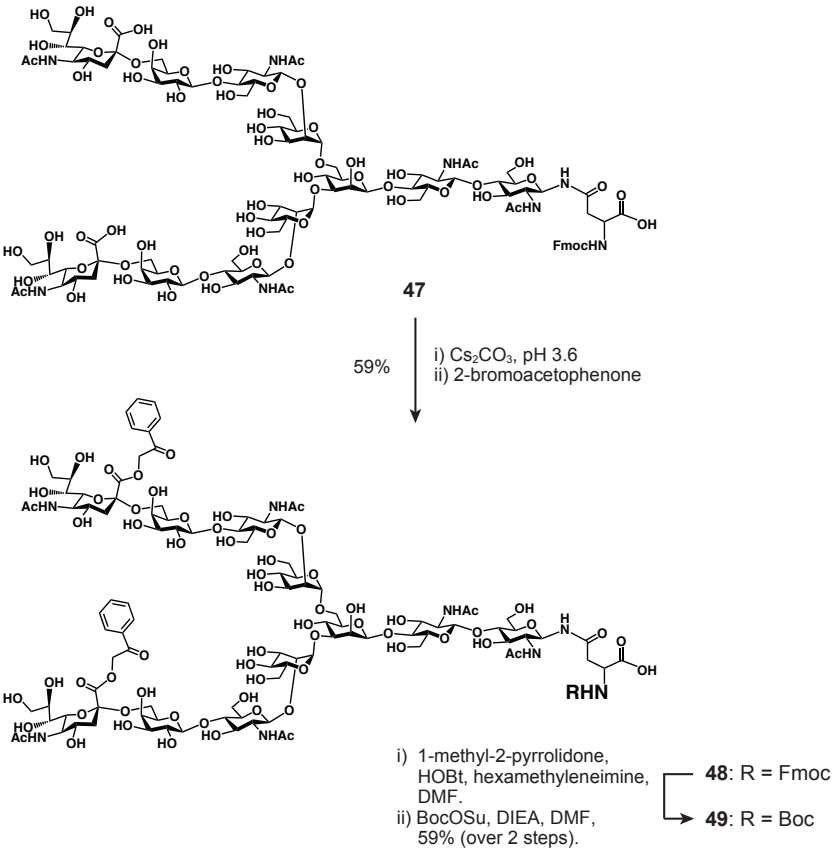
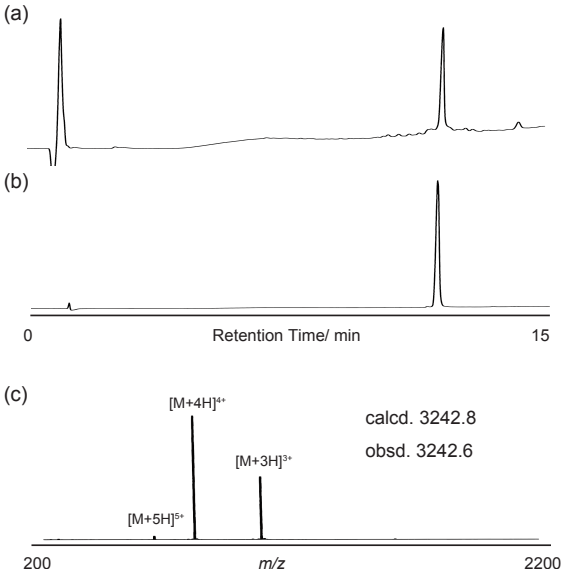


Figure 5-2. Preparation of phenacyl esterified oligosaccharyl Asn.

5-4. Preparation of peptides and glycopeptides

Seg 3 (**50**) was prepared by Boc-SPPS as shown in Figure 5-3. *S*-trityl-3-mercaptopropionic acid was linked with amino PEGA resin as a linker. After deprotection of trityl group by 5% TIPS in TFA, the first amino acid coupling was performed by Boc-Ala-OH (4 equiv, 200 mM), HBTU (3.8 equiv), and DIEA (8 equiv) in DMF. Following TFA treatment of the resin to remove Boc group, the amino acid elongation was repeated. After the completion of the all coupling steps, the resin was treated with low acidic cocktail [TFA/ DMS/m-cresol/TfOH (5:3:1:1)] and highly acidic cocktail [TFA/thioanisole/EDT/TfOH/ (20:2:1:2)] at 0 ^{o}C to remove protecting groups for *N*-terminus and side chains on resin. Then, the resulting unprotected peptide was cleaved from the resin by thioester exchange reaction using 300 mM sodium 2-meraptoethanesulfonate (MESNa) in phosphate buffer to give peptide-^{a}thioester **50** (ca. 15%).



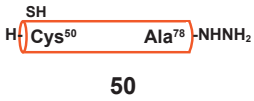


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Figure 5-3. (a) HPLC analysis of the crude peptide Seg3 after TfOH cleavage. The LC trace in (b) is peptide **50** after purification, and (c) is the ESI-MS for the major peak. Calculated mass was based on average isotope composition.

Glycopeptide Seg 2 was also prepared by standard Boc SPPS, except for amino acid coupling in diluted conditions after incorporating oligosaccharide, according to Murakami's procedure. After the elongation of peptide on resin, phenacyl esterified sialyloligosaccharyl asparagine **49** was coupled by PyBOP as a coupling reagent. It is known that non-diluted coupling causes esterification of hydroxy groups in oligosaccharide as reported by Yamamoto *et al*., therefore amino acid coupling steps were conducted in a diluted condition (50 mM Boc-amino acids).[17] In addition, deprotection of side chain protecting groups was performed by low acidic cocktail because highly acidic cocktail cleaves glycoside linkages. MESNa treatment, followed by removal of Pac groups, gave glycopeptide **51** in ca. 10%.

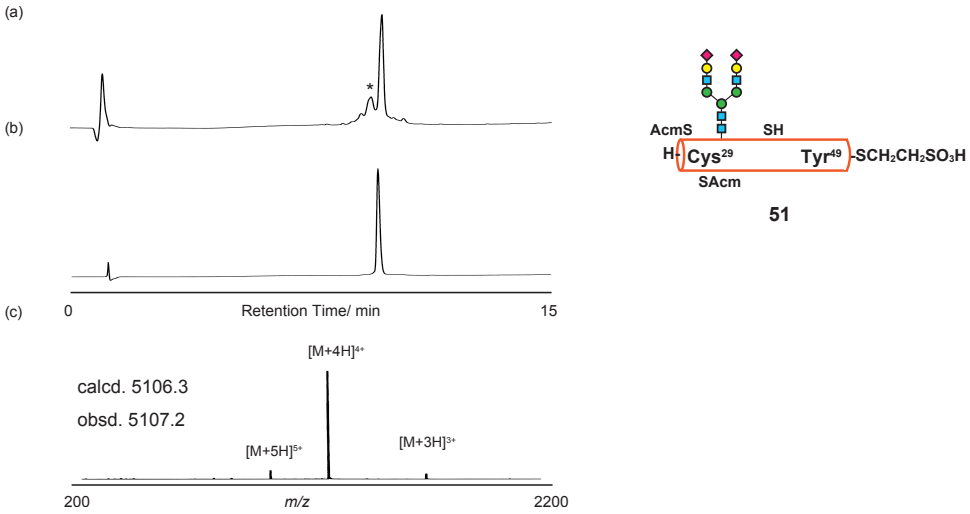

Figure 5-4. (a) HPLC analysis of the crude glycopeptide Seg 2 after TfOH cleavage. The LC trace in (b) is glycopeptide **51** after purification, and (c) is the ESI-MS for the major peak. Calculated mass was based on average isotope composition.

Glycopeptide Seg 1 (**52**) was synthesized by segment coupling reaction on resin because it is known that *N*-terminal peptide of EPO is difficult to be synthesized. Seg 1a: Boc-[Ala^{1}-Ala^{19}]-OH with protecting groups for side chains was prepared by Fmoc SPPS. First, 4-(4-Hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) was coupled to amino PEGA resin as a linker, which enables generation of fully protected peptide acid. The first amino acid was introduced to HMPB-PEGA resin by 2,4,6-mesitylene- sulfonyl-3-nitro-1,2,4-triazolide (MNST). This coupling reagent facilitates the efficient esterification of alcohol with suppressing epimerization as reported by Blankemeyer-Menge *et al*.[18] The peptide was elongated by use of Fmoc-AA-OH (4 equiv, 200 mM), HBTU (3 equiv), HOBt (3 equiv), and DIEA (6 equiv) in DMF. After the completion of peptide construction, the fully protected peptide was cleaved from the resin by AcOH/trifluoroethanol (1:1).

Seg 1b: H-[K^{20}-Gly^{28}] was constructed on amino PEGA resin by Boc SPPS as described above and used for peptide segment coupling. After the removal of the *N*-terminal Boc group, the resulting amine was coupled with Seg 1a in a solution of phenol/chloroform containing HODhBt and DIC.[19] The resulting resin was treated with 2.5% TIPS and 2.5% H_2O in TFA to remove protecting groups for Fmoc SPPS and with TFA/DMS/*m*-cresol/TfOH (5:3:1:1) to remove protecting groups for Boc SPPS. The unprotected peptide was then cleaved from the resin by MESNa to give glycopeptide **52** in ca. 6%.

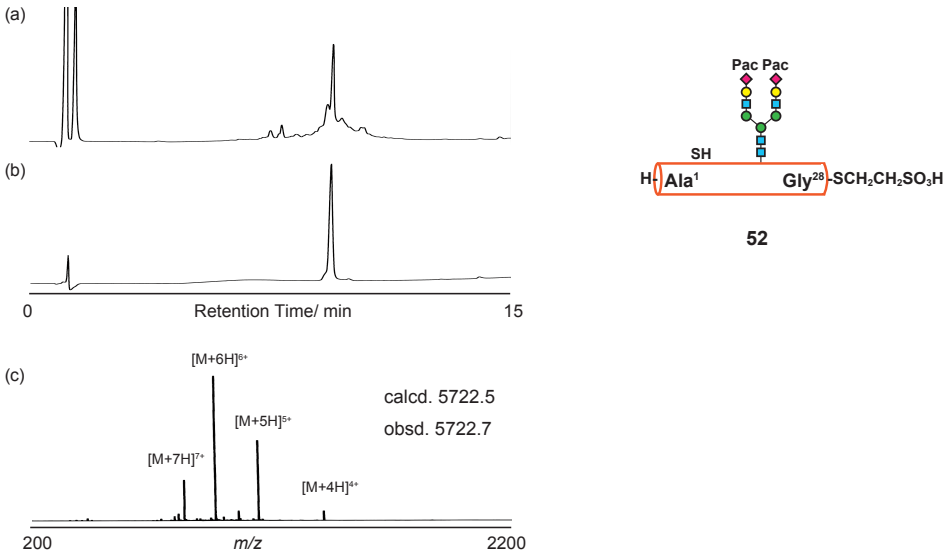

Figure 5-5. (a) HPLC analysis of the crude glycopeptide Seg1 after TfOH cleavage. The LC trace in (b) is glycopeptide **52** after purification, and (c) is the ESI-MS for the major peak. Calculated mass was based on monoisotopic mass.

5-5. Peptide elongation by NCL and hydrazide ligation.

All peptide segments in hand, I examined ligation reactions to obtain a full length of EPO, which began with NCL of Seg 2 and Seg 3. First, peptide **51** was activated by the treatment with 4-mercaptophenylacetic acid (MPAA) to give peptide MPAA-thioester. Thioester exchange with peptide **50**, followed by S-to-N acyl shift, produced peptide **53** with native sequence.[14, 20-21] After the completion of the ligation, pH value of the mixture was adjusted to 9.9 for removing Pac ester. RP-HPLC purification yielded desired Seg 23 (**54**) in 50% yield.

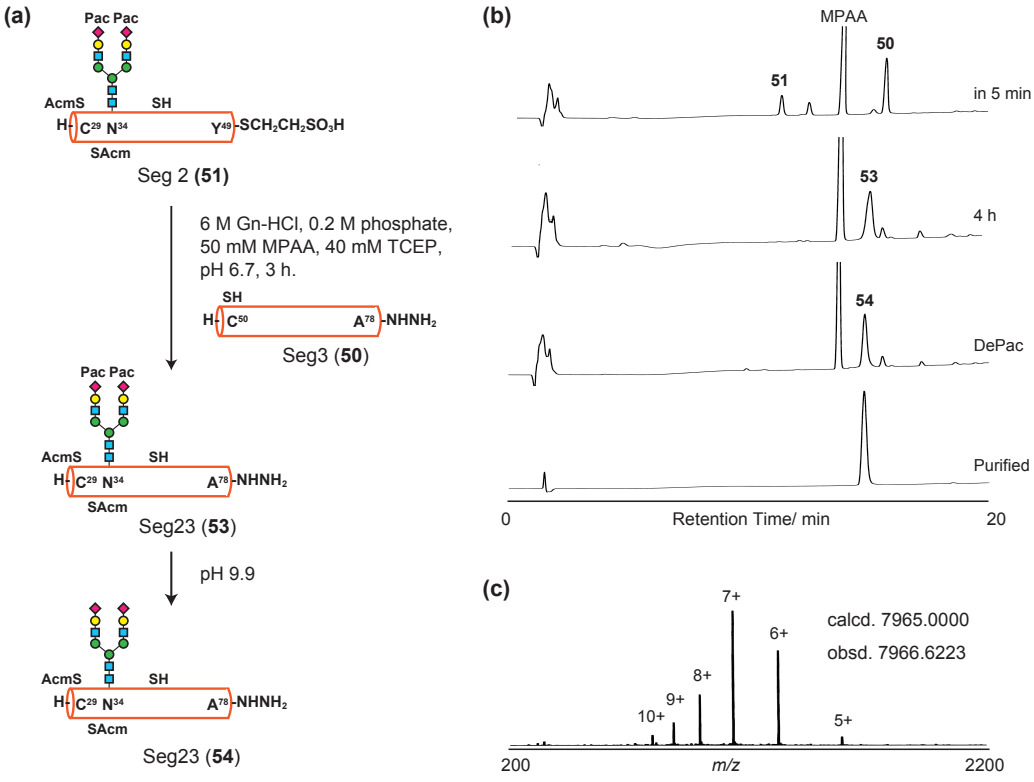

Figure 5-6. (a) Scheme of the NCL reaction. (b) LC trace of the reaction. (c) ESI-MS of purified **54**. Calculated mass was based on monoisotope mass.

Next, Seg 4 bearing a synthesized triantennary oligosaccharide (**33**) was coupled with obtained Seg 23 (**54**) by hydrazide ligation to give a Seg 234 having a triantennary oligosaccharide and a biantennary oligosaccharide (**56**). Glycopeptide hydrazide **54** was activated by $NaNO_2$ (10 equiv) at pH 3.5, followed by the addition of MPAA to allow conversion into glycopeptide thioester. Following NCL reaction with glycopeptide hydrazide **33** gave desired glycopeptide **56** in 68% yield.

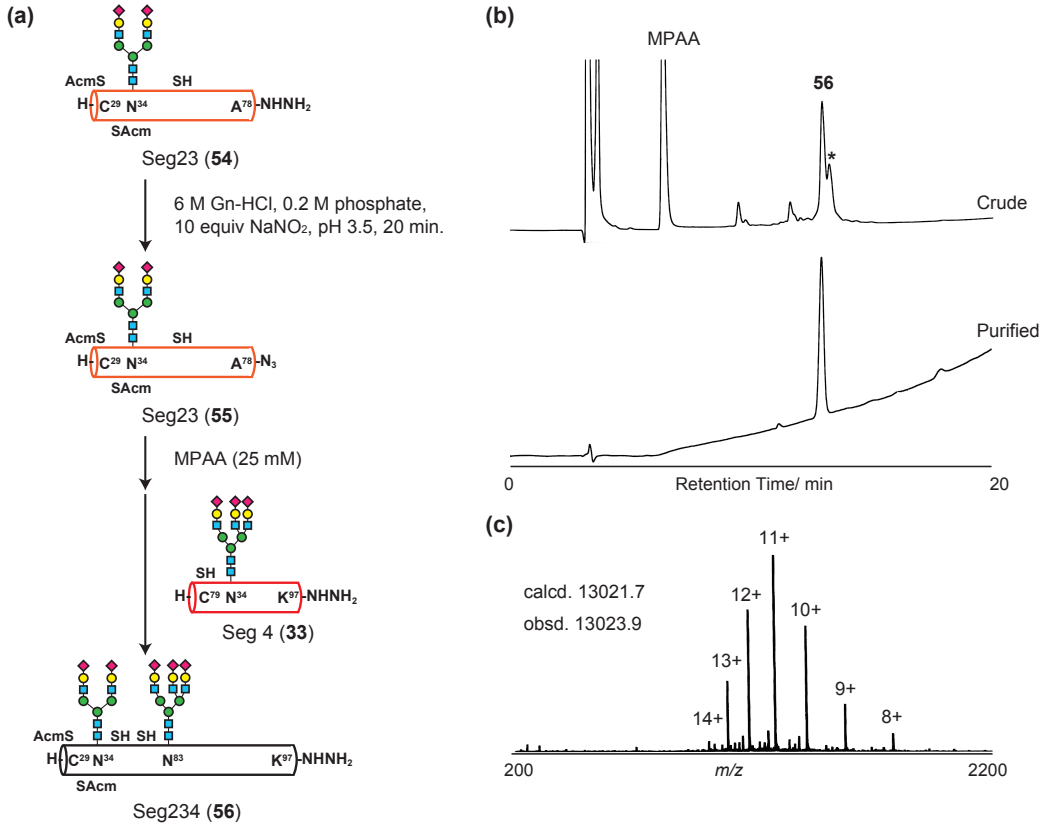

Figure 5-7. (a) Scheme of hydrazide ligation of Seg 23 (**54**) and Seg 4 (**33**). (b) HPLC trace of the hydrazide ligation. * is the peak of **56** having disulfide bridge with MPAA. (c) ESI-MS of purified Seg 234 (**56**). Calculated mass was based on average isotope composition.

To ligate Seg 234-hydrazide (**56**) with Seg 56, activation/ligation conditions were investigated, because peptidyl hydrazide having *C*-terminal Lys sometimes shows undesired lactamization during activation and/or ligation. Brik *et al.* reported that in some cases ε-amine of the *C*-terminal Lys shows nucleophilic attack to the activated *C*-terminal to result in lactamization.[22] They prevented this undesired reaction by using 6-nitroveratryloxycarbonyl (Nvoc) group for the protection of Lys side chain. Thus, I also prepared Fmoc-Lys(Nvoc)-OH (**62**) and investigated to incorporate it into Seg 4, which has triantennary oligosaccharide and is prepared by Fmoc-SPPS as described in *Chapter 4*.[23-25] However, this protecting group was not stable during final TFA cleavage and was difficult to be coupled to HMPB-resin using MNST during Seg 4 synthesis. Therefore, I investigated to suppress lactamization of *C*-terminal Lys without protecting group for the amine. I used peptidyl hydrazide **57**, which is a *C*-terminal peptide of Seg4, as a model, even though it did not contain oligosaccharide. When hydrazide **57** was activated by $NaNO_2$ at pH3.5 and the pH value of the mixture was elevated to 6.5, more than half of azide **56** resulted in lactam formation (Figure 5-7c). Elevation at higher pH (8.2) showed complete conversion of azide **58** into lactam **61** (Figure 5-7d). Investigation of direct hydrazide ligation with excess of MPAA and *C*-terminal peptide also resulted in lactamization and unidentified side products, because lactamization is superior to MPAA conversion. However, once azide **58** is converted to peptide MESNa-thioester **60**, elevation of pH did not yield lactamized product (Figure 5-7d), in other words, MESNa-thioester **60** is suitably stable while **58** has unprotected Lys on its *C*-termini. Conversion to thioester at lower pH needed excess of thiol reagent because of lower nucleophilicity around pH 3.5. Furthermore, rapid conversion was also required due to a risk of sialoside cleavage. MPAA is not well soluble at lower pH, and thus conversion of azide into MESNa-thioester, followed by thioester exchange with MPAA, was found to be a suitable synthetic strategy in terms of this ligation site.

Therefore, obtained Seg234 hydrazide (**56**) was converted into glycopeptide MESNa-thioester (**63**) and isolated by RP-HPLC in 74% (Figure 5-9). After treatment of hydrazide **56** with $NaNO_2$ at -15 ^{o}C, emulsion of MESNa buffer was added to the mixture (ca. 200 equiv) and the mixture was kept at room temperature to allow for acyl azide converting to MESNa thioester **63**. Addition of MPAA and Seg56 to the crude mixture was examined, however the one-pot NCL did not show suitable conversion. Thus, MESNa-thioester **63** was purified by RP-HPLC.

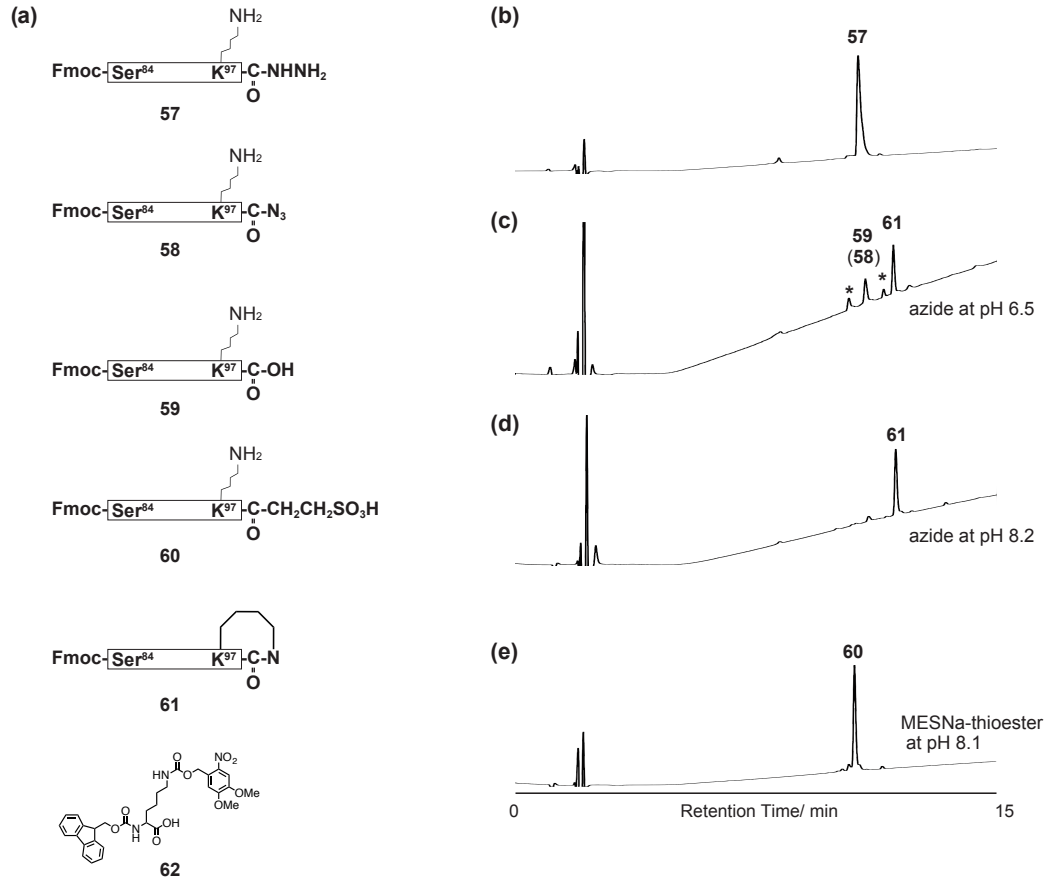

Figure 5-8. Model experiments to investigate activation of peptide-Lys-hydrazide **57**. (a) Structures of model peptide **57**, the resulting products **58**-**61**, and Fmoc-Lys(Nvoc)-OH (**62**). LC data of starting material (b) and the results (c-e) are shown. (c) After treatment of **57** with $NaNO_2$, the pH value was adjusted to 6.5, which was analyzed by LC-MS. Peptude acid **59** was observed instead of azide **58** possibly due to decomposition during MS analysis. * are Fmoc-cleaved peptides due to impurity of SM in this experiment. (d) After conversion to **58**, pH value was elevated to 8.2, resulting in lactam formation. (e) After conversion of azide **58** into MESNa-thioester **60**, pH value was adjusted to 8.1, however this did not show lactam formation.

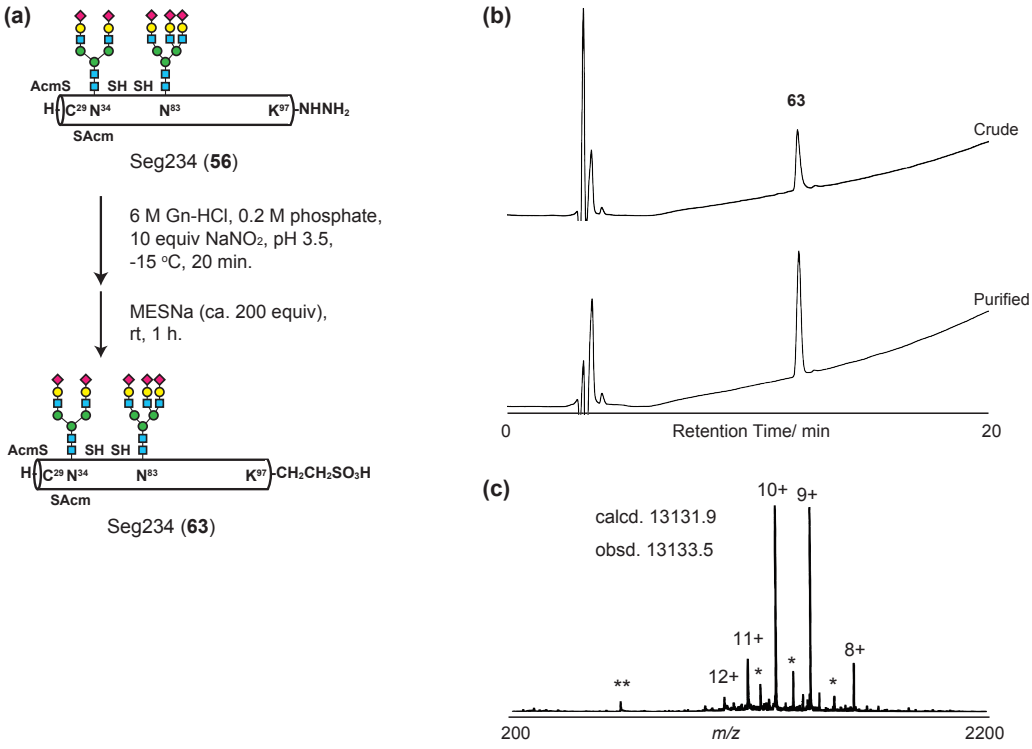

Figure 5-9. Hydrazide to MESNa-ester conversion. (a) The scheme of the reaction. (b)RP-HPLC data. (c) ESI-MS of purified **61**. * are fragmentation peaks and ** corresponds to Sia-Gal-GlcNAc fragmentation peak. Calculated mass was based on average isotope composition.

The obtained Seg 234 thioester (**63**) was then ligated with Seg 56 to give Seg 23456 (**65**) in 26% yield. MESNa-thioester was converted to MPAA-thioester at pH 6.6, which then reacted with Seg56 (**64**) to give Seg23456 (**65**) as shown in Figure 5-10. During this ligation, lactam formation was fortunately not observed.

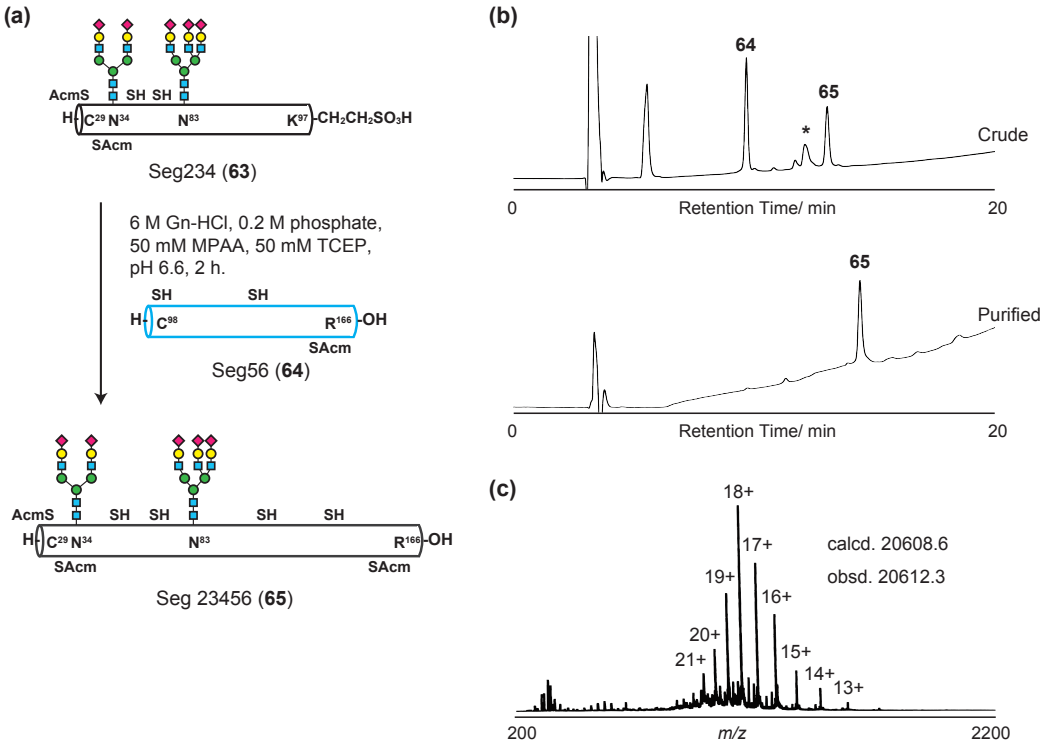

Figure 5-10. (a) Scheme of the NCL reaction. (b) RP-HPLC trace of the reaction. Retention time of the desired product differs because different gradient was used. * is an unidentified peptide. (c) ESI-MS of purified **65**. Calculated mass was based on average isotope composition.

Ligated product Seg23456 (**65**) in hand, I examined desulfurization of free four Cys residues and deprotection of three Acm groups as shown in Figure 5-11. Glycopeptide **65** was treated with VA-044 (2,2'-azobis[2-(2- imidazolin-2-yl)propane]dihydrochloride, which is a water-soluble radical initiator, in 6 M Gn-HCl buffer containing 250 mM TCEP and 2-methyl-2-propanetiol at 37 ^{o}C for 3 h. Addition of the thiol reagent enhances the radical reaction to reduce Cys residues to Ala residues. Purification by RP-HPLC afforded the desired glycopeptide **66**. Then, obtained **66** was dissolved in 90% AcOH containing AgOAc to remove Acm groups. After treatment with DTT to remove undesired disulfide bonds, the resulting mixture was purified by RP-HPLC to give Seg23456 (**67**).

Coupling of Seg 23456 (**67**) and Seg 1 (**52**) was performed by NCL reaction. Seg 1 (**52**) in 50 mM MPAA buffer containing 6 M Gn-HCl, 0.2 M phosphate, 40 mM TCEP (pH 6.5) was added to the obtained Seg 23456 (**67**). After 2 hours, pH value of the mixture was adjusted to 10.0 in order to remove Pac ester to give a full length of glycosylated EPO (**68**) as shown in Figure 5-12.

Finally, step-wise dialysis folding of EPO full length (**68**) was performed based on previous EPO folding. EPO polypeptide (**68**) was first dissolved in 6 M Gn-HCl buffer, which was then set into dialysis tube. The dialysis tube was kept in 3 M Gn-HCl buffer containing 100 mM Tris-HCl, 4 mM cysteine, and 0.5 mM cysteine, where the redox environment allows disulfide bond formation under the equilibrium manner. Decreasing the concentration of Gn-HCl enabled folding of EPO polypeptide. Aliquot of the reaction mixture was analyzed by LC-MS. Protonation/ charge pattern of the ESI-MS clearly changed compared with the polypeptide MS pattern. Major MS peaks shifted from low *m/z* to higher *m/z*, which is a typical pattern of folding experiment. Some undesired MS peaks were observed, however it possibly because of small scale and low intensity of the desired product peaks. This tentative analysis suggested that folding experiment successfully yielded the target EPO (**69**) even though some impurities were included.

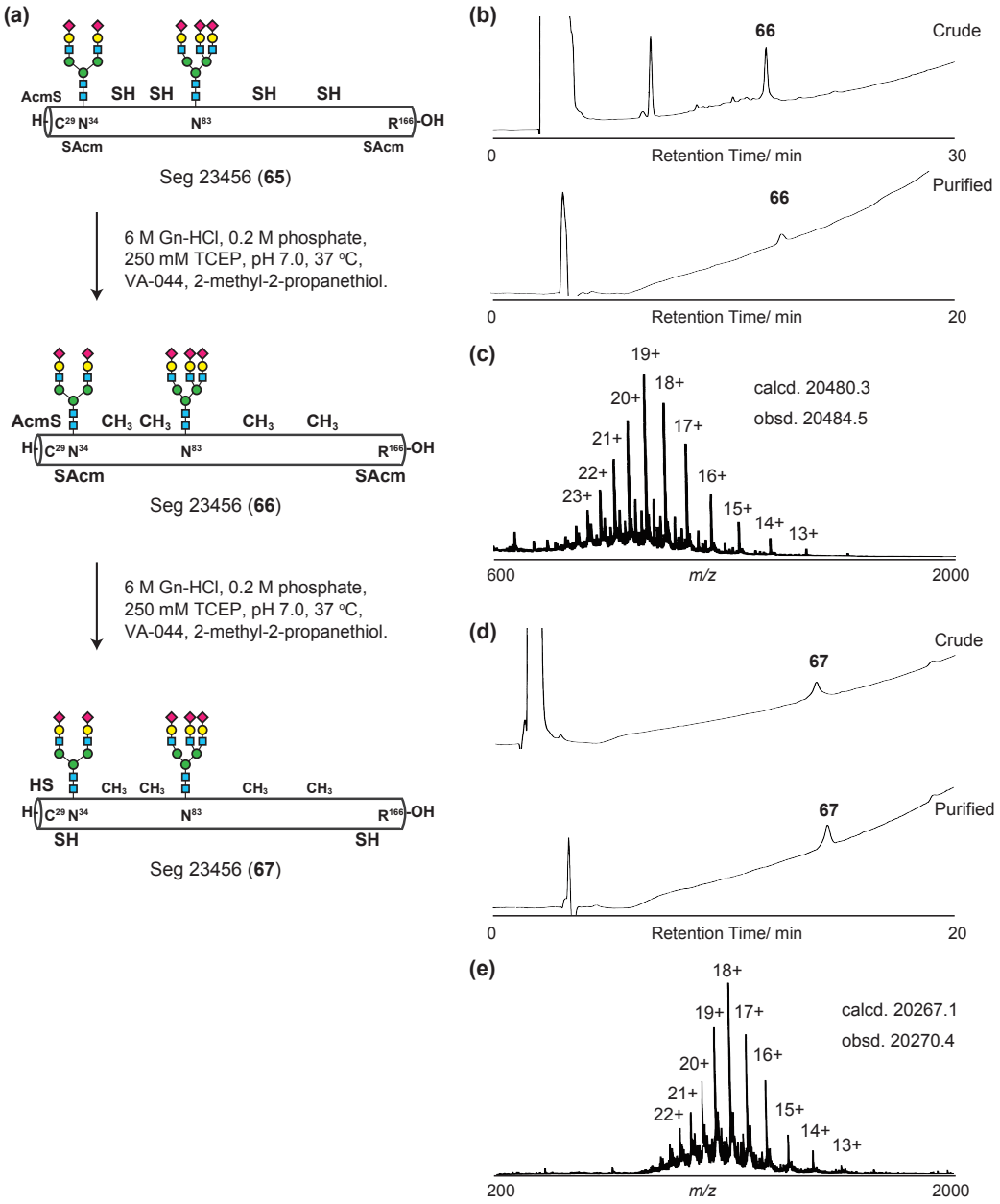

Figure 5-11. (a) Scheme of desulfurization and Acm deprotection reactions. (b) RP-HPLC data for desulfurization reaction. (c) ESI-MS of purified **66**. (d) RP-HPLC data for Acm deprotection reaction. (c) ESI-MS of purified **67**. Calculated masses were based on average isotope composition.

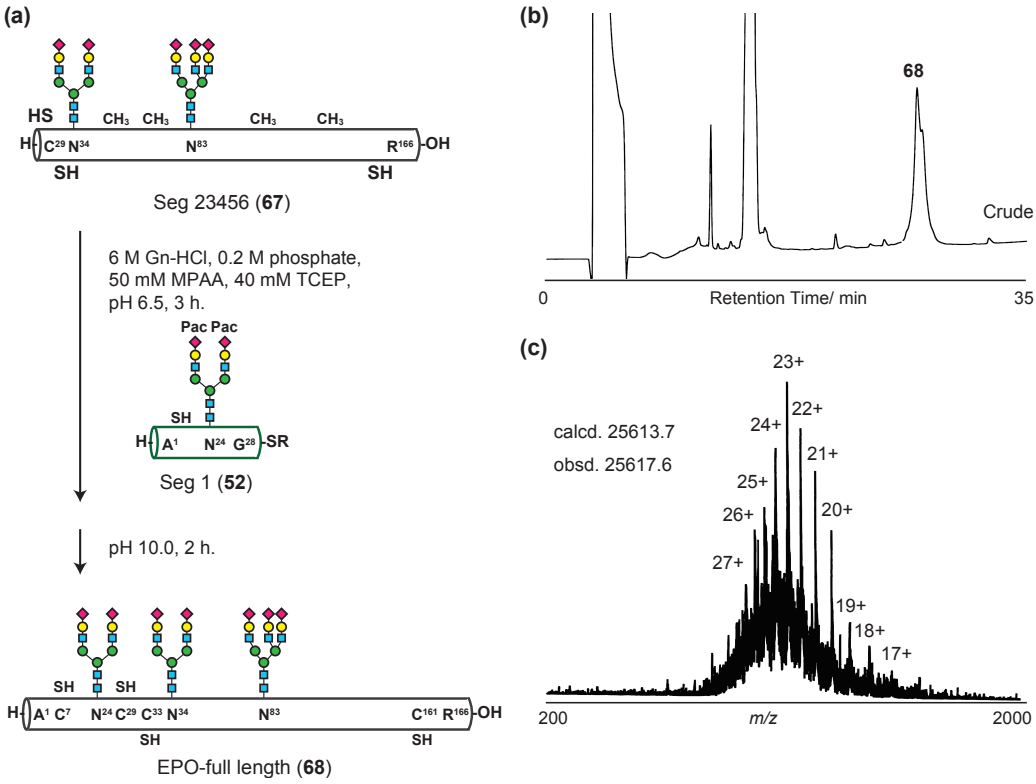

Figure 5-12. (a) Scheme of the final NCL reaction. (b) RP-HPLC data for purification of **68**. Major peak was the desired product and right and left shoulders were undesired products. (c) ESI-MS of purified **68**. Calculated mass was based on average isotope composition.

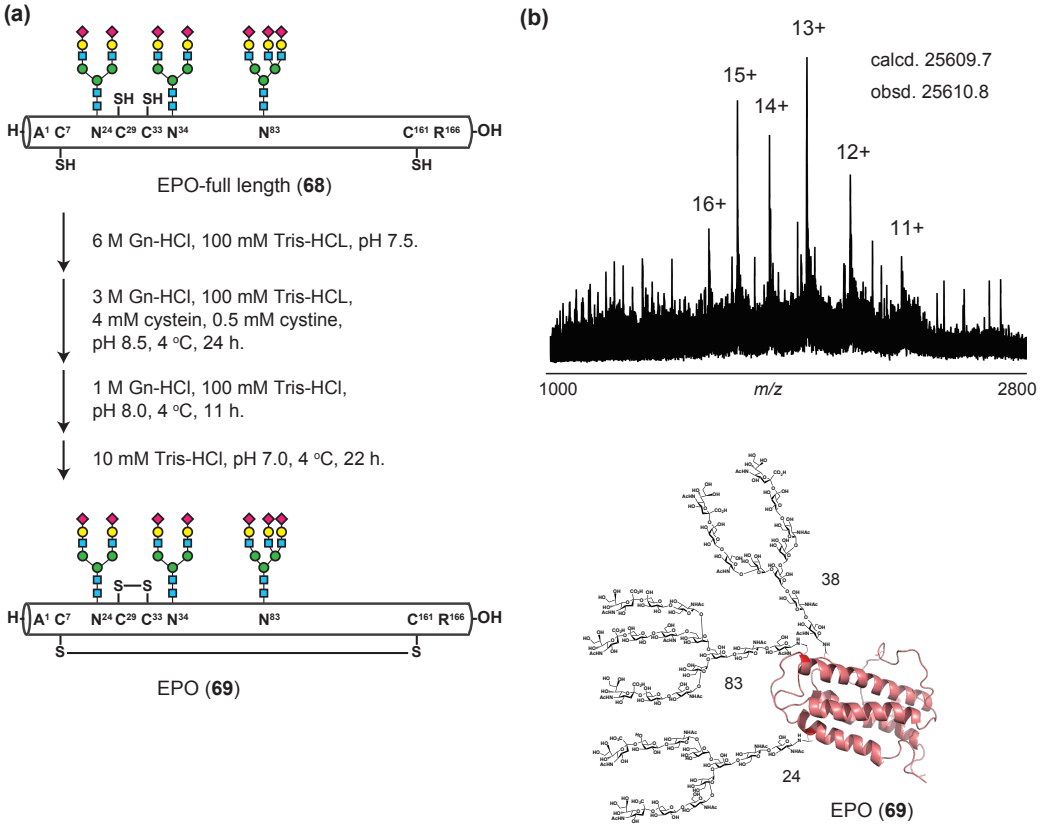

Figure 5-13. Folding of EPO-polypeptide **68**. (a) Scheme of the folding experiment by dialysis. (b) Tentatively picked up MS data from LC-MS analysis. Calculated mass was based on average isotope composition.

5-6. Summary of *Chapter 5*

I demonstrated the chemical ligation reactions and folding experiment based on newly established glycopeptide synthesis, thus allowing access to the EPO glycoform having one triantennary oligosaccharide and two biantennary oligosaccharides. The glycopeptide having the synthesized triantennary oligosaccharide was applicable to EPO (166 amino acids) synthesis, along with overcoming a difficulty in hydrazide ligation at Lys^{97}-$Cys(Ala)^{98}$. Sialyl glycopeptide having a triantennary oligosaccharide (0.5 mg), which was derived from 2.0 mg Fmoc-Asn(Triantennary asialooligosaccharide)-OH, was subjected to sequential ligation to give a full length of glycosylated polypeptide, followed by folding experiment.

Though final folded EPO has not been isolated and structurally confirmed by circular dichroism (CD) and disulfide mapping yet, ESI-MS showed a typical MS pattern of folded EPO.

I established a practical synthetic strategy for the synthesis of EPO glycoforms, which can efficiently vary the oligosaccharide structure on Asn^{83}. Introducing another type of triantennary oligosaccharide, more branched oligosaccharides, and poly-LacNAc containing oligosaccharide will reveal the effect and necessity of carbohydrate diversity.

5-7. References

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Conclusion

I established an unprecedented strategy for the semisynthesis of two kinds of complex-type triantennary oligosaccharides from a biantennary nonasaccharide, which was isolated from hen egg yolk. Finding of the unprecedented protection protocol toward 24 hydroxy groups of a biantennary nonasaccharide enabled me to examine chemical glycosylation with Gal-β-1,4-GlcNAc donor to obtain human type triantennary oligosaccharides. This semisynthesis enabled 9- and 10-step chemical conversion into the desired triantennary oligosaccharides. This strategy significantly reduced the synthetic steps compared with a conventional oligosaccharide synthesis (71 steps for one of triantennary sialyloligosaccharide).

During the course of the synthesis of triantennary oligosaccharides, I found a new selectivity of benzylidene acetal deprotection between Man and Gal, and then studied the selectivity using monosaccharides in order to elucidate what factors regulated this selective deprotection. Thermodynamic parameters obtained by the Arrhenius and Eyring plot revealed entropic difference, which came from the stereochemistry at 4-positions.

Segment coupling of oligosaccharyl asparagine and protected peptides was found to more efficiently yield glycopeptide segment than standard SPPS preparation. Overcoming a difficulty in dealing with protected peptides allowed me to synthesize EPO-glycopeptide having a synthesized triantennary oligosaccharide in good yield. The idea to couple an oligosaccharyl asparagine with pure *C*- and *N*-terminal peptides in solution was critical.

Finally, I demonstrated the synthetic study of EPO having a triantennary and two biantennary oligosaccharides. The synthesized glycopeptide having triantennary sialyloligosaccharide was elongated at its *C*- and *N*-terminus by sequential NCL and hydrazide ligation to give a full length of glycosylated EPO. With monitoring by LC-MS analysis, stepwise dialysis folding produced the folded EPO bearing a triantennary oligosaccharide at 83rd Asn and biantennary oligosaccharides at 24th and 38th Asn.

In summary, I developed the robust synthetic methodology for the preparation of the triantennary oligosaccharides, the glycopeptides, and the glycoprotein having a triantennary oligosaccharide. These all syntheses have been extremely difficult by conventional and traditional methodologies. My strategy is furthermore applicable to synthesize various glycoforms of glycoproteins to shed a light on carbohydrate diversity.

Experimental Information

Table of Contents:

1. General methods and abbreviation

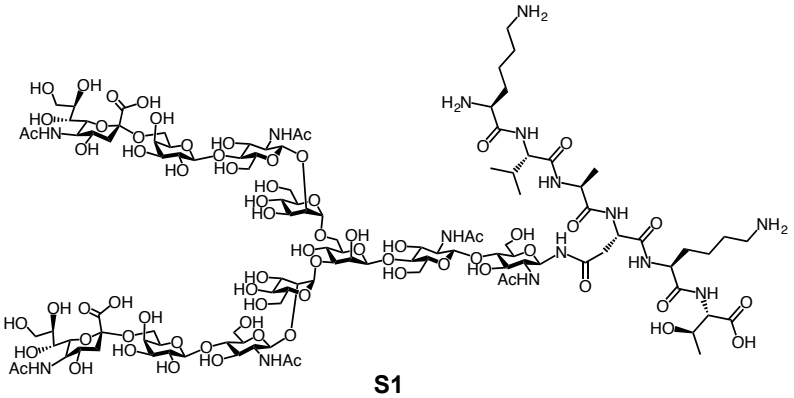
Hydroxybenzotriazole (HOBt), Boc-Lys(Cl-Z), Boc-Glu(OBzl), Boc-Ala, Boc-Ile・1/2H_2O, Boc-Thr(Bzl), Boc-Gly, Boc-Cys(Acm), Boc-Ser(Bzl), Boc-Leu, Boc-Val, Boc-Pro, Boc-Asp(OBzl), Boc-Phe, Boc-Trp(CHO), Boc-Met, Boc-Gln were purchased from PEPTIDE INSTITUDE. INC. *O*-(1*H*-Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU), 1*H*-Benzotriazol-1-yloxy-tri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HODhbt), Boc-Asn(Xan)-OH, Boc-Tyr(Br-Z)-OH, Boc-Arg(Mtr)-OH, and Boc-Arg($Z)_2$-OH were purchased from WATANABE CHEMICAL INDUSTRIES, LTD. Boc-OSu was purchased from Combi-Blocks. 2,2,2-Trifluoroethanol, *N*-methylimidazole, Triisopropylsilane (TIPS), *N,N*-Diisopropylethylamine, Tris(2-carboxyethyl)phosphine Hydrochloride (TCEP), Sodium 2-Mercaptoethanesulfonate (MESNa), tert-Butyl Carbazate, Methyl α-D-Galactosyl Monohydrate, Zinc Chloride, Hexamethyleneimine, Methyl Sulfide (DMS), Trifluoromethanesulfonic acid (TfOH), *tert*-Butyldiphenylchlorosilane (TBDPSCl), and 1,2-Ethanedithiol (EDT), were purchased from TOKYO CHEMICAL INDUSTRY CO., LTD. Sodium Nitrite, Acetic Acid, Piperidine, Thioanisol, *O*-Methylhydroxylamine Hydrochloride, 3-Mercaptopropionic Acid, and HEPES{2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic Acid} were purchased from nacalai tesque. 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] Dihydrochloride (VA-044), (±)-Dithiothreitol (DTT), Hydrazine Monohydrate, Silver Acetate, Dimethyl Sulfoxide (DMSO), 1-Methyl-2-pyrrolidone, *N,N'*-Diisopropylcalbodiimide (DIC), Ninhydrin, Cesium Carbonate, 1,4-Dioxane, Ammonium Acetate, 2-Amino-2-hydroxymethyl-1,3-propanediol (Tris), Sodium Hydrogen Carbonate, Sodium Chloride, Molecular Sieves 4A 1/16, Guanidine Hydrochloride (Gn-HCl), (±)-Camphor-10-sulfonic Acid, 4-Dimethylaminopyridine (DMAP), Magnesium Sulfate, Trifluoroacetic Acid (TFA), Thiophenol, p-Mercaptophenylacetic acid (MPAA), Sodium 2-Mercaptoethanesulfonate (MESNa), Disodium Hydrogenphosphate Dodecahydrate, Chloroform-d, Acetonitrile-d3 were purchased from Wako Chemical Industries, Ltd. Boc-L-thiazolidine-4-carboxylic acid was purchased from BACHEM. *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU), 1-(2-Mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MNST), Amino PEGA resin, 4-(4-Hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB), Boc-His(Dnp)-OH isopropanol, Fmoc-Asn(Trt)-OH, Fmoc-Trp(Boc)-Oh, Fmoc-His(Trt)-OH, Fmoc-Glc(Trt)-OH,

Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, Fmoc-Ser-OH, Fmoc-Leu-OH, and Fmoc-Ile-OH were purchased from Novabiochem. Aminomethyl ChemMatrix® resin, Methyl α-D-glucopyranoside, METHYL α-D-MANNOPYRANOSIDE, Tetrabutylammonium hydrogensulfate, *N*-Iodosuccinimide, 2-Bromoacetophenone, Trityl chloride, ALBUMIN BOVINE, and Acetic acid-d were purchased from SIGMA-ALDRICH. Acetonitrile, *N,N'*-Dimethylformamide, Dichloromethane, and Chloroform were purchased from KANTO CHEMICAL CO., INC.

^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz or 700 MHz spectrometer (Bruker Avance III). All ^{1}H chemical shifts are reported in parts per million (ppm) relative to TMS in $CDCl_3$ (0 ppm), H_2O in CD_3CN (2.13 ppm), CD_3CN (1.94 ppm), or D_2O (4.79 ppm). All ^{13}C chemical shifts are assigned according to CD_3Cl (77.16 ppm), CD_3CN (1.32 or 113.26 ppm), d3-AcOD (2.03), or 1,4-dioxane in D_2O as the external standard (67.19 ppm). All NMR signals were assigned on the basis of ^{1}H NMR, DQF-COSY, HSQC, HMBC, and HSQC-TOCSY. High-resolution mass spectrometry was performed by FTICR (Bruker solariX XR), and other mass spectra were recorded on a Bruker esquire 3000^{plus}, or an amaZon ETD mass spectrometer. TLC-analysis was conducted on Silicagel 60 F_{254} (Merck TLC plates), and visualizations were performed with UV light (254 nm) and/or sulfuric acid stain (5% H_2SO_4 in Methanol). RP-HPLC analyses were performed using 0.1% aq. TFA and 90% aq. CH_3CN containing 0.1% TFA, and RP-UHPLC analyses were performed using 0.1% aq. HCOOH and 90% aq. CH_3CN containing 0.09% HCOOH. RP-HPLC analyses were performed on a Waters 2996 HPLC system equipped with a multi wavelength detector, and RP-UHPLC analyses were performed on a DIONEX UltiMate 3000 UHPLC system equipped with a variable wavelength detector. An XBridge^{TM} prep column (Waters, 10 × 250 mm) was used for preparative HPLC using 50 mM aq. NH_4OAc and CH_3CN. A Cadenza CD-C18 (Imtakt, 4.6 mm × 75 mm) column or a Proteonavi (Shiseido, 4.6 × 250 mm) column were used for analytical HPLC using 0.1% aq. TFA and 90% aq. CH_3CN containing 0.1% TFA, or 50 mM aq. NH_4OAc and CH_3CN, respectively. For LC-MS system, a Cadenza CD-C18 (Imtakt, 100 or 50 × 2 mm) column or a Proteonavi (Shiseido, 2.0 mm× 150 mm) column was used using 0.1% HCOOH and 10% acetonitrile containing 0.1% HCOOH.

2. Experimental Procedures and Characterizations

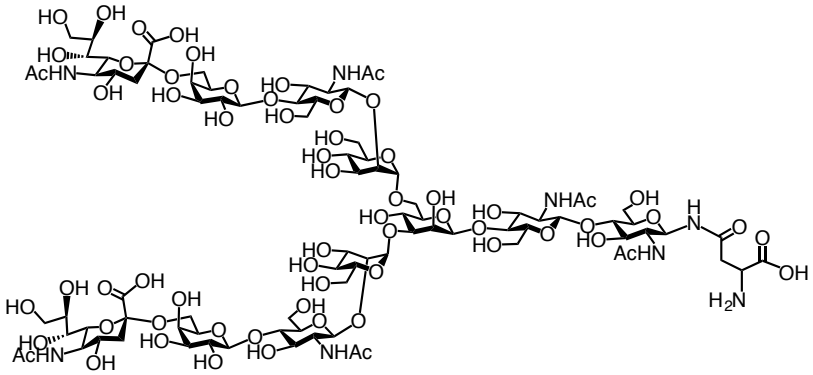
2-1. Isolation of oligosaccharides from hen egg yolk[1-2]



S1

Sialylglycopeptide (SGP) (S1)

Distilled water (1 L) was added to fresh hen egg yolk obtained from 60 eggs. To the solution of egg yolk, phenol/water (100 g: 10 g) was added. The mixture was vigorously stirred at 4 ºC for 2 hours. To the obtained emulsion, distilled water (400 mL) was added, and the mixture was centrifuged at 12500 g for 30 min. The supernatant was collected, and the oil layer was removed. The resulting mixture was concentrated under reduced pressure and centrifuged at 12500 g for 30 min again to remove insoluble material. The supernatant was then subjected thrice to gel filtration chromatography using a column (ϕ= 4.0 cm, l= 1.0 m) packed with Sephadex^{TM} G-50 Fine (GE Healthcare), equilibrated and eluted with 0.1 M distilled water. Fractions containing SGP were collected, concentrated, and lyophilized to afford SGP with some impurities (419 mg).



S2

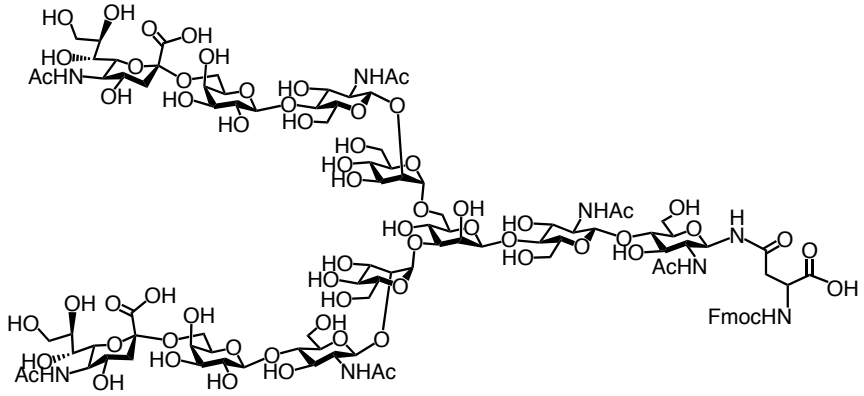
Biantennary sialylundecasaccharyl asparagine (S2)

Actinase-E (32 mg) was added to a solution of sialylglycopeptide (201 mg) containing some impurities, such as egg yolk peptides, in 50 mM Tris-HCl buffer (10 mM $CaCl_2$, 0.5% NaN_3, pH 7.5, 10 mL), and this mixture was incubated for 3 days at 37°C. During incubation, the pH value of the mixture was kept at 7.5. After 4 days, to the mixture was added Actinase-E (36 mg) and this reaction mixture was further incubated for 3 days. This reaction was monitored by TLC (1 M NH_4OAc/isopropanol, 1:1) and ESI-MS analyses. On the completion of the reaction, the mixture was lyophilized, and purification of the resulting residue by gel permeation (Sephadex-G 25, ϕ= 2.0 cm, l= 1.0 m) afforded asparagine-linked sialyloligosaccharide **S2** (135 mg, 83%).

^{1}H NMR (400 MHz, D_2O, HDO 4.79 ppm) δ 5.13 (s, 1 H), 5.07 (d, *J* = 9.65 Hz, 1 H), 4.95 (s, 1 H), 4.77 (s, 1 H), 4.67-4.55 (m, 3 H), 4.44 (d, *J* = 7.78 Hz, 1 H×2), 4.26 (brdd, 1 H), 4.20 (brdd, 1 H), 4.12 (brdd, 1 H), 4.08-3.42 (m), 3.0-2.81 (m, 2 H), 2.67 (brd, 1 H×2), 2.08 (s, 3 H), 2.07 (s, 3 H), 2.06 (s, 3 H), 2.03 (s, 3 H×2), 2.01 (s, 3 H), 1.72 (dd, *J* = 12.1, 12.1 Hz, 1 H×2).

^{13}C NMR (100 MHz, D_2O, external standard 1,4-dioxane 66.54 ppm) 174.91, 174.83, 174.71, 174.63, 173.49, 172.84, 172.48, 103.53, 101.28, 100.46, 100.14, 100.12, 99.55, 99.32, 99.26, 96.94, 80.69, 80.61, 80.48, 79.63, 78.70, 78.06, 76.40, 76.18, 74.42, 73.68, 73.53, 72.78, 72.53, 72.41, 72.14, 72.04, 71.97, 71.70, 70.73, 70.18, 69.46, 68.39, 68.19, 67.32, 65.87, 65.68, 63.32, 62.65, 61.70, 61.61, 60.22, 59.96, 59.86, 54.92, 54.63, 53.55, 51.87, 50.85, 40.05, 34.92, 22.41, 22.23, 22.04.

ESI-MS: calcd for $C_{88}H_{144}N_8O_{64}$ $[M-2H]^{2-}$ 1167.4, $[M-3H]^{3-}$ 779.9, found for *m*/z 1167.0, 777.7.



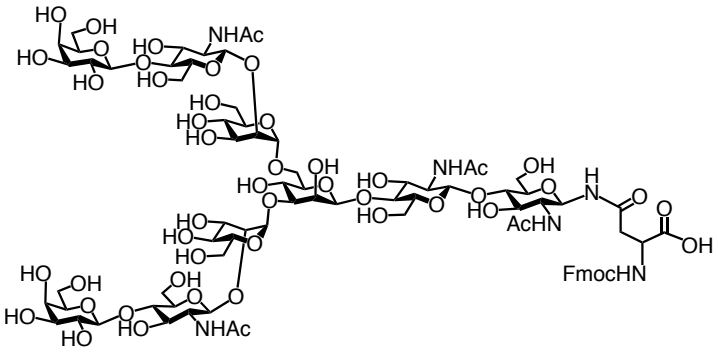
Fmoc-Asparagine(Biantennary sialylundecasaccharide)-OH (**46**)

To a solution of Asn-linked sialyloligosaccharide **S2** (213 mg, 91μmol) in acetone/H_2O (1: 1, 5.4 mL) was added $NaHCO_3$ (22 mg) and 9-fluorenylmethyl-*N*-succimidylcarbonate (61 mg, 180μmol), which was stirred at rt for 21 hours. The resulting mixture was evaporated to remove acetone, which was then purified by reverse-phased column packed with Cosmosil 75C_{18}-OPN (ϕ= 30 mm, l= 300 mm; eluted with i) distilled water, ii) 5% aq. acetonitrile). Fractions containing the desired product were collected, evaporated, and lyophilized to afford a pure Fmoc-Asn-linked sialyl-undecasaccharide **46** (133 mg, 52 μmol, 57%).

^{1}H NMR[3] (400 MHz, D_2O, HDO 4.79 ppm) d 7.87-7.69 (m, 2 H), 7.69-7.50 (m, 2 H), 7.50-7.26 (m, 4 H), 5.14 (s, 1 H), 4.98 (d, *J* = 9.37 Hz, 1 H), 9.45 (s, 1 H), 4.75 (s, 1 H), 4.66-4.49 (m, 3 H), 4.49-4.31 (m, 4 H), 4.31-4.23 (m, 2 H), 4.20 (brdd, 1 H), 4.17-4.05 (m), 4.05-3.23 (m), 2.82-2.40 (m, 4 H), 2.08 (s, 3 H), 2.07 (s, 3 H×2), 2.03 (s, 3 H×2), 2.00 (s, 3 H), 1.88 (s, 3 H), 1.73 (d, *J* = 12.1 Hz, 1 H×2).

^{13}C NMR (100 MHz, D_2O, external standard 1,4-dioxane 67.19 ppm) 179.59, 177,91, 177.73, 175.53, 175.35, 175.16, 174.13, 173.96, 158.11, 144.38, 144.24, 141.46, 128.62, 128.05, 125.71, 120.78, 104.14, 101.90, 101.07, 100.78, 100.76, 100.16, 99.97, 99.92, 97.55, 81.27, 81.19, 81.05, 80.24, 79.34, 78.81, 77.07, 76.90, 76.73, 75.05, 74.96, 74.30, 74.16, 73.45, 73.29, 73.15, 73.04, 72.76, 72.66, 72.59, 72.34, 71.35, 70.80, 70.06, 69.02, 68.82, 67.93, 67.04, 66.48, 66.33, 63.95, 63.29, 62.32, 62.23, 60.86, 60.57, 60.38, 55.50, 55.25, 54.36, 53.18, 52.52, 47.40, 40.68, 39.08, 23.07, 22.90, 22.70, 22.64, 22.47, 21.93.

ESI-MS: calcd for $C_{103}H_{154}N_8O_{66}$ $[M-2H]^{2-}$ 12778.4, found for *m*/z 1278.6.



3

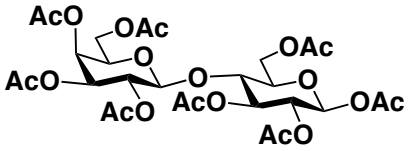
Fmoc-Asparagine(Biantennary asialononasaccharide)-OH (3)

Fmoc-Asn-linked sialyl-undecasaccharide **S3** (172 mg, 67 μmol) was treated with 40 mM HCL (20 mL) at 50 ºC for 20 hours. On the completion of sialic acid removal, the mixture was neutralized with aq. $NaHCO_3$ and lyophilized. Purification of the residue with revers-phased column packed with Cosmosil 75C_{18}-OPN (ϕ= 30 mm, l= 300 mm; eluted with i) distilled water, ii) 20% aq. MeOH) afforded Asn-linked asialo-nonasaccharide **3** (111 mg, 56 μmol, 84%).

^{1}H NMR (700 MHz, D_2O, HDO 4.79 ppm) δ 7.89-7.78 (m, 2 H), 7.73-7.54 (m, 2 H), 7.47-7.40 (m, 2 H), 7.40-7.28 (m, 2 H), 5.16 (s, 1 H), 4.99 (d, *J* = 9.03 Hz, 1 H), 4.75 (s, 1 H), 4.68-4.54 (m, 3 H), 4.54-4.41 (m, 3 H), 4.24 (brs, 1 H), 4.23-4.16 (m, 2 H), 4.11 (brs, 1 H), 4.05-3.39 (m), 2.80-2.61 (m, 2 H), 2.07 (s, 3 H), 2.05 (s, 3 H), 2.04 (s, 3 H), 1.89 (s, 3 H).

ESI-MS: calcd for $C_{81}H_{120}N_6O_{50}$ $[M+2H]^{2+}$ 989.4, found for *m*/z 989.2

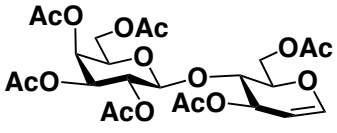
2.2 Synthesis of glycosyl donor[4]



2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (14). A suspension of sodium acetate (4.57 g, 55.7 mmol) in acetic anhydride (100 mL) was heated at reflux. D-(+)-Lactose monohydrate **13** (20.1 g, 55.8 mmol) was then added into the mixture by small portions. The reaction mixture gradually became a clean solution, which was stirred under reflux for 2 additional hours. The solution was then poured into a beaker containing ice water (1.3 L), which was then vigorous stirred until the ice melted. The resulting mixture was filtered and recrystallized from EtOH and petroleum ether to afford Octa-*O*-acetyl-β-D-lactopyranose (**14**) as a white crystal (29 g, 43 mmol, 78%).

^{1}H(400 MHz, CD_3Cl, TMS) δ 5.72-5.65 (d, *J* = 8.27 Hz, 1 H), 5.39-5.34 (dd, *J* = 3.51, 0.81 Hz, 1 H), 5.30-5.22 (dd, *J* = 9.17 Hz, 1 H), 5.16-5.09 (dd, *J* = 10.34, 7.91 Hz, 1 H), 5.09-5.02 (dd, *J* = 9.71, 8.36 Hz, 1 H), 4.99-4.93 (dd, *J* = 10.61, 3.42 Hz, 1 H), 4.52-4.43 (m, 2 H), 4.20-4.05 (m, 3 H), 3.93-3.82 (m, 2 H), 3.81-3.73 (ddd, *J* = 9.80, 4.86, 1.89 Hz, 1 H) 2.18-2.16 (s, 3 H), 2.14-2.13 (s, 3 H), 2.12-2.10 (s, 3 H), 2.09-2.08 (s, 3 H), 2.07-2.06 (s, 3 H), 2.06-2.05 (s, 3 H), 1.99-1.97 (s, 3H).

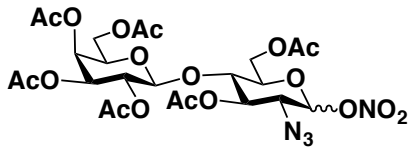
^{13}C(100 MHz, CD_3Cl, TMS) δ 170.52, 170.46, 170.29, 170.22, 169.77, 169.71, 169.16, 169.00, 101.11, 91.67, 75.82, 73.63, 72.77, 71.10, 70.88, 70.64, 69.13, 66.72, 61.87, 60.97, 20.99, 20.97, 20.90, 20.79, 20.75, 20.65.



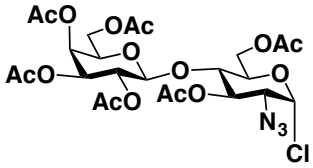
2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-acetyl-D-glucal (16). To a solution of **14** (6.46 g, 9.52 mmol) in AcOH (6.5 mL) was slowly added 30% hydrogen bromide in acetic acid (6.46 mL, ca. 27.8 mmol) and stirred at rt for 5 hours. The resulting mixture was slowly added to Zinc (powder) (7.47 g, 114 mmol, 12 equiv), copper (IV) sulfate anhydrous

(457 mg, 2.86 mmol) in 50 % aq. AcOH (64 mL) and stirred at rt for 2 h. During this reaction, the temperature was carefully kept at 0 ~ 5 ºC. Then the reaction mixture was filtered through celite-charcoal and extracted with CH_2Cl_2. The organic phase was washed with H_2O twice, sat. $NaHCO_3$ twice, dried over $MgSO_4$, filtered, and concentrated to give **16** (4.9 g, 8.8 mmol, 92%).

^{1}H(400 MHz, CD_3Cl, TMS) δ 6.44-6.39 (dd, *J* = 6.04, 1.01 Hz, 1 H), 5.43-5.39 (ddd, 1 H), 5.39-5.35 (dd, *J* = 3.63, 0.97 Hz, 1 H), 5.24-5.17 (dd, *J* = 10.50, 7.87 Hz, 1 H), 5.04-4.98 (dd, *J* = 10.58, 3.53 Hz, 1 H), 4.87-4.82 (dd, *J* = 6.13, 3.37 Hz, 1 H), 4.69-4.64 (d, *J* = 8.1 Hz, 1 H), 4.48-4.41 (dd, *J* = 11.43, 2.29 Hz, 1 H), 4.23-4.13 (m, 3 H), 4.12-4.05 (dd, *J* = 11.24, 7.43 Hz, 1 H), 4.03-3.97 (dd, *J* = 7.27, 5.46, 1 H), 3.94-3.88 (ddd, 1 H), 2.17-2.16 (s, 3 H), 2.13-2.12 (s, 3 H), 2.10-2.09 (s, 3H), 2.08-2.06 (s, 3 H), 2.06-2.05 (s, 3 H), 1.99-1.98 (s, 3 H).
^{13}C(100 MHz, CD_3Cl, TMS) δ 170.61, 170.59, 170.36, 170.29, 170.11, 169.45, 145.59, 101.19, 99.13, 74.81, 74.30, 70.97, 70.84, 69.00, 68.99, 66.83, 61.98, 61.10, 21.24, 21.01, 20.81, 20.78, 20.71.

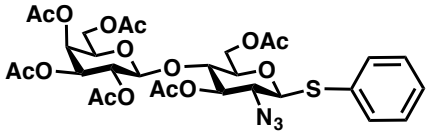


2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-azido-2-deoxy-D-gluco- and mannopyranosyl nitrate (17).[5-6] To a solution of **16** (10.5 g, 18.8 mmol) in dry acetonitrile (45 mL) was added diammonium cerium (IV) nitrate (23.4 g, 42.7 mmol), sodium azide (1.71 g, 26.3 mmol) at -15 ºC and stirred at the same temperature under argon gas for 15 hours. Then, the reaction mixture was poured into Brine and extracted with diethylether thrice. Organic phase was dried over $MgSO_4$, filtered, and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc = 2:1 to 3:2, ϕ = 6.5 cm, h= 15 cm) to give **16** (6.59 g, 9.91 mmol, 53%, α-gluco:β-gluco:α-manno = 1:2:1) as a white powder.



2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl chloride (18). To the obtained compound **16** (6.59 g, 9.91 mmol) in dry acetonitrile (31.5 mL) was added tetraethylammonium chloride (7.29 g, 44.0 mmol) and stirred at rt for 15 hours. Then, the reaction mixture was diluted with CH_2Cl_2, washed with Brine twice, dried over $MgSO_4$, filtered, and concentrated. The crude residue was recrystallized from ethanol-petroleum ether to give glycosyl chloride **18** as a white powder (2.39 g, 3.75 mmol, 38%).

^{1}H(400 MHz, CD_3Cl, TMS) δ 6.09-6.307 (d, *J* = 3.74 Hz, 1 H), 5.54-5.47 (dd, *J* = 9.73 Hz, 1 H), 5.38-5.35 (d, *J* = 3.15 Hz, 1 H), 5.16-5.10 (dd, *J* = 10.37, 7.99 Hz, 1 H), 4.99-4.94 (dd, *J* = 10.37, 3.47 Hz, 1 H), 4.52-4.47 (m, 2 H), 4.28-4.22 (ddd, *J* = 10.09, 4.29, 1.32 Hz, 1 H), 4.22-4.16 (m, 2 H), 4.11-4.05 (dd, *J* = 11.10, 7.40 Hz, 1 H), 3.92-3.87 (dd, *J* = 6.80 Hz, 1 H), 3.87-3.80 (dd, *J* = 9.64 Hz, 1 H), 3.75-3.70 (dd, *J* = 10.37, 3.88, 1 H), 2.18-2.17 (s, 3 H), 2.15-2.14 (s, 3 H), 2.14-2.13 (s, 3H), 2.08-2.07 (s, 3 H), 2.07-2.05 (s, 3 H), 1.98-1.96 (s, 3 H).
^{13}C(100 MHz, CD_3Cl, TMS) δ 170.50, 170.31, 170.29, 170.24, 169.32, 169.05, 100.97, 91.89, 75.40, 71.80, 71.11, 70.95, 70.01, 69.15, 66.70, 62.69, 61.33, 60.95, 20.98, 20.97, 20.80, 20.66.



Phenyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-3,6-di-*O*-acetyl-2-azido-2-deoxy-1-thio-β-D-glucopyranoside (19).

To a solution of **18** (1.91 g, 3.0 mmol) in ethylacetate (24 mL) was added tetrabutylammonium hydrogensulfate (1.01 g, 2.98 mmol), thiophenol (911 μL, 8.93 mmol), and 1 M aqueous sodium carbonate (24 mL). The resulting mixture was stirred at 40 ºC for 2 hours.[7] The mixture was diluted with ethylacetate, washed by 0.1 M aq. $NaHCO_3$ twice, dried over Mg_2SO_4, filtered, and concentrated. The resulting residue was purified by silica gel column chromatography (hexane/EtOAc = 5:1 to 1:1, ϕ= 5.0 cm, h= 15 cm). Fractions containing the desired thioglycoside were combined and evaporated. The resulting residue was recrystallized

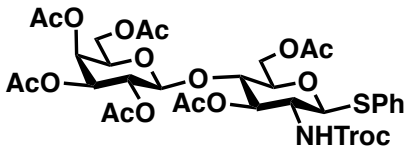
from ethanol and petroleum ether to give **19** (539 mg, 0.78 mmol, 25%).

$[\alpha]_D^{17}$ -32.3 (*c* 0.42 $CHCl_3$)

^{1}H NMR (400 MHz, $CDCl_3$, TMS): 7.60-7.54 (m, 2 H), 7.39-7.31 (m, 3 H), 5.35 (brd, 1 H, *J* = 3.29 Hz), 5.13-5.04 (m, 2 H), 4.94 (dd, *J* = 10.31, 3.42 Hz, 1 H), 4.52 (dd, *J* = 12.00, 1.41 Hz, 1 H), 4.47 (d, *J* = 10.13 Hz, 1 H), 4.44 (d, *J* = 7.91 Hz, 1 H), 4.16 (dd, *J* = 11.04, 6.18 Hz, 1 H), 4.11 (dd, *J* = 11.98, 4.96 Hz, 1 H), 4.11 (dd, *J* = 7.49, 11.08 Hz, 1 H), 3.86 (bddd *J* = 6.72 Hz, 1 H), 3.67-3.56 (m, 2 H), 3.32 (dd, *J* = 10.07, 10.02 Hz, 1 H), 2.15 (s, 3 H), 2.12 (s, 3 H), 2.11 (s, 3 H), 2.04 (s, 3 H), 2.03 (s, 3 H), 1.96 (s, 3 H).

^{13}C NMR (100 MHz, $CDCl_3$): 20.64, 20.74, 20.77, 20.98, 60.93, 62.15, 63.24, 66.73, 69.21, 70.86, 71.12, 73.93, 75.87, 77.36, 77.01, 86.01, 101.07, 128.88, 129.17, 130.81, 134.06, 169.03, 169.49, 170.21, 170.26, 170.35, 170.43.

HRMS (ESI): calcd for $C_{30}H_{37}Cl_3O_{17}S$ $[M+Na]^+$ 734.1838, found for *m/z* 734.1834 $[M+Na]^+$.



Phenyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-thio-β-D-glucopyranoside (10).

To a solution of **19** (530 mg, 0.74 mmol) in tetrahydrofuran (7.4 mL) was slowly added AcOH (2.2 mL, 37 mmol) and activated zinc (4.8 g, 74 mmol). Zinc was activated as previously reported. The reaction mixture was stirred at rt for 30 min and diluted with CH_2Cl_2. The resulting mixture was filtered over celite, washed by sat. aq. $NaHCO_3$ thrice, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. Triethylamine (267 μL, 1.92 mmol) and 2,2,2-trichloroethyl chloroformate were slowly added to a solution of crude amine in CH_2Cl_2 (5.3 mL) and stirred at rt under argon gas for 5 hours. This reaction mixture was diluted with CH_2Cl_2, washed by sat. aq. $NaHCO_3$ thrice and Brine, dried over $MgSO_4$, filtered, and concentrated. The crude reside was purified by silica gel column chromatography (hexane/EtOAc = 1:2.5 to 2:3, ϕ= 3.5 cm, h= 15 cm). Fractions containing the desired product were combined and evaporated. The resulting residue was recrystallized from ethanol and petroleum ether to give glycosyl donor **10** (224 mg, 0.26 mmol, 35%)[4].

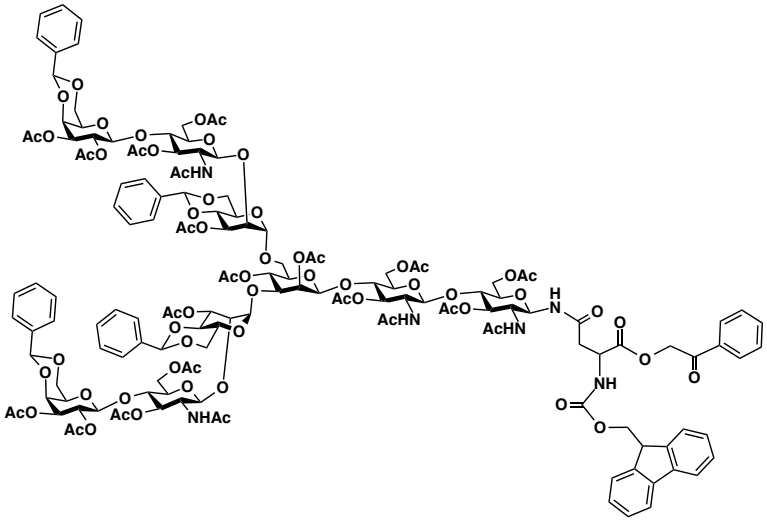
$[\alpha]_D^{17}$ -7.63 (*c* 1.0 $CHCl_3$)

^{1}H NMR[8] (400 MHz, $CDCl_3$, TMS): 7.52-7.45 (m, 2 H), 7.33-7.27 (m, 3 H), 5.35 (brd, *J* = 3.22 Hz, 1 H), 5.27 (d, *J* = 9.57 Hz, 1 H), 5.14 (dd, *J* = 9.40, 8.76 Hz, 1 H), 5.10 (dd, *J* = 10.30, 7.85 Hz, 1 H), 4.96 (dd, *J* = 10.44, 3.37 Hz, 1 H), 4.81 (d, *J* = 12.06 Hz, 1H), 4.74 (d, *J* =12.12 Hz, 1 H), 4.70 (d, *J* = 10.39 Hz, 1 H), 4.55-4.45 (m, 2 H), 4.18-4.02 (m, 3 H), 3.87 (brddd, *J* = 6.84 Hz, 1 H), 3.82-3.70 (m, 2 H), 3.64 (ddd, *J* = 9.51, 5.74, 1.83 Hz, 1 H), 2.13(s, 3 H), 2.11 (s, 3 H), 2.06 (s, 3 H), 2.05 (s, 3 H), 2.04 (s, 3 H), 1.97 (s, 3 H).

^{13}C NMR (100 MHz, $CDCl_3$): 20.65, 20.75, 20.78, 20.94, 20.98, 55.35, 60.98, 62.44, 66.76, 69.24, 70.89, 71.07, 73.58, 74.74, 76.34, 77.36, 87.11, 95.53, 101.23, 128.29, 129.06, 132.46, 132.82, 154.30, 169.24, 170.19, 170.25, 170.43, 170.48, 170.55.

HRMS (ESI): calcd for $C_{33}H_{40}Cl_3NO_{17}S$ $[M+Na]^+$ 882.0975; found for *m/z* 882.0962 $[M+Na]^+$.

2-3. Synthesis of a triantennary oligosaccharide having β-1,6-branching



N^2-(9-Fluorenylmethyloxycarbonyl)-N^4-{*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→3)-*O*-[(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→6)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)}-L-asparagine phenacyl ester (5).

To a solution of Asn-linked biantennary asialononasaccharide **3** (139 mg, 70.4 μmol) in DMF (14 mL) were added camphor-10-sulfonic acid (162 mg, 697 μmol) and benzaldehyde dimethyl acetal (420 μL, 2.71 mmol). The reaction mixture was stirred under an atomosphere of Ar at rt, and the reaction was monitored with RP-HPLC. After 17 hours, the reaction mixture was poured into ice-cold diethylether to form a precipitate. The suspension was centrifuged, and diethylether was decanted. The precipitate was then washed twice more with ice-cold diethylether and air-dried to give crude tetra-benzylidene derivative **4**. The resultant **4** was then used for the next reaction without further purification; ESI-MS calcd for $C_{109}H_{136}N_6O_{50}$ $[M+2H]^{2+}$ 1165.4, found for *m/z* 1165.4.

To a solution of crude tetra-benzylidene derivative **4** in DMF (14 mL) were added *N,N'*-diisopropylethylamine (62 μL, 354 μmol) and 2-bromoacetophenone (42 mg, 211 μmol). The reaction mixture was stirred at rt for 3 hours, and RP-HPLC showed complete conversion to the phenacyl ester (**S2**). The mixture was treated with ice-cold diethylether and centrifuged to

give crude ester **S2**. The resulting **S2** was then used for the next reaction without further purification; ESI-MS calcd for $C_{117}H_{142}N_6O_{51}$ $[M+2H]^{2+}$ 1224.4, found for *m/z* 1224.5.

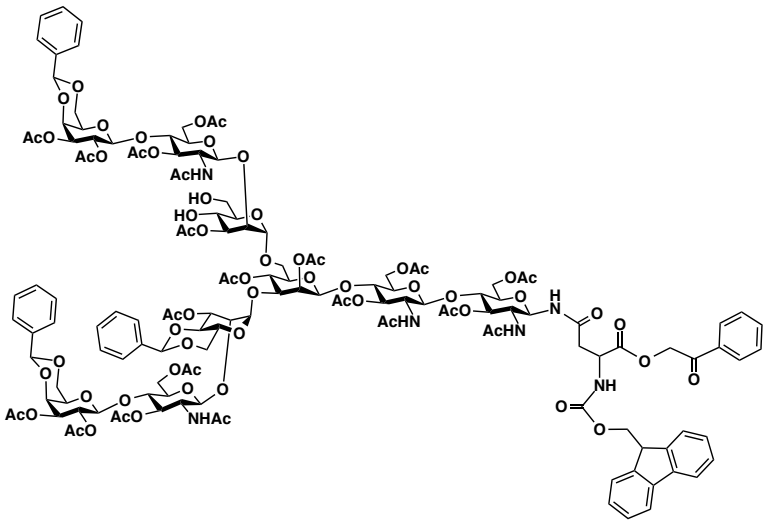
To a solution of crude ester **S2** in acetic anhydride (1.4 mL) and pyridine (1.4 mL), *N,N'*-dimethyl-4-aminopyridine (9 mg, 70 μmol) was added, and the resulting mixture was stirred at rt for 3 hours. The reaction was monitored with RP-HPLC and TLC. After the completion of the reaction, ice-cold MeOH was added to the mixture on an ice bath. The mixture was then allowed to warm up to rt, after which it was concentrated, and the resulting residue was azeotropically dried with toluene several times. The resulting residue was dissolved in aq. CH_3CN, filtered, and purified by preparative RP-HPLC (XBridge, 50 mM aq. NH_4OAc:CH_3CN = 40:60 to 30:70 over 90 min at 4 mL/min). Fractions containing the desired product were combined, treated with Dowex to remove ammonium acetate salt, filtered, and lyophilized to provide fully protected oligosaccharide **5** (52 mg, 24%) as a white form.

$[\alpha]_D^{24}$ -10.3 (*c*: 0.93, $CHCl_3$)

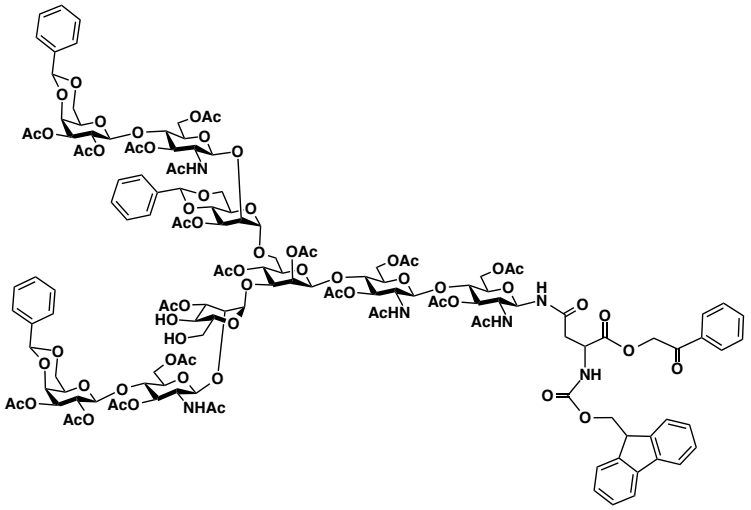
^{1}H NMR (400 MHz, CD_3CN, HDO 2.13 ppm) δ 7.52 (d, *J* = 7.75 Hz, 2 H), 7.42 (d, *J* = 7.49 Hz, 2 H), 7.29-7.20 (m, 3 H), 7.18-7.09 (m, 3 H), 7.09-6.88 (m, 23 H), 6.34-6.25 (m, 2 H), 6.25-6.18 (m, 2 H), 6.04 (d, *J* = 8.61 Hz, 1 H), 5.18 (s, 1 H), 5.14 (s, 3 H), 5.06-4.94 (m, 3 H), 4.89 (dd *J* = 9.95, 9.85.Hz, 1 H), 4.78-4.54 (m, 10 H), 4.47 (s, 1 H), 4.45 (dd, *J* = 6.21, 3.19 Hz, 1 H), 4.39 (s, 1 H), 4.33 (s, 1 H), 4.33-4.28 (m, 1 H), 4.23-4.15 (m, 3 H), 4.09 (d, *J* = 8.39 Hz, 1 H), 4.03 (d, *J* = 8.61 Hz, 1 H), 4.01-3.65 (m, 19 H), 3.64-3.41 (m, 9 H), 3.41-3.10 (m, 17 H), 2.45-2.30 (m, 2 H), 1.75 (s, 3 H), 1.72 (s, 3 H), 1.69 (s, 3 H), 1.69 (s, 6 H), 1.67 (s, 3 H), 1.66 (s, 3 H), 1.65 (s, 3 H), 1.62 (s, 3 H), 1.61 (s, 3 H), 1.60 (s, 3 H), 1.58 (s, 9 H), 1.56 (s, 6 H), 1.54 (s, 3 H), 1.46 (s, 3 H), 1.45 (s, 3 H), 1.43 (s, 3 H), 1.36 (s, 3 H).

^{13}C NMR (100 MHz, CD_3CN 118.26 ppm) δ 193.32, 171.97, 171.84, 171.57, 171.54, 171.47, 171.41, 171.21, 171.17, 171.13, 171.08, 171.02, 170.91, 170.57, 170.54, 170.49, 156.88, 145.01, 144.09, 139.28, 139.25, 138.74, 135.01, 134.98, 130.04, 129.97, 129.87, 129.19, 129.13, 129.06, 128.74, 128.68, 128.12, 127.41, 127.32, 127.25, 126.14, 120.95, 102.75, 102.55, 101.64, 101.55, 101.32, 101.02, 100.60, 99.68, 99.21, 79.52, 79.10, 78.49, 77.84, 77.61, 76.78, 76.42, 76.37, 76.14, 75.61, 75.55, 75.26, 74.43, 74.37, 74.01, 73.78, 73.68, 73.54, 73.29, 73.00, 72.84, 72.22, 71.63, 70.31, 69.97, 69.92, 69.87, 69.34, 69.21, 69.13, 68.38, 67.94, 67.42, 67.19, 67.11, 65.65, 64.91, 63.45, 63.35, 63.25, 62.24, 55.00, 54.90, 54.15, 53.78, 51.51, 47.93, 38.26, 23.24, 23.18, 23.05, 21.57, 21.54, 21.22, 21.19, 21.16, 21.12, 21.08, 21.05, 21.01, 20.98, 20.89.

HRMS (ESI): calcd for $C_{149}H_{174}N_6O_{67}$ $[M+2H]^{2+}$ 1560.5269, found for *m/z* 1560.5242 $[M+2H]^{2+}$.

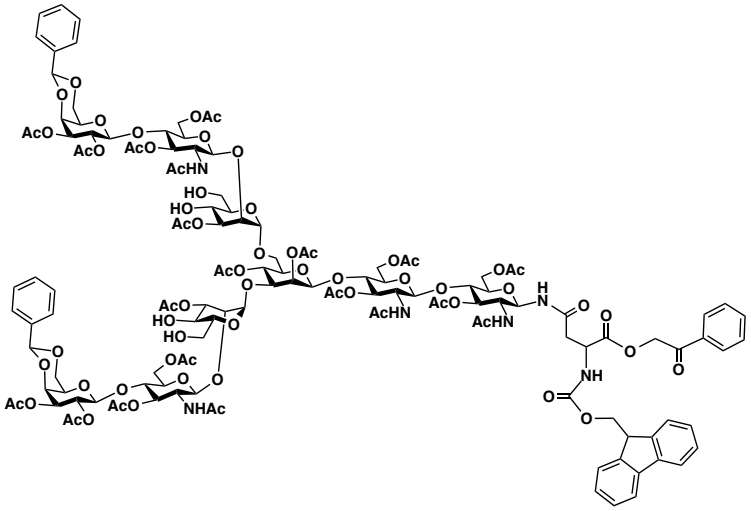


N^2-(9-Fluorenylmethyloxycarbonyl)-N^4-{*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→3)-*O*-[(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-α-D-mannopyranosyl)-(1→6)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)}-L-asparagine phenacyl ester (6).



N^2-(9-Fluorenylmethyloxycarbonyl)-N^4-{*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-

galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-*O*-[(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→6)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)}-L-asparagine phenacyl ester (7).



N^2-(9-Fluorenylmethyloxycarbonyl)-N^4-{*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-*O*-[(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-α-D-mannopyranosyl)-(1→6)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)}-L-asparagine phenacyl ester (8).

The fully protected oligosaccharide **5** (21.3 mg, 6.83 μmol) was dissolved in ice-cold 60% aq. AcOH and stirred with allowing the temperature to arise from 0 ^{o}C to rt. The reaction was monitored with RP-HPLC and TLC. After 10 hours, the mixture was then concentrated *in vacuo* and azeotropically dried with toluene. The resulting residue was dissolved in aq. CH_3CN, filtered, and purified by preparative HPLC (X-Bridge, 50 mM aq. NH_4Ac:CH_3CN = 47:53 to

37:63 over 90 min at 4 mL/min). Fractions containing dibenzylidene derivative **8**, tribenzylidene derivatives **6** and **7**, and remaining starting material **5** were individually collected, and then treated with Dowex in order to remove ammonium acetate salt. Lyophilization of them gave dibenzylidene derivative **8** (1.8 mg, 9%), tribenzylidene derivative **6** (2.7 mg, 13%), tribenzylidene derivative **7** (3.3 mg, 15%), and starting material **5** (3.4 mg, 16%) as white forms. The recovered starting material was used in deprotection of benzylidene acetal group repetitively. Benzylidene acetalation was performed to the dibenzylidene derivative by benzylidene dimethyl acetal and camphor-10-suphonic acid in CH_3CN to yield tetra- and tribenzylidene derivatives again.

Tribenzylidene derivative **6**;

^{1}H NMR (400 MHz, CD_3CN, HOD 2.13 ppm) δ 7.79-7.71 (d, *J* = 7.42 Hz, 2H), 7.69-7.63 (dd, *J* = 7.42, 2.78 Hz, 2H), 7.51-7.46 (m, 3H), 7.39-7.32 (m, 2H), 7.31-7.11 (m, 19H), 6.51-6.41 (m, 2H), 6.39-6.33 (d, *J* = 9.29 Hz, 1H), 6.22-6.14 (d, *J* = 8.77 Hz, 1H), 5.39-5.37 (s, 2H), 5.37-5.35 (s, 1H), 5.31-5.16 (m, 3H), 5.02-4.93 (m, 2H), 4.93-4.77 (m, 8H), 4.73-4.69 (s, 1H), 4.69-4.62 (m, 2H), 4.59-4.50 (m, 3H), 4.47-4.40 (m, 2H), 4.39-4.31 (m, 2H), 4.27-3.87 (m, 18H), 3.87-3.80 (m, 4H), 3.80-3.56 (m, 12H), 3.56-3.31 (m, 12H), 2.69-2.52 (m, 2H), 2.00-1.97 (s, 3H), 1.94-1.92 (s, 6H), 1.91-1.89 (s, 6H), 1.89 (s, 3H), 1.89-1.87 (s, 6H), 1.86-1.84 (s, 3H), 1.84-1.82 (s, 9H), 1.81-1.80 (s, 3H), 1.80-1.79 (s, 6H), 1.79-1.78 (s, 3H), 1.70-1.68 (s, 6H), 1.68-1.66 (s, 3H), 1.64-1.59 (s, 3H).

^{13}C NMR (100 MHz, CD_3CN, 118.26 ppm) δ 193.27, 172.49, 172.09, 171.98, 171.82, 171.56, 171.49, 171.45, 171.22, 171.11, 171.06, 170.94, 170.89, 170.55, 170.50, 156.85, 144.98, 142.06, 139.26, 139.23, 138.70, 134.97, 130.02, 129.96, 129.85, 129.18, 129.05, 128.72, 128.66, 128.09, 127.28, 127.24, 126.13, 120.94, 102.52, 101.97, 101.59, 101.50, 101.10, 100.48, 98.77, 98.34, 79.53, 77.92, 77.79, 77.38, 76.85, 76.10, 75.67, 75.47, 75.13, 74.39, 74.35, 74.08, 73.94, 73.79, 73.68, 73.59, 73.45, 72.96, 72.77, 72.73, 72.18, 71.37, 69.92, 69.86, 69.81, 69.11, 68.92, 67.92, 67.51, 67.39, 67.15, 67.08, 65.62, 64.60, 63.40, 63.29, 63.22, 62.42, 54.87, 54.73, 54.20, 53.68, 51.45, 47.89, 38.21, 23.46, 23.32, 23.21, 23.13, 23.03, 21.54, 21.49, 21.25, 21.17, 21.14, 21.11, 21.03, 20.98, 20.94, 20.88, 20.60.

HRMS (ESI): calcd for $C_{142}H_{170}N_6O_{67}$ $[M+2H]^{2+}$ 1516.5113, found for *m/z* 1516.5156 $[M+2H]^{2+}$.

Tribenzylidene derivative **7**;

^{1}H NMR (400 MHz, CD_3CN) δ 7.80 (d, *J* = 7.46 Hz, 2 H), 7.69 (d, *J* = 7.54 Hz, 2 H), 7.56-7.49(m, 3 H), 7.43-7.37 (m, 2 H), 7.37-6.15 (m, 19 H), 6.44-6.30 (m, 4 H), 6.17 (d, *J* = 8.79, 1 H), 5.44 (s, 1 H), 5.42 (s, 1 H), 5.41 (s, 1 H), 5.33-5.19 (m, 3 H), 5.12 (dd, *J* = 9.89, 9.77 Hz, 1 H), 5.03-4.80 (m, 10 H), 4.64 (s, 2 H), 4.61-4.53 (m, 2 H), 4.52-4.40 (m, 4 H), 4.31-3.84 (m, 21 H), 3.84-3.35 (m, 26 H), 2.71-2.55 (m, 2 H), 2.02 (s, 3 H), 1.96 (s, 3 H), 1.95 (s, 3 H), 1.92 (s, 9 H), 1.91 (s, 6 H), 1.87 (s, 6 H), 1.86 (s, 3 H), 1.85 (s, 3 H), 1.84 (s, 6 H), 1.82 (s, 3 H), 1.73 (s, 3 H), 1.71 (s, 6 H), 1.68 (s, 3 H), 1.62 (s, 3 H).

^{13}C NMR (100 MHz, CD_3CN, 118.26 ppm) δ 193.30, 171.97, 171.90, 171.83, 171.65, 171.57, 171.52, 171.46, 171.39, 171.32, 171.19, 171.17, 171.16, 171.07, 171.01, 170.98, 170.89, 170.58, 170.48, 174.98, 156.85, 144.98, 142.07, 139.26, 139.22, 138.71, 130.02, 129.85, 129.17, 129.12, 128.73, 128.66, 128.10, 127.40, 127.25, 127.23, 126.12, 120.94, 102.71, 101.61, 101.55, 101.53, 101.48, 100.95, 100.44, 99.55, 99.20, 79.48, 77.71, 77.65, 77.55, 75.73, 76.33, 76.28, 75.81, 75.44, 75.19, 74.77, 74.40, 74.35, 73.94, 73.71, 73.47, 73.20, 73.05, 72.96, 72.64, 72.19, 71.43, 70.30, 69.87, 69.81, 69.31, 69.18, 69.11, 68.46, 67.93, 67.39, 67.15, 67.08, 64.83, 64.31, 63.27, 63.13, 62.05, 54.89, 54.66, 54.12, 53.76, 51.47, 47.90, 38.22, 23.42, 23.33, 23.19, 23.15, 23.02, 21.58, 21.51, 21.22, 21.17, 21.14, 21.11, 21.06, 20.99, 20.95, 20.90, 20.87.

HRMS (ESI): calcd for $C_{142}H_{170}N_6O_{67}$ $[M+2H]^{2+}$ 1516.5113, found for *m*/*z* 1516.5163 $[M+2H]^{2+}$.

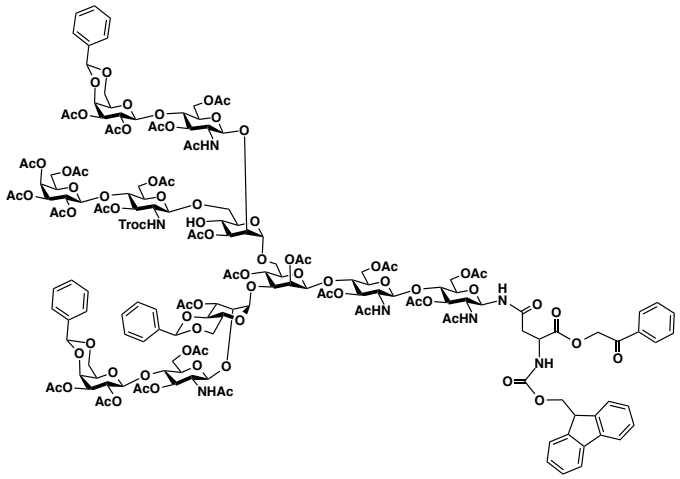
Dibenzylidene derivative **8**;

^{1}H NMR (700 MHz, CD_3CN) δ 7.93 (d, *J* = 7.53 Hz, 2 H), 7.83 (d, *J* = 7.56 Hz, 2 H), 7.69-7.64 (m, 3 H), 7.56-7.51 (m, 2 H), 7.46-7.39 (m, 12 H), 7.36-7.31 (m, 2 H), 7.24 (d, *J* = 8.66 Hz, 1 H), 6.51-6.46 (m, 2 H), 6.44 (d, *J* = 9.31 Hz, 1 H), 6.40 (d, *J* = 9.11 Hz, 1 H), 6.24 (d, *J* = 8.80 Hz, 1 H), 5.55 (s, 2 H), 5.43 (d, *J* = 16.56 Hz), 5.37 (d, *J* = 16.56 Hz, 1 H), 5.34 (d, *J* = 3.06 Hz, 1 H), 5.14-4.94 (m, 10 Hz), 4.80 (dd, *J* = 9.89, 3.27 Hz, 1 H), 4.75 (s, 1 H), 4.73-4.64 (m, 3 H), 4.62-4.56 (m, 3 H), 4.54 (d, *J* = 8.34 Hz, 1 H), 4.42-4.29 (m, 9 H), 4.29-4.20 (m, 3 H), 4.19-4.05 (m, 6 H), 4.02 (dd, *J* = 12.12 5.23 Hz, 1 H), 3.98 (brs, 1 H), 3.95 (dd, *J* = 9.80, 3.13 Hz), 3.90-3.72 (m, 11 H), 3.72-3.50 (m, 15 H), 3.47 (dd, *J* = 10.53, 2.44 Hz), 3.36 (brs, 1 H), 3.17 (brs, 1 H), 2.97 (brs, 1 H), 2.79 (dd, *J* = 16.07, 4.83 Hz, 1 H), 2.73 (dd, *J* = 16.12, 6.44 Hz, 1 H), 2.13 (s, 3 H), 2.09 (s, 6 H), 2.08 (s, 3 H), 2.06 (s, 3 H), 2.05 (s, 6 H), 2.03 (s, 3 H), 2.02 (s, 3 H), 2.01 (s, 6 H), 2.00 (s, 3 H), 1.99 (s, 3 H), 1.96 (s, 6 H), 1.95 (s, 3 H), 1.85 (s, 6 H), 1.83 (s, 3 H), 1.75 (s, 3 H).

^{13}C NMR (100 MHz, CD_3CN 118.26 ppm) δ 193.28, 172.02, 171.95, 171.92, 171.82, 171.65,

171.52, 171.48, 171.41, 171.38, 171.26, 171.23, 171.19, 170.99, 170.92, 170.88, 170.55, 170.55, 170.50, 156.84, 144.98, 142.06, 139.25, 139.23, 134.97, 130.02, 129.85, 129.17, 128.72, 128.65, 129.09, 127.23, 126.12, 120.93, 101.91, 101.62, 101.58, 101.51, 101.46, 100.30, 98.74, 98.47, 79.49, 77.53, 77.41, 77.21, 76.75, 75.69, 75.15, 74.99, 74.76, 74.38, 74.34, 74.10, 73.99, 73.86, 73.69, 73.56, 73.49, 73.45, 73.18, 73.01, 72.75, 72.57, 72.18, 71.21, 69.85, 69.80, 69.15, 69.10, 69.06, 67.92, 67.53, 67.38, 67.13, 67.08, 64.62, 64.28, 63.31, 63.27, 63.15, 62.45, 62.06, 57.87, 54.69, 54.20, 53.69, 51.45, 47.89, 38.20, 23.43, 23.40, 23.13, 23.02, 21.56, 21.50, 21.25, 21.14, 21.11, 20.99, 20.93, 20.87, 20.58, 18.67.

HRMS (ESI): calcd for $C_{135}H_{166}N_6O_{67}$ $[M+2H]^{2+}$ 1472.4956, found for *m*/*z* 11472.4956 $[M+2H]^{2+}$.



***N*²-(9-Fluorenylmethyloxycarbonyl)-*N*⁴-{*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→3)-*O*-[(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(3,6-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxy)-carbonylamino-β-D-glucopyranosyl)-(1→6)]-(3-*O*-acetyl-α-D-mannopyranosyl)-(1→6)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)}-L-asparagine phenacyl ester (11).**

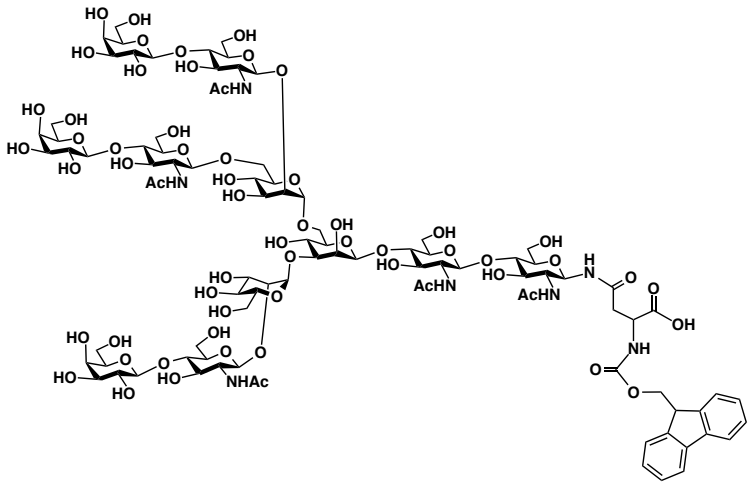
A mixture of acceptor **6** (8.4 mg, 2.8 μmol) and donor **10** (23.9 mg, 28 μmol, 10 equiv) was dried *in vacuo* for 3 hours, after which the mixture was dissolved in distilled CH_2Cl_2 (560

μL) under an atmosphere of Ar, followed by addition of freshly activated 4 A molecular sieves (56 mg). The reaction mixture was cooled to 0 ^{o}C, and NIS (5.0 mg, 22 μmol) was added. Then, TfOH (2.07 μmol, with dilution by CH_2Cl_2) was added to the mixture, which was stirred for 1 hour at the same temperature. After the completion of the reaction, the mixture was diluted with chloroform, which was directly applied to silica gel column chromatography to remove a remaining glycosyl donor and its derivatives (ϕ 1.0 × 7.0 cm, Hexane:EtOAc (1:1), EtOAc, EtOAc:MeOH (7:1)). The fractions containing oligosaccharides were combined and evaporated. The resulting residue was dissolved in aq. CH_3CN, filtered, and purified by preparative HPLC (XBridge, 50 mM aq. NH_4OAc:CH_3CN = 40:60 to 30:70 over 90 min at 4 mL/min). Fractions containing the desired product were collected, treated with Dowex, filtered and lyophilized to give the protected triantennary oligosaccharide **11** (4.9 mg, 1.3 μmol, 47%) as a white form.

^{1}H NMR (400 MHz, CD_3CN, HOD 2.13 ppm) δ 7.79 (d, *J* = 7.54 Hz, 2 H), 7.69 (d, *J* = 7.54 Hz, 2 H), 7.55-7.49 (m, 3 H), 7.43-7.35 (m, 2 H), 7.35-7.16 (m, 19 H), 7.04 (d, *J* = 7.96 Hz, 1 H), 6.53 (d, *J* = 9.65 Hz, 1 H), 6.47-6.32 (m, 3 H), 6.18 (d, *J* = 8.68 Hz, 1 H), 5.41 (m, 2 H), 5.40 (s, 1 H), 5.33-5.20 (m, 4 H), 5.17 (brd, *J* = 3.62 Hz, 1 H), 5.04-4.77 (m, 13H), 4.77-4.66 (m, 3 H), 4.66-4.52 (m, 5 H), 4.39-4.32 (m, 2 H), 4.30 (d, *J* = 8.82 Hz, 1 H), 4.27-3.81 (m, 26 H), 3.81-3.29 (m, 26 H), 3.06 (dd, *J* = 18.95, 8.58 Hz, 1 H), 2.71-2.54 (m, 2 H), 1.99 (s, 3 H), 1.96 (s, 9 H), 1.95 (s, 3 H), 1.92 (s, 3 H), 1.91 (s, 9 H), 1.90 (s, 3 H), 1.88 (s, 6 H), 1.87 (s, 3 H), 1.86 (s, 9 H), 1.82 (s, 6 H), 1.81 (s, 3 H), 1.79 (s, 3 H), 1.76 (s, 3 H), 1.74 (s, 3 H), 1.70 (s, 6 H), 1.62 (s, 3 H).

Chemical shift of ^{13}C (CD_3CN, chemical shift picked up from HSQC except for quaternary carbon) δ 134.92, 129.93, 129.76, 128.81, 128.63, 128.01, 127.16, 126.00, 120.86, 102.41, 101.74, 101.49, 101.25, 101.09, 100.94, 100.71, 100.40, 98.21, 98.06, 79.38, 77.88, 77.66, 77.43, 76.70, 76.00, 75.40, 75.08, 74.84, 74.53, 74.29, 74.05, 73.99, 73.76, 73.59, 73.43, 73.35, 73.27, 72.98, 72.66, 72.11, 71.55, 71.40, 71.25, 69.93, 69.85, 69.76, 69.06, 68.68, 68.05, 67.82, 67.35, 67.04, 66.73, 65.56, 63.99, 63.36, 63.28, 63.18, 63.13, 62.98, 61.96, 57.49, 54.84, 54.59, 54.44, 53.60, 51.40, 47.81, 38.20, 23.46, 23.23, 23.02, 21.50, 21.17, 21.04, 20.88, 20.73.

HRMS (ESI): calcd for $C_{169}H_{204}Cl_3N_7O_{84}$ $[M+2H]^{2+}$ 1891.0559, found for *m*/*z* 1891.0580 $[M+2H]^{2+}$.



N^2-(9-Fluorenylmethyloxycarbonyl)-N^4-{*O*-β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-*O*-[β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-[β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)]-*O*-α-D-mannopyranosyl-(1→6)]-*O*-β-D-mannopyranosyl-*O*-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)}-L-asparagine (1).

The protected triantennary oligosaccharide **11** (2.3 mg, 0.61 μmol) was dried *in vacuo* for 3 hours, which was then dissolved in THF (300 μL), AcOH (200 μL), and Ac_2O (100 μL) on an ice bath. To the mixture, freshly activated zinc powder (161 mg, 2.45 mmol) was slowly added. After 1 hour, the resulting mixture was allowed to arise from 0 ^oC to rt and then stirred for additional 15 hours. The reaction was monitored by RP-HPLC and TLC analyses. After completion of the reaction, the mixture was diluted with CH_3CN and filtered through a pad of Celite, followed by addition of MeOH, the resulting mixture was concentrated to give crude **S3**; ESI-MS calcd for $C_{160}H_{199}N_7O_{82}$ $[M+2H]^{2+}$ 1766.1, found for *m/z* 1766.2.

To a solution of crude **S3** in ice-cold MeOH (256 μL), ice-cold 5 M aq. NaOH (256 μL) was added slowly, and the mixture was stirred for 30 min on ice bath and stirred for additional 2 hours at rt. After completion of the reaction, the resulting mixture was subjected directly to gel filtration chromatography (φ1.4×33 cm, Sephadex^{TM} G-15, eluent: distilled water). Fractions containing oligosaccharide were collected and lyophilized to give crude oligosaccharide **S4**; ESI-MS calcd for $C_{101}H_{145}N_7O_{58}$ $[M+2H]^{2+}$ 1192.9, found for *m/z* 1193.0.

To an ice-cold solution of the crude oligosaccharide **S4** in H_2O (250 μL) containing

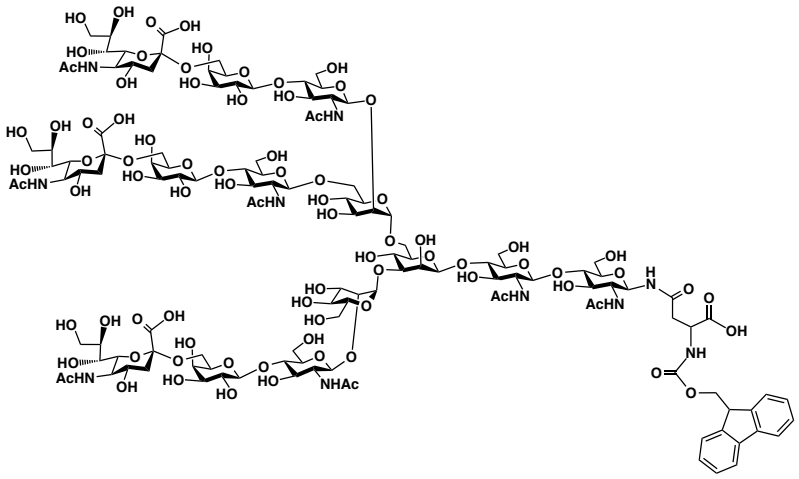
$NaHCO_3$ (10 μmol) were added a solution of FmocOSu (1.7 mg, 5.0 μmol) in acetone (250 μL) slowly. The reaction mixture was stirred for 30 min on an ice bath, which was stirred for additional 3 hours at rt. The resulting mixture was subjected directly to gel filtration chromatography (φ1.4×33 cm, Sephadex^{TM} G-25, eluent: distilled water). Fractions containing oligosaccharide were collected and lyophilized to give crude **S5**; ESI-MS calcd for $C_{116}H_{155}N_7O_{60}$ $[M+2H]^{2+}$ 1304.0, found for *m/z* 1303.9.

Ice-cold 90% aq. TFA (255 μL) was slowly added to crude **S5** on ice bath. The mixture was kept at 0 ^oC for 10 min, and concentrated at rt, after which distilled water was added. The resulting mixture was frozen by treatment of liquid N_2 and lyophilized immediately. The resulting residue was purified by RP-HPLC (Proteonavi C4, 0.1% aq. TFA:90% aq. CH_3CN containing 0.1% TFA = 80:20 to 50:50 over 30 min at 1 mL/min). Fractions containing the desired product were collected and lyophilized to give Asparagine-linked β-1,6-branched triantennary asialo-undecasaccharide **1** (0.9 mg, 0.38 μmol, 63%).

^{1}H NMR (400 MHz, D_2O, 4.79 ppm) δ7.92 (d, *J* = 7.35 Hz, 2 H), 7.71 (brd, *J* = 6.50 Hz, 2 H), 7.50 (brt, *J* = 7.34 Hz, 2 H), 7.47-7.40 (m, 2 H), 5.13 (s, 1 H), 5.01 (d, *J* = 9.55 Hz, 1 H), 4.87 (s, 1 H), 4.77 (s, 1 H), 4.62-4.57 (m, 3 H), 4.56 (d, J = 7.77 Hz, 1 H), 4.52-4.42 (m, 3 H), 4.39-4.31 (m, 2 H), 4.25 (brs, 1 H), 4.24-4.16 (m, 2 H), 4.10 (s, 1 H), 4.05-3.69 (m, 45 H), 3.69-3.65 (m, 3 H), 3.65-3.45 (m, 13 H), 3.41 (t, *J* = 9.75 Hz, 1 H), 2.72 (dd, *J* = 15.62, 3.96 Hz, 1 H), 2.61 (dd, *J* = 15.62, 8.07 Hz, 1 H), 2.08 (s, 3 H), 2.06 (s, 3 H), 2.04 (s, 6 H), 1.90 (s, 3 H).

Chemical shift of ^{13}C (D_2O, chemical shift picked up from HSQC except for quaternary carbon) δ128.96, 128.46, 126.01, 121.08, 103.87, 102.56, 102.29, 101.28, 100.45, 98.13, 81.26, 80.09, 79.56, 79.45, 79.11, 77.50, 77.33, 77.10, 76.29, 75.65, 74.48, 73.65, 73.45, 73.19, 72.88, 72.49, 71.92, 71.28, 71.14, 70.33, 69.47, 68.48, 68.23, 67.76, 67.73, 66.49, 66.10, 62.67, 61.98.

HRMS (ESI): calcd for $C_{95}H_{143}N_7O_{60}$ $[M+2H]^{2+}$ 1171.9250, found for *m/z* 1171.9246 $[M+2H]^{2+}$.



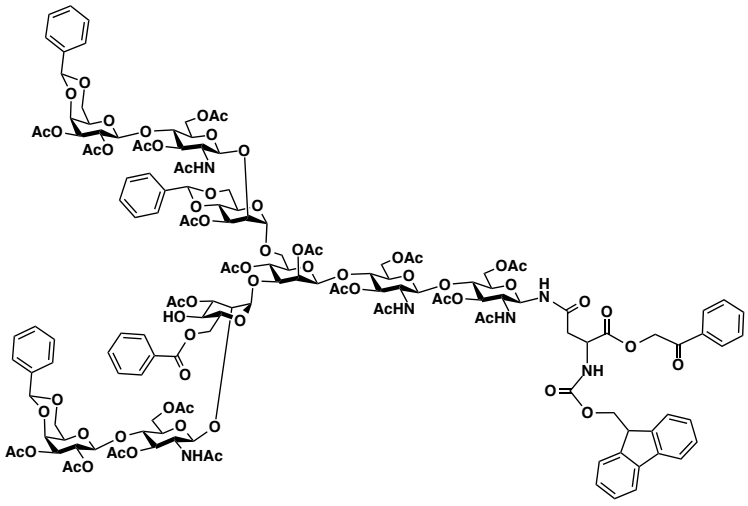
N^2-(9-Fluorenylmethyloxycarbonyl)-N^4-{*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-*O*-β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-*O*-[(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-[(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)]-*O*-α-D-mannopyranosyl-(1→6)]-*O*-β-D-mannopyranosyl-*O*-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)}-L-asparagine (20).

Asialooligosaccharide **1** (0.13 μmol) and CMP-Neu5Ac (1.2 μmol, 9.1 equiv) were dissolved in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 6.1) containing BSA (0.1 mg/ mL) and alkaline phosphatase (100 mU). To the reaction mixture, α-2,6-sialyltransferase (3 U) was added (total volume = 65 μL), and the reaction mixture was incubated at rt. The reaction was monitored with LC/MS. After 48 hours, Additional CMP-Neu5Ac (1.6 μmol), Alkaline phosphatase (100 mU) and α-2,6-sialyltransferase (1.5 U) were added and then incubated for another 2 days at rt. The resulting mixture was filtered, and the filtrate was purified by RP-HPLC (Proteonavi C4, 50 mM aq. MH_4OAc:CH_3CN = 90:10 to 80:20 over 30 min at 1 mL/min). Fractions containing desired product were collected and lyophilized, which was further purified by RP-HPLC (Proteonavi C4, distilled water over 30 min at 1 mL/min). Fractions containing desired product were collected and lyophilized to provide trisialylated oligosaccharide **20** (isolated; ca. 30 nmol, conversion yield; 86% based on HPLC analysis).

^{1}H NMR (D_2O) δ 7.83 (d, *J* = 7.68 Hz, 2 H), δ7.92 (d, *J* = 7.35 Hz, 2 H), 7.65-7.61 (m, 2 H), 7.44-7.40 (m, 2 H), 7.37-7.32 (m, 2 H), 5.04 (s, 1 H), 4.38-4.33 (m), 4.29-4.24 (m), 4.20-4.15 (m), 4.15-4.10 (m), 4.03-3.99 (m), 3.95-3.38 (m), 2.65-2.55 (m, 4 H), 2.47 (m, 1 H), 1.98 (s, 9 H), 1.94 (s, 9 H), 1.82 (s, 3 H), 1.80 (s, 3 H), 1.67-1.60 (m, 3 H).

HRMS (ESI): calcd for $C_{128}H_{194}N_{10}O_{84}$ $[M-3H]^+$ 1070.6989, found for *m*/*z* 1070.7012 $[M-3H]^{3-}$.

2-4. Synthesis of a triantennary oligosaccharide having β-1,4-branching



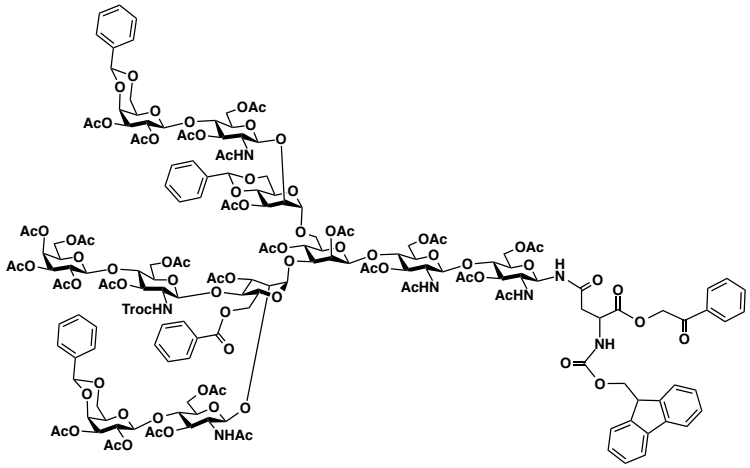
N^2-(9-Fluorenylmethyloxycarbonyl)-N^4-{*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-6-*O*-benzoyl-α-D-mannopyranosyl)-(1→3)-*O*-[(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→6)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)}-L-asparagine phenacyl ester (9).

The tribenzylidene **7** (11.8 mg, 3.9 μmol) was dried *in vacuo* for 2 hours, which was then dissolved in CH_2Cl_2 (780 μL), followed by addition of *N, N'*-diisopropylethylamine (10 μL, 59 μmol) and BzCN (4.6 μL, 38.8 μmol), the resulting mixture was stirred at rt for 2 hours. The reaction was monitored by HPLC and TLC analyses. After a completion of the reaction, the mixture was diluted with chloroform and directly purified by silica gel chromatography to remove reagents (φ1.0×8.0 cm, EtOAc, EtOAc:MeOH (5:1)). The fractions containing oligosaccharides were collected and then evaporated. The resulting residue was dissolved in aq. CH_3CN, filtered, and purified by preparative RP-HPLC (XBridge, 50 mM aq. NH_4Ac:CH_3CN = 45:55 to 35:65 over 90 min at 4 mL/min). Fractions containing the desired product was collected, treated with Dowex, and lyophilized to give glycosyl acceptor **9** (5.8 mg, 1.9 μmol, 48%).

^{1}H NMR (400 MHz, CD_3CN, HOD 2.13 ppm) δ 7.79 (d, *J* = 7.36 Hz, 2 H), 7.66 (d, *J* = 7.72 Hz, 2 H), 7.55 (d, *J* = 7.59 Hz, 2 H), 7.42-7.35 (m, 4 H), 7.33-7.22 (m, 4 H), 7.22-7.01 (m, 19 H), 6.36 (d, *J* = 9.73 Hz, 1 H), 6.29 (d, *J* = 9.20 Hz, 1 H), 6.25 (d, *J* = 9.51 Hz), 6.14 (d, *J* = 9.28 Hz, 1 H), 6.08 (d, *J* = 8.82 Hz, 1 H), 5.30 (s, 1 H), 5.27 (s, 2 H), 5.20-5.06 (m, 3 H), 4.99 (dd, *J* = 9.91, 9.84 Hz, 1 H), 4.89-4.66 (m, 10 H), 4.60 (s, 1 H), 4.50 (s, 1 H), 4.47-4.36 (m, 3 H), 4.35-4.26 (m, 3 H), 4.24-3.77 (m, 22 H) , 3.77-3.70 (m, 2 H), 3.67-3.20 (m, 23 H), 2.57-2.42 (m, 2 H), 1.88 (s, 3 H), 1.82 (s, 3 H), 1.81 (s, 3 H), 1.78 (s, 9 H), 1.75 (s, 6 H), 1.74 (s, 3 H), 1.73 (s, 3 H), 1.72 (s, 3 H), 1.68 (s, 12 H), 1.61 (s, 3 H), 1.58 (s, 3 H), 1.55 (s, 3 H), 1.48 (s, 3 H), 1.39 (s, 3 H).

^{13}C NMR (100 MHz, CD_3CN, 118.26 ppm) δ193.39, 172.07, 171.91, 171.74, 171.66, 171.57, 171.49, 171.41, 171.33, 171.27, 171.23, 171.20, 171.15, 171.11, 170.98, 170.67, 170.63, 170.57, 167.19, 159.73, 145.06, 142.14, 139.33, 139.30, 38.79, 135.07, 135.04, 134.24, 131.08, 130.58, 130.10, 129.92, 129.85, 129.26, 129.24, 129.19, 128.80, 128.73, 128.17, 127.47, 127.31, 126.20, 121.01, 102.79, 101.68, 101.61, 101.18, 101.05, 100.77, 99.65, 99.04, 79.55, 78.20, 77.90, 77.72, 76.78, 76.41, 76.02, 75.57, 75.51, 75.31, 74.48, 74.43, 73.99, 73.79, 73.69, 73.31, 73.06, 72.36, 72.27, 71.71, 70.41, 69.97, 69.92, 69.38, 69.20, 68.56, 68.00, 67.47, 67.23, 67.15, 64.92, 64.35, 63.36, 55.03, 54.80, 54.21, 53.83, 51.57, 47.98, 38.31, 23.27, 23.23, 23.09, 21.68, 21.57, 21.25, 21.20, 21.14, 21.07, 21.02, 20.97, 20.94.

HRMS (ESI): calcd for $C_{149}H_{174}N_6O_{68}$ $[M+2H]^{2+}$ 1568.5244, found for *m*/*z* 1568.5292 $[M+2H]^{2+}$.



***N*^{2}-(9-Fluorenylmethyloxycarbonyl)-*N*^{4}-{*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-**

galactopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-*O*-(3,6-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxy)-carbonylamino-β-D-glucopyranosyl)-(1→4)]-*O*-(3-*O*-acetyl-6-*O*-benzoyl-α-D-mannopyranosyl)-(1→3)-*O*-[(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galacto-pyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→6)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)}-L-asparagine phenacyl ester (12).

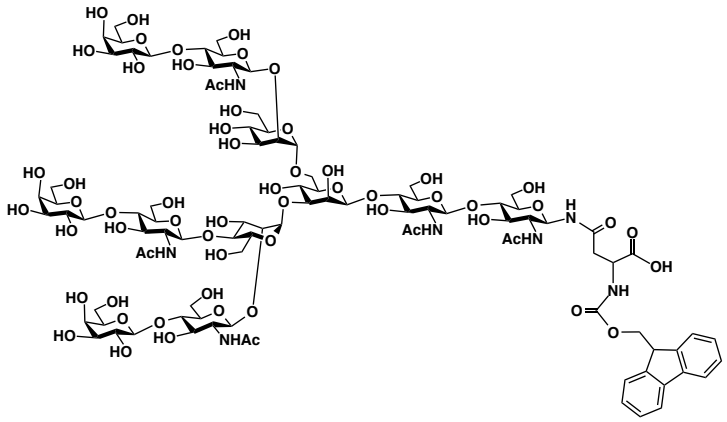
A mixture of glycosyl acceptor **9** (5.3 mg, 1.7 μmol) and glycosyl donor **10** (29.1 mg, 33.8 μmol) was *in vacuo* for 3 hours, after which this mixture was dissolved in distilled CH_2Cl_2 (320 μL) under an atmosphere of Ar, followed by addition of freshly activated 4 A molecular sieves (35 mg). The reaction mixture was allowed to cool to 0 ^oC, followed by addition of NIS (6.1 mg, 27.1 μmol). Then, TfOH (1.27 μmol, dilution with CH_2Cl_2) was added to the mixture and stirred for 1.5 hours at the same temperature, after which the temperature was elevated to rt and stirred for additional 30 min. The resulting mixture was directly applied on silica gel column chromatography to remove glycosyl donor derivatives (ϕ 1.0×7.0 cm, Hexane:EtOAc (1:1), EtOAc, EtOAc:MeOH (5:1)). The fractions containing oligosaccharides were collected and then evaporated. The resulting residue was dissolved in CH_3CN-H_2O, filtered, and purified by preparative RP-HPLC (XBridge, 50 mM aq. NH_4OAc:CH_3CN = 45:55 to 35:65 over 90 min at 4 mL/min). Fractions containing the desired product were collected, treated with Dowex, filtered, and lyophilized to yield protected triantennary oligosaccharide **12** (2.9 mg, 0.75 μmol, 44%) as a white form.

^{1}H NMR (400 MHz, CD_3CN, HOD 2.13 ppm) δ 7.98 (d, *J* = 7.46 Hz, 2 H), 7.86 (d, *J* = 7.52 Hz, 2 H), 7.75 (d, *J* = 7.51 Hz, 2 H), 7.61-7.55 (m, 4 H), 7.52-7.42 (m, 4 H), 7.42-7.20 (m, 19 H), 6.45 (d, *J* = 9.86 Hz, 1 H), 6.39 (d, *J* = 9.40 Hz, 1 H), 6.36 (d, *J* = 9.70 Hz, 1 H), 6.21 (d, *J* = 8.71 Hz, 1 H), 6.11 (d, *J* = 9.24 Hz, 1 H), 6.03 (d, *J* = 9.79 Hz, 1 H), 5.49 (s, 1 H), 5.47 (s, 2 H), 5.39-5.25 (m, 3 H), 5.23-5.14 (m, 2 H), 5.08-4.85 (m, 12 H), 4.84-4.71 (m, 4 H), 4.69 (s, 1 H), 4.66-4.59 (m, 3 H), 4.57-4.44 (m, 5 H), 4.39 (d, *J* = 8.40 Hz, 1 H), 4.36-3.87 (m, 28 H), 3.87-3.63 (m, 11 H), 3.63-3.33 (m, 14 H), 2.76-2.60 (m, 2 H), 2.06 (s, 3 H), 2.03 (s, 3 H), 2.01 (s, 6 H), 1.99 (s, 6 H), 1.98 (s, 6 H), 1.93 (s, 15 H), 1.92 (s, 3 H), 1.91 (s, 3 H), 1.90 (s, 3 H),

1.89 (s, 12 H), 1.88 (s, 3 H), 1.85 (s, 3 H), 1.81 (s, 3 H), 1.76 (s, 3 H), 1.74 (s, 3 H), 1.68 (s, 3 H).

Chemical shift of ^{13}C (CD_3CN, chemical shift was picked up from HSQC except for quaternary carbon) δ134.53, 130.56, 129.92, 129.59, 128.88, 128.65, 127.97, 127.21, 126.03, 120.86, 102.61, 101.50, 101.43, 101.34, 100.89, 100.63, 100.45, 99.53, 98.85, 79.37, 78.13, 77.58, 76.92, 76.60, 76.25, 75.90, 75.42, 75.25, 75.18, 74.74, 74.31, 73.87, 73.65, 73.58, 73.52, 73.44, 73.10, 72.92, 72.14, 71.51, 71.40, 70.26, 69.89, 69.82, 69.19, 69.08, 68.48, 68.04, 67.86, 67.37, 67.04, 64.82, 63.78, 63.22, 63.17, 61.88, 57.33, 54.83, 54.12, 54.07, 53.70, 51.45, 47.85, 38.18, 23.03, 21.53, 21.01, 20.63.

HRMS (ESI): calcd for $C_{176}H_{208}Cl_3N_7O_{85}$ $[M+2H]^{2+}$ 1943.0690, found for *m*/*z* 1943.0666 $[M+2H]^{2+}$.



***N*^{2}-(9-Fluorenylmethyloxycarbonyl)-*N*^{4}-{*O*-β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-[β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)]-*O*-α-D-mannopyranosyl-(1→3)-*O*-[β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-α-D-mannopyranosyl-(1→6)]-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)}-L-asparagine (2).**

The protected triantennary oligosaccharide **12** (3.0 mg, 0.77 μmol) was dried *in vacuo* for 2 hours, which was dissolved in THF (384 μL), AcOH (256 μL), and Ac_2O (128 μL) on an ice bath. To the mixture, freshly activated zinc powder (200 mg, 3.06 mmol) was slowly added.

The resulting mixture was stirred at 0 °C for 1 hour and stirred at rt for additional 17 hours. The reaction was monitored by RP-HPLC and TLC analyses. After completion of the reaction, the mixture was diluted with CH_3CN and filtered through a pad of celite, followed by addition of MeOH, the resulting mixture was concentrated to give crude **S6**; ESI-MS calcd for $C_{167}H_{203}N_7O_{83}$ $[M+2H]^{2+}$ 1818.1, found for *m/z* 1818.1.

To a solution of crude **S6** in ice-cold MeOH (385 μL), ice-cold 5 M aq. NaOH (385 μL) was added slowly, and the mixture was stirred for 30 min on an ice bath, after which the reaction temperature was allowed to warm up to rt and stirred for additional 90 min. After completion of the reaction, the resulting mixture was directly subjected to gel filtration chromatography (φ1.4×33 cm, Sephadex™ G-15, eluent: distilled water). Fractions containing oligosaccharide were collected and lyophilized to give crude oligosaccharide **S7**; ESI-MS calcd for $C_{103}H_{147}N_7O_{59}$ $[M+2H]^{2+}$ 1192.9, found for *m/z* 1192.9.

To an ice-cold solution of the crude oligosaccharide **S7** in H_2O (385 μL) containing $NaHCO_3$ (16 μmol) were added a solution of FmocOSu (2.6 mg, 7.7 μmol) in acetone (385 μL). The reaction mixture was stirred for 30 min on ice bath, after which the reaction mixture was allowed to warm up to rt and stirred at the same temperature for additional 2.5 hours. The resulting mixture was subjected directly to gel filtration chromatography (φ1.4×33 cm, Sephadex™ G-25, eluent: distilled water). Fractions containing oligosaccharide were collected and lyophilized to give crude **S8**; ESI-MS calcd for $C_{116}H_{155}N_7O_{60}$ $[M+2H]^{2+}$ 1304.0, found for *m/z* 1304.0.

Ice-cold 90% aq. TFA (385 μL) was slowly added to crude **S8** on an ice bath. The mixture was kept at 0 °C for 10 min, and concentrated at rt, after which distilled water was added. The solution was immediately frozen by the treatment of liquid N_2 and then lyophilized. The resulting residue was purified with RP-HPLC (Proteonavi C4, 0.1% aq. TFA:90% aq. CH_3CN containing 0.1% TFA = 80:20 to 50:50 over 30 min at 1 mL/min). Fractions containing the desired product were collected and lyophilized to give Asn-linked triantennary asialo-undecasaccharide **2** (0.8 mg, 0.34 μmol, 44%).

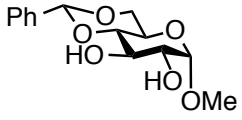
^{1}H NMR (700 MHz, D_2O, 4.76 ppm)[9] δ 7.92 (d, *J* = 7.34 Hz, 2 H), 7.71 (d, *J* = 6.11 Hz, 2 H), 7.51 (t, *J* = 7.33 Hz, 2 H), 7.47-7.37 (m, 2 H), 5.12 (s, 1H), 5.01 (d, *J* = 9.23 Hz, 1 H), 4.92 (s, 1

H), 4.75 (s, 1 H), 4.64-4.50 (m, 6 H), 4.50-4.43 (m, 3 H), 4.39-4.28 (m, 2 H), 4.22 (brs, 2 H), 4.11 (brs, 1 H), 4.05 (d, J = 9.28 Hz, 1 H), 4.03-3.94 (m, 4 H), 3.94-3.65 (m, 42 H), 3.65-3.51 (m, 14 H), 3.51-3.43 (m, 2 H), 2.77-2.67 (dd, J = 15.40, 2.66 Hz, 1 H), 2.63-2.52 (dd, J = 15.47, 8.73 Hz, 1 H), 2.11-2.06 (s, 6 H), 2.06-2.01 (s, 6 H), 1.93-1.86 (s, 3 H).

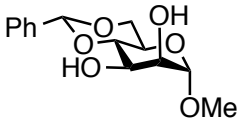
Chemical shift of ^{13}C (D_2O, chemical shift was picked up from HSQC except for quaternary carbon) δ128.97, 128.45, 126.02, 121.08, 103.88, 102.52, 120.23, 101.36, 100.45, 100.08, 98.00, 81.33, 80.33, 79.55, 79.46, 79.11, 79.04, 77.26, 77.13, 76.94, 76.31, 75.75, 75.65, 75.33, 73.79, 73.68, 73.44, 73.04, 72.91, 71.93, 71.10, 70.38, 69.48, 69.02, 69.27, 67.35, 66.68, 62.59, 62.19, 61.95, 60.87, 60.70, 56.09, 55.81, 54.72, 53.02, 47.92, 39.12, 23.47, 23.29, 22.96.

HRMS (ESI): calcd for $C_{95}H_{143}N_7O_{60}$ $[M+2H]^{2+}$ 1171.9250, found for *m/z* 1171.9263 $[M+2H]^{2+}$.

2-5. Synthesis and investigation of benzylidene monosaccharides



24



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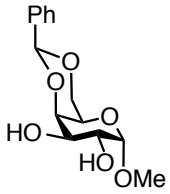

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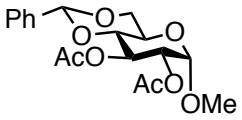
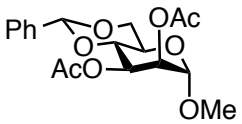
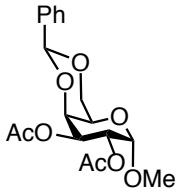


27



28

All above methyl 4,6-*O*-benzylidene-α-D-glycosides (**24**, **25**, and **26**) and their acetate derivatives (**27** and **28**) used for the experiment were prepared as previously reported.[10-12] Methyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-galactoside (**28**) was synthesized as follow.



Methyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-galactoside (28)

Methyl 4,6-*O*-benzylidene-α-D-galactoside **26** (495 mg, 1.8 mmol) was dissolved in 17.6 mL of Ac_2O/pyridine (1:1), and the resulting mixture was stirred at rt. After 6 hours, MeOH (10 mL) was added to the mixture on an ice bath. After elevation of the temperature to rt, the mixture was evaporated and azeotropically dried with toluene. The resulting residue was purified by silica gel column chromatography (hexane/EtOAc = 1:2, ϕ = 1.8 cm, h= 15 cm). The resulting residue was further recrystallized from ethanol and petroleum ether to give **28** (525 mg, 1.43 mmol, 82 %).

$[a]_D^{25}$ 189.5 (*c* 0.50 $CHCl_3$)

^{1}H NMR (400 MHz, $CDCl_3$): 7.57-7.47 (m, 2H), 7.42-7.32 (m, 3H), 5.52 (s, 1H), 5.37 (dd, 1H, *J* = 10.88, 3.1 Hz), 5.32 (dd, 1H, *J* = 10.87, 3.11 Hz), 5.09 (d, 1H, *J* = 3.02 Hz), 4.47 (dd, 1H, *J* = 3.24, 1.11 Hz), 4.28 (dd, 1H, *J* = 12.51, 1.56 Hz), 4.07 (dd, 1H, *J* = 12.56, 1.68 Hz), 3.75

(brddd, 1H), 3.43 (s, 3H), 2.09 (s, 3H), 2.08 (s, 3H).

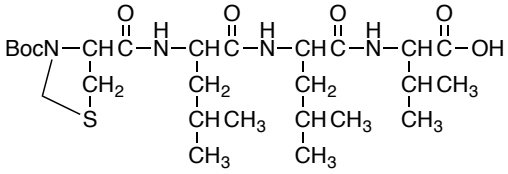
^{13}C NMR (100 MHz, $CDCl_3$): 170.77, 170.33, 137.68, 129.15, 128.31, 126.36, 100.99, 97.89, 74.05, 69.22, 68.68, 68.22, 62.16, 55.67, 21.08, 20.98.

HRMS (ESI): calcd for $C_{18}H_{22}O_8$ $[M+Na]^+$ 389.1207, found for *m/z* 389.1206 $[M+Na]^+$.

Six substrates prepared above were dissolved 60 % d_3-AcOD in D_2O, respectively, and the concentration was adjusted to 10 mM substrate. The resulting mixtures were kept at 4, 23, or 33 ^oC. ^1H NMR spectra were recorded time-dependently (number of scan: 16 or 32, dummy scan: 4, 400 MHz NMR). Hydrolysis yield (percentage) was determined as "the integration of ^1H signal of a product / (the integration of ^1H signal of a product + the integration of ^1H signal of a starting material)". An anomeric proton region was used for the estimation of benzylidene derivatives without acetyl groups, and a methyl proton region of methyl glycoside was used for the estimation of its acetate derivatives.

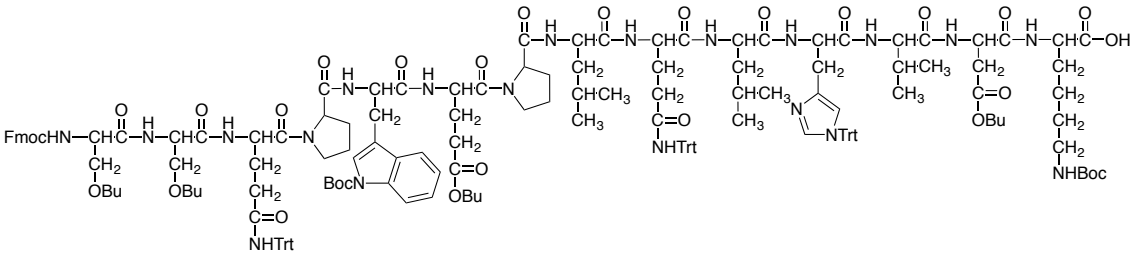
Competitive hydrolysis experiments were performed using a mixture of glucoside, mannoside, and galactoside (10 μmol, respectively) in 1.0 mL of 60% d_3-AcOD in D_2O, and the reaction was monitored at 23 ^oC as above. Because of the signal overlap in ^1H NMR, I did not estimate the hydrolysis yield in these experiments.

2-6. Synthesis of glycopeptide having a triantennary oligosaccharide



N-terminal peptide of EPO Seg4: **Boc-[Thz^{79}-Leu^{80}-Leu^{81}-Val^{82}]-OH** (**35**)

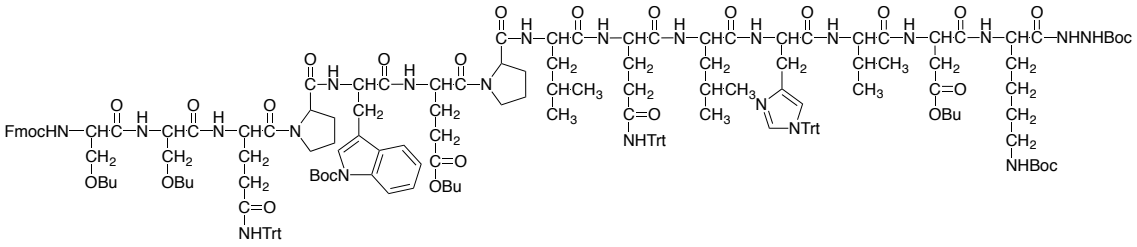
The peptide assembly was performed manually on a 500 μmol scale by use of HMPB-PEGA resin. Fmoc-Val-OH (5 equiv) was attached to the resin in the presence of MSNT (5 equiv) and *N*-methylimidazole (3.75 equiv) in DCM (10 mL). Then the resin was treated with 20% Piperidine/DMF for 20 min at the ambient temperature to remove Fmoc group. Peptide elongation was carried out as follows. Coupling of Fmoc-AA-OH (2.0 mmol) was performed by HOBt (1.5 mmol), HBTU (1.5 mmol), and DIEA (3 mmol) in DMF (10 mL) for 30 min at the ambient temperature. Fmoc-AA-OH used was Fmoc-Leu-OH. After the Fmoc-AA-OH coupling, Boc-Thz-OH was coupled by the same manner. Coupling reactions were confirmed by ninhydrin test. After completion of all coupling steps, the protected peptide was released from the resin by treatment with AcOH/TFE (1:1, 16 mL) for 6 hours. The solution was filtered, and then the filtrate was dried in vacuo. This manipulation was repeated twice and combined, which was dissolved in 10% aq. acetonitrile containing 0.1% TFA (5 mL) and purified by preparative RP-HPLC (CAPCELL PAK, Φ10 mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 60:40 to 30:70 over 30 min at 2.5 mL/min) to give a desired product (70 mg, 31%). ESI-MS: *m/z* calcd for $C_{26}H_{46}N_4O_7S$: $[M+H]^+$ 559.3, found: 559.5.



N-terminal peptide of EPO Seg4: **Fmoc-[Ser(**^{t}**Bu)^{84}-Ser(**^{t}**Bu)-Gln(Trt)-Pro-Trp(Boc)-Glu(**^{t}**Bu)-Pro-Leu-Gln(Trt)-Leu-His(Trt)-Val-Asp(**^{t}**Bu)-Lys(Boc)^{97}]-OH** (**37**)

The peptide assembly was performed manually on a 100 μmol scale by use of HMPB-PEGA resin. Fmoc-Lys-OH (5 equiv) was coupled twice to the resin in the presence of

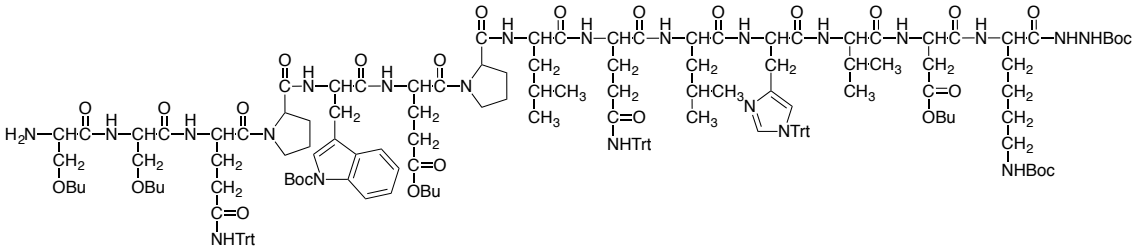
MSNT (5 equiv) and *N*-methylimidazole (3.75 equiv) in DCM (2 mL) to ensure quantitative coupling. Then the resin was treated with 20% Piperidine/DMF for 20 min at the ambient temperature to remove Fmoc group. Peptide elongation was carried out as follows. Coupling of Fmoc-AA-OH (400 μmol) was performed by HOBt (300 μmol), HBTU (300 μmol), and DIEA (600 μmol) in DMF (2 mL) for 30 min at the ambient temperature. Fmoc-AA-OH used were Fmoc-Ser(^{t}Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Pro-OH, Fmoc-Trp(Boc)-OH, Fmoc-Glu(^{t}Bu)-OH, Fmoc-Leu-OH, Fmoc-His(Trt)-OH, Fmoc-Val-OH, Fmoc-Asp(^{t}Bu)-OH, and Fmoc-Lys(Boc)-OH. Coupling reaction steps were confirmed by ninhydrin test. After all Fmoc-AA-OH coupling steps, the protected peptide was released from the resin by treatment with AcOH/TFE (1:1, 6 mL) at the ambient temperature for 3 hours. The solution was filtered, and then the filtrate was dried in vacuo. This cleavage manipulation was repeated four times, and the mixture was combined, which was purified by gel filtration column (Φ1.8×30 cm, LH-20, MeOH). The resulting residue was further purified by silica gel column chromatography (CH_3Cl/MeOH/AcOH = 80:2:5 then 80:5:5) to afford a desired protected peptide **37** (62 mg, 20%). HRMS: *m/z* calcd for $C_{173}H_{214}N_{20}O_{29}$ (protected form): $[M+H]^+$ 3036.5958, found: 3036.5995. ESI-MS *m/z* calcd for $C_{90}H_{124}N_{20}O_{25}$ (deprotected form): $[M+H]^+$ 1885.9, found: 1885.9.



N-terminal peptide of EPO Seg4b: **Fmoc-[Ser(^{t}Bu)^{84}-Ser(^{t}Bu)-Gln(Trt)-Pro-Trp(Boc)-Glu(^{t}Bu)-Pro-Leu-Gln(Trt)-Leu-His(Trt)-Val-Asp(^{t}Bu)-Lys(Boc)^{97}]-$^{\alpha}$NHNH-Boc** (**38**)

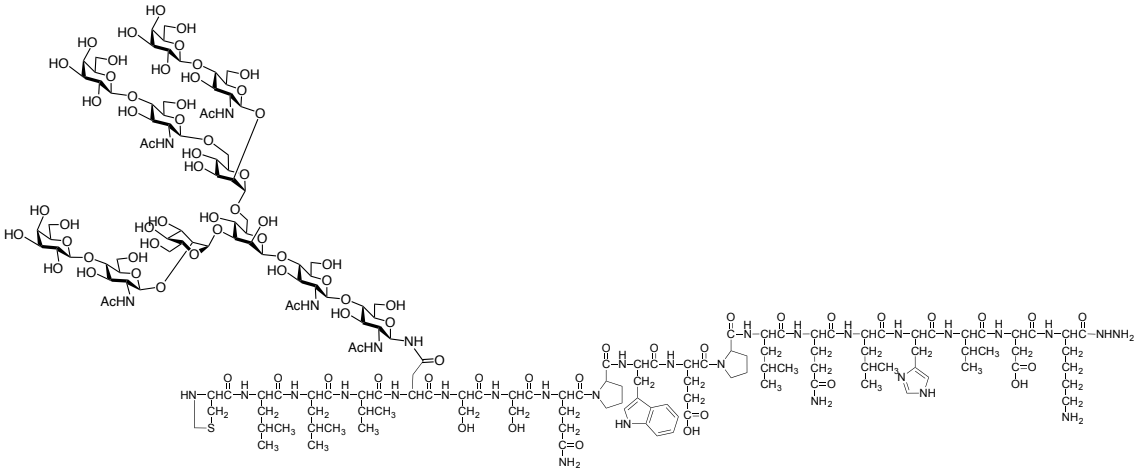
tert-Butyl carbazate (295 mg, 2.2 mmol) and Fmoc-[Ser(^{t}Bu)^{84}-Ser(^{t}Bu)-Gln(Trt)-Pro-Trp(Boc)-Glu(^{t}Bu)-Pro-Leu-Gln(Trt)-Leu-His(Trt)-Val-Asp(^{t}Bu)-Lys(Boc)^{97}]-OH (**37**) (65.7 mg, 21.6 μmol) was dissolved in DMF (1.1 mL), and the mixture was stirred at -20 ^{o}C for 10 min. PyBOP (228 mg, 0.44 mmol) and DIEA (95.9 μL, 0.55 mmol) were added to the mixture, which was stirred at the same temperature for 6 hours. During this reaction, an aliquot of the mixture (5 μL) was separated, and TFA/TIPS/H_2O (95:2.5:2.5, 40 μL) was added to the mixture,

which was kept at room temperature to remove protecting groups. Ice-cold ether (1 mL) was added to the mixture, and precipitate was collected by centrifuge and air-dried. This precipitate was dissolved in 10% acetonitrile containing 0.1% TFA, which was used for the HPLC analysis to monitor the reaction. On the completion of the reaction, the resulting mixture was directly subjected to gel filtration over Sephadex LH-20 (ϕ: 30 mm, l: 25 cm, eluent MeOH). Fractions containing the desired product were combined, evaporated, dissolved in 1,4-dioxane, and lyophilized to give the desired peptide **38** (62.1 mg, 91%). ESI-MS: *m/z* calcd for $C_{90}H_{126}N_{22}O_{24}$ (deprotected form): $[M+H]^+$ 1889.9, found: 1899.9.



N-terminal peptide of EPO Seg4b: **H-[Ser(^{t}Bu)^{84}-Ser(^{t}Bu)-Gln(Trt)-Pro-Trp(Boc)-Glu(^{t}Bu)-Pro-Leu-Gln(Trt)-Leu-His(Trt)-Val-Asp(^{t}Bu)-Lys(Boc)^{97}]-$^{\alpha}$NHNH-Boc (36)**

Fmoc-[Ser(^{t}Bu)^{84}-Ser(^{t}Bu)-Gln(Trt)-Pro-Trp(Boc)-Glu(^{t}Bu)-Pro-Leu-Gln(Trt)-Leu-His(Trt)-Val-Asp(^{t}Bu)-Lys(Boc)^{97}]-$^{\alpha}$NHNH-Boc **38** (62.1 mg, 19.7 μmol) was dissolved in 20% Piperidine in DMF (2 mL), which was stirred at room temperature for 20 min. Then the resulting mixture was directly subjected to gel filtration over Sephadex LH-20 (ϕ: 30 mm, l: 25 cm, eluent MeOH). Fractions containing the desired product were combined, evaporated, dissolved in 1,4-dioxane, and lyophilized to give the desired product (40.1 mg, 69%). HRMS: *m/z* calcd for $C_{163}H_{214}N_{22}O_{28}$ (protected form): [M+H]+ 2928.6071, found 2928.6117. ESI-MS: *m/z* calcd for $C_{75}H_{116}N_{22}O_{22}$ (deprotected form): $[M+H]^+$ 1677.9, found: 1678.1.



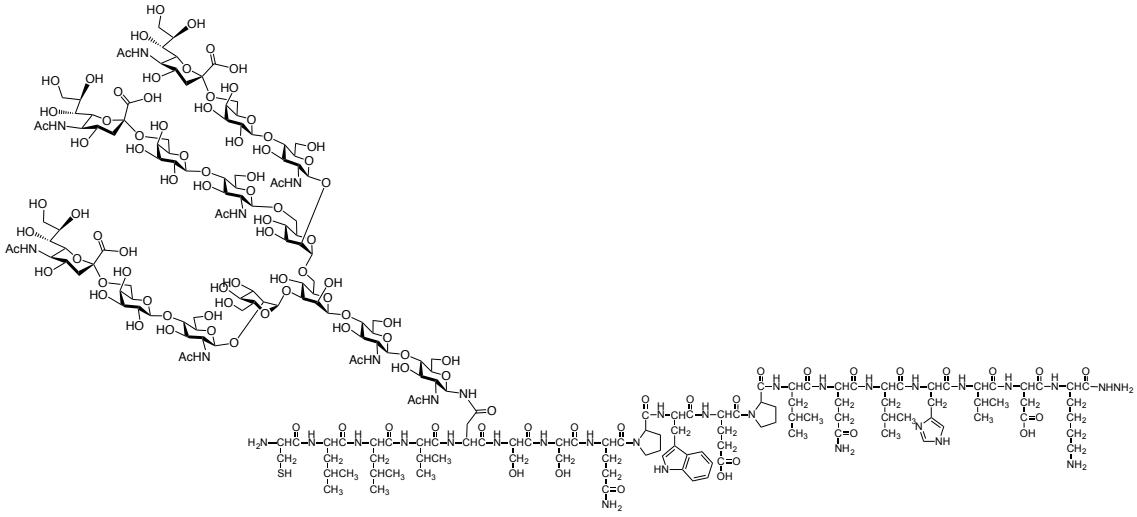
Seg4 having a triantennary asialoundecasaccharide; **H-[Thz^{79}-Leu-Leu-Val- Asn^{83} (Triantennary Asialoundecasaccharide)-Ser-Ser-Gln-Pro-Trp-Glu-Pro-Leu-Gln-Leu-His-Val-Asp-Lys^{97}]-$^{\alpha}$NHNH$_2$ (34)**

To a solution of Fmoc-Asn(triantennary asialoundecasaccharide)-OH **1** (2.0 mg, 0.85 μmol) in DMF/DMSO (1:1, 142 uL), were added H-[Ser(^{t}Bu)^{84}-Ser(^{t}Bu)-Gln(Trt)-Pro-Trp(Boc)-Glu(^{t}Bu)-Pro-Leu-Gln(Trt)-Leu-His(Trt)-Val-Asp(^{t}Bu)-Lys(Boc)^{97}]-$^{\alpha}$NHNHBoc **36** (4.5 mg, 1.5 μmol), PyBOP (2.2 mg, 4.2 μmol), and DIEA (0.97 μL. 5.6 μmol). The resulting mixture was stirred at room temperature for 5 hours. The reaction was monitored by RP-HPLC as above and TLC analysis (EtOAc/MeOH/H_2O, 3: 2: 1). On the completion of the reaction, the mixture was directly subjected to gel filtration over Sephadex LH-20 (ϕ: 10 mm, l: 28 cm, eluent MeOH) to remove the solvent and reagents. Fractions containing the desired product and the remaining peptide were combined, evaporated, dissolved in 1,4-dioxane, and lyophilized. This mixture was directly used for the next reaction without further purification. (ESI-MS: *m/z* calcd for $C_{170}H_{257}N_{29}O_{81}$ (deprotected form): $[M+H]^+$ 4001.7, found: 4004.2.

To the obtained mixture, 20% piperidine in DMF (150 μL) was added, and the mixture was stirred at room temperature for 20 min. Then the resulting mixture was directly subjected to gel filtration over Sephadex LH-20 (ϕ: 10 mm, l: 28 cm, eluent MeOH) to remove the solvent. Fractions containing the desired product and the remaining peptide were combined, evaporated, dissolved in 1,4-dioxane, and lyophilized. This mixture was directly used for the next reaction without further purification. (ESI-MS: *m/z* calcd for $C_{155}H_{247}N_{29}O_{79}$ (deprotected form): $[M+H]^+$ 3779.6, found: 3780.8).

To the obtained mixture, Boc-[Thz^{79}-Leu^{80}-Leu^{81}-Val^{82}]-OH **35** (9.5 mg, 17 μmol),

HODhbt (1.5 mg, 9.2 μmol), and DIC (1.3 μL, 8.3 μmol) were added. Then the resulting mixture was stirred at room temperature for 9 hours. Then the resulting mixture was directly subjected to gel filtration over Sephadex LH-20 (ϕ: 10 mm, l: 28 cm, eluent MeOH) to remove the solvent and reagents. Fractions containing the desired product and the remaining peptides were combined, evaporated, dissolved in 1,4-dioxane, and lyophilized. To the obtained mixture, Boc-[Thz^{79}-Leu^{80}-Leu^{81}-Val^{82}]-OH **35** (8.7 mg, 16 μmol), HODhbt (1.7 mg, 10 μmol), and DIC (1.3 μL, 8.3 μmol) were added again. Then, the resulting mixture was stirred at room temperature for 5 hours. Then the resulting mixture was directly subjected to gel filtration over Sephadex LH-20 (ϕ: 10 mm, l: 28 cm, eluent MeOH) to remove the solvent and reagents. Fractions containing the desired product and the remaining peptides were combined, evaporated, dissolved in 1,4-dioxane, and lyophilized. The resulting residue was dissolved in DMF (50 μL), and TFA/TIPS/H_2O (95:2.5:2.5, 2 mL) was added to the mixture, which was kept at room temperature for 2 hours. Ice-cold ether (40 mL) was added to the resulting mixture, and a precipitate was collected by centrifuge and air-dried. The precipitate was dissolved in 10% acetonitrile containing 0.1% TFA, which was kept at room temperature for 2 hours to allow for decarboxylation. This is because carboxylate on the peptide was observed by HPLC analysis right after dissolving peptides in 10% acetonitrile containing 0.1% TFA. The resulting mixture was then purified by preparative RP-HPLC (Proteonavi, Φ 10 mm × 250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 85:15 over 10 min, then 85:15 to 45:55 over 60 min at 2.5 mL/min) to yield H-[Thz^{79}-Asn^{83}(Triantennary Asialoundecasaccharide)-Lys^{97}]-$^{\alpha}$NHNH$_2$ **34** (1.0 mg, 0.24 μmol, 28%, calculated based on Fmoc-Asn(glycan)-OH **1**) as a white form; ESI-MS: *m/z* calcd for $C_{176}H_{283}N_{33}O_{83}S$: $[M+H]^+$ 4219.87, found: 4219.98.

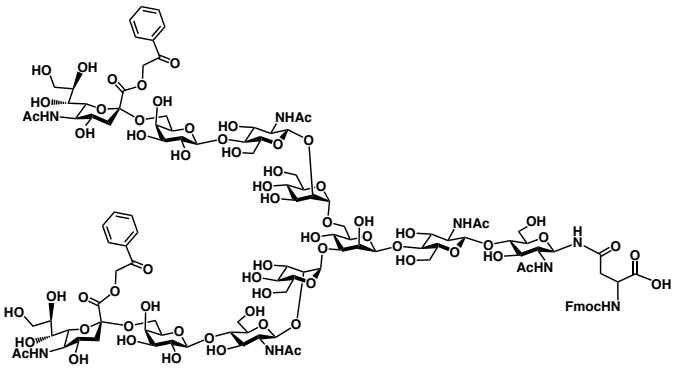


Seg4 having a triantennary sialyltetradecasaccharide: **H-[Cys^{79}-Leu-Leu-Val-Asn^{83} (Triantennary sialyltetradecasaccharide)-Ser-Ser-Gln-Pro-Trp-Glu-Pro-Leu-Gln-Leu-His-Val-Asp-Lys^{97}]-$^{\alpha}$NHNH$_2$ (33)**

To H-[Thz^{79}-Leu-Leu-Val- Asn^{83}(Triantennary asialoundecasaccharide)-Ser-Ser-Gln-Pro-Trp-Glu-Pro-Leu-Gln- Leu-His-Val-Asp-Lys^{97}]-$^{\alpha}$NHNH$_2$ **34** (0.9 mg, 0.2 μmol), 500 mM HEPES buffer (pH 5.9, 16.8 μL), bovine serum albumin (BSA) in water (0.8 μL, 10 mg/mL), alkaline phosphatase（Calf intestine）(9 μL, 0.9 U), α-2,6 sialyltransferase (45 μL, 6.7 U), CMP-Neu5Ac (4.0 mg, 6.5 μmol), and H_2O (12.3 μL) were added (final concentration of glycopeptide was 2 mM). After incubation at room temperature for 2 days, CMP-Neu-5Ac (1.9 mg, 3.1 μmol), α -2,6 sialyltransferase (20 μL, 3 U), and alkaline phosphatase（Calf intestine）(9 μL, 0.9 U) were added. After 1 day, CMP-Neu-5Ac (4.0 mg, 6.5 μmol), α -2,6 sialyltransferase (20 μL, 3 U), and alkaline phosphatase（Calf intestine）(9 μL, 0.9 U) were added again, which was further incubated for 1 day. The resulting mixture was diluted with 0.2 M phosphate buffer containing 6 M Gn-HCl (100 μL). The pH value of the mixture was adjusted to 4.3 by the addition of 25% (w/v) *O*-methylhydroxylamine hydrochloride in 0.2 M phosphate buffer containing 6 M Gn-HCl to remove the Thz group. After 5 hours, 200 mM TCEP buffer containing 0.2 M phosphate and 6 M Gn-HCl (pH 7.0, 200 μL) was added to the mixture, which was left at room temperature for 30 min. The resulting mixture was purified by preparative RP-HPLC (Proteonavi, Φ 10 mm × 250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 90:10 over 10 min, then 90:10 to 50:50 over 60 min at 2.5 mL/min) to produce H-[Cys^{79}-Asn^{83}(Triantennary Sialyl-tetradecasaccharide)-Lys^{97}]- $^{\alpha}$NHNH$_2$

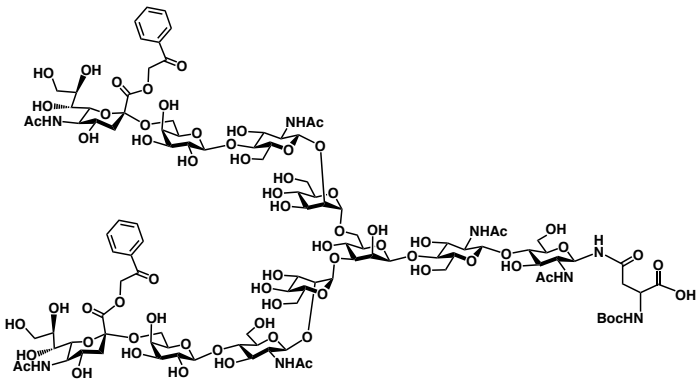
33 (0.6 mg, 0.12 μmol, 55%) as a white form; HR-MS (ESI): *m/z* calcd for $C_{176}H_{283}N_{33}O_{83}S$: $[M+H]^+$ 5081.1594, found: 5081.1591.

2-7. Synthesis of an erythropoietin glycoform



Fmoc-asparagine(phenacyl-esterified biantennary sialylundecasaccharide)-OH (48)[13]

Fmoc-Asn(disialyl-undecasaccharide)-OH **47** (290 mg, 113 μmol) was dissolved in distilled water (10 mL), which was passed through DOWEX™ 50Wx8 100-200 resin. The pH value of the resulting mixture was adjusted to 3.6 by the addition of aq. Cs_2CO_3 (2.5 mg/mL), and then the mixture was lyophilized. A solution of 2-bromoacetophenone (90 mg, 453 μmol) in DMF (50 mL) was added to the resulting residue, and the mixture was stirred at the ambient temperature for 29 hours. Ice-cold ether (400 mL) was added to the mixture to form a precipitate. The precipitate was collected by centrifuge and air-dried. The resulting precipitate was dissolved in 20% aq. acetonitrile and purified by preparative RP-HPLC (CAPCELL PAK, Φ 10 mm × 250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 85 :15 to 45:55 over 30 min at 2.5 mL/min) to give a desired product (169 mg, 59%); ESI-MS: *m/z* calcd for $C_{119}H_{166}N_8O_{68}$: $[M+H]^+$ 2796.0, found: 2796.2.

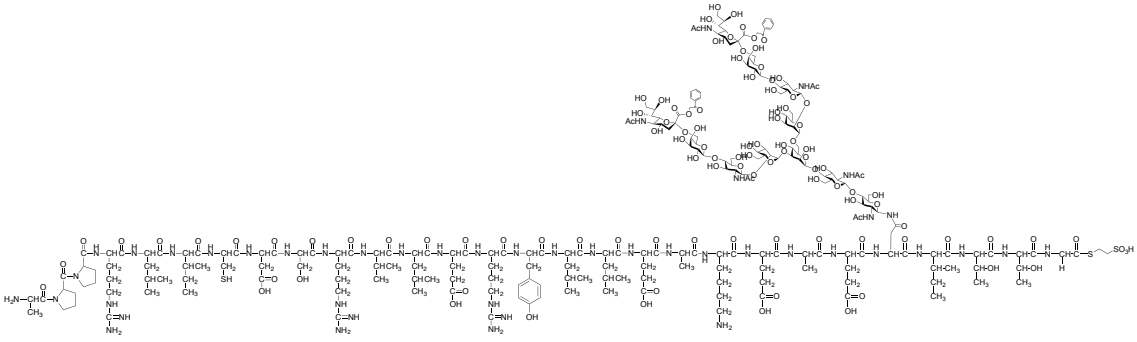


Boc-Asn(phenacyl esterified biantennary disialyl-oligosaccharide)-OH (49)[13]

1-methyl-2-pyrrolidone (383 μL, 3.98 mmol), HOBt (11.0 mg, 81 μmol), and

hexamethyleneimine (44.2 μL, 392 μmol) were added to Fmoc-Asn(diPac-biantennary disilalyoligosaccharide)-OH **48** (111 mg, 39.7 μmol) in DMF (10 mL), and the mixture was stirred at the ambient temperature for 1 h. Ice-cold ether (40 mL) was added to the mixture to form a precipitate. The precipitate was collected by centrifuge and air-dried. The resulting residue was dissolved in DMF (5.3 mL), and BocOSu (53.8 mg, 250 μmol) and DIEA (62.3 μL, 358 μmol) were added to the mixture. The resulting mixture was stirred at the ambient temperature for 5 hours. Then, ice-cold ether (45 mL) was added to form a precipitate. The precipitate was collected by centrifuge and air-dried. The resulting residue was dissolved in 20% acetonitrile (5 mL) and purified by preparative RP-HPLC (CAPCELL PAK, Φ 10 mm × 250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 95:05 to 50:50 over 30 min at 2.5 mL/min) to give a desired product (63 mg, 59%); ESI-MS: *m/z* calcd for $C_{109}H_{164}N_8O_{68}$: $[M+H]^+$ 2674.0, found: 2674.2.

^{1}H-NMR (700 MHz, 25°C in D_2O, HOD = δ 4.79) δ 8.05-7.90, 7.82-7.71, 7.67-7.55 (m, phenyl), 5.82-5.60 (m, 2H), 5.13 (s, 1H), 5.05 (d, *J* = 9.55 Hz, 1H), 4.94 (s, 1H), 4.76 (s, 1H), 4.66-4.54 (m, 2H), 4.47-4.39 (m, 2H), 4.25 (brd, 1H), 4.19 (brd, 1H), 4.16-4.07 (m), 4.07-3.42 (m), 2.91-2.68 (m), 2.07 (s, 3H), 2.51 (s, 3H ×2), 2.04 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.97-1.91 (m, 2H), 1.42 (s, 9H).



EPO Seg1: **H-[Ala^1-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-Glu-Ala-Glu-Asn^{24}(Glycan)-Ile-Thr-Thr-Gly^{28}]-$^{\alpha}$thioester (52)**[14]

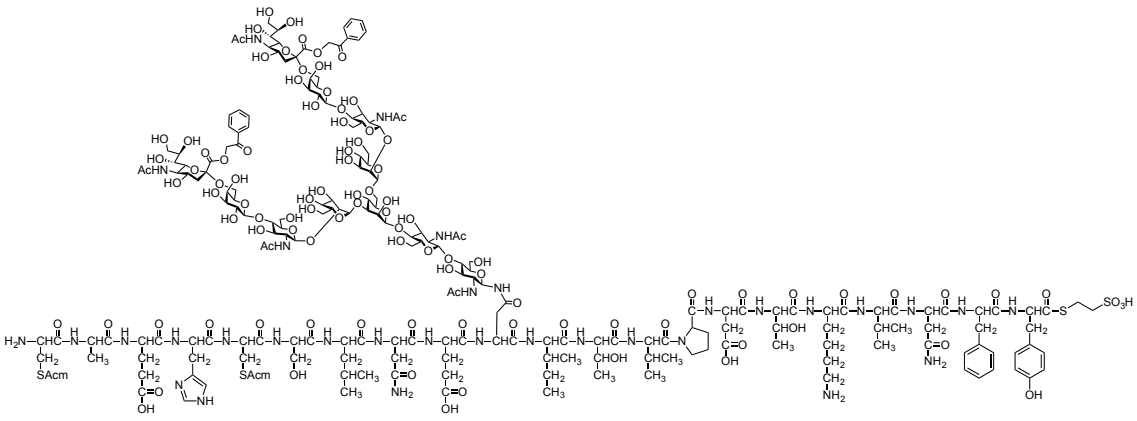
The side chain protected peptide Boc-[Ala^1-Pro-Pro-Arg(Pbf)-Leu-Ile-Cys(Trt)-Asp(^{t}Bu)-Ser(^{t}Bu)-Arg(Pbf)-Val-Leu-Glu(^{t}Bu)-Arg(Pbf)-Tyr(^{t}Bu)-Leu-Leu-Glu(^{t}Bu)-Ala]-OH was synthesized by Fmoc SPPS. First, Fmoc-Ala-OH (500 μmol) was coupled twice to HMPB-PEGA resin (100 μmol) in the presence of MSNT (500 μmol) and *N*-methylimidazole

(375 μmol) in DCM (2 mL) to ensure quantitative coupling. Then the resin was treated with 20% Piperidine/DMF for 20 min at the ambient temperature to remove Fmoc group. Peptide elongation was carried out as follows. Coupling of Fmoc-AA-OH (400 umol) was performed by HOBt (300 μmol), HBTU (300 μmol), and DIEA (600 μmol) in DMF (2 mL) for 30 min at the ambient temperature. Fmoc-AA-OH used were Fmoc-Ala-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asp(^{t}Bu)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Val-OH, Fmoc-Glu(^{t}Bu)-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Glu(^{t}Bu)-OH. Coupling reaction steps were confirmed by ninhydrin test. After all Fmoc-AA-OH coupling steps, Boc-Ala-OH was coupled in the same manner, and then the protected peptide was released from the resin by treatment with AcOH/TFE (1:1, 6 mL) at the ambient temperature for 12 hours. The solution was filtered, and then the filtrate was dried in vacuo to afford a crude peptide (221 mg).

The *C*-terminal side chain protected glycopeptide-^{a}thioester (H-[Lys^{20}(2-Cl-Z)-Glu(OBn)-Ala-Glu(OBn)-Asn(Glycan)-Ile-Thr(Bn)-Thr(Bn)-Gly^{28}] was synthesized on the resin by *in situ neutralization* Boc SPPS. A $HSCH_2CH_2CONH$-PEGA resin (PEGA resin: poly[acryloyl-bis8aminopropyl]polyethylene glycol)) (50 μmol) was used for the synthesis. Side chain protection of Boc amino acids was as follows: Boc-Lys(Cl-Z), Boc-Glu(OBzl), Boc-Ala, Boc-Ile, Boc-Boc-Thr(Bzl), Boc-Gly. The resin was washed with DMF, DCM, and DMF. *S*-trityl-3-mercaptopropionic acid (200 μmol) was pre-activated with HBTU (190 μmol) and DIEA (400 μmol) in DMF (1 mL) for 30 sec, and then the solution was poured onto the resin. The resulting suspension was gently shaken at the ambient temperature for 20 min. After washing the resin with DMF and DCM thoroughly, the resin was treated twice with TFA/TIPS (95:5) at the ambient temperature for 1 min to remove trityl group. Next, Boc-Gly (200 μmol) was pre-activated with HBTU (190 μmol) and DIEA (400 μmol) in DMF (1 mL) for 30 min, which was poured onto the resin. The resulting mixture was gently shaken at the ambient temperature for 20 min. After washing the resin with DMF and DCM, the resin was treated with neat TFA at the ambient temperature for 2 min. After filtration of TFA, the resin was treated with TFA again for 5 min to completely remove Boc group. Subsequent peptide elongation was performed in the same manner. Then, the resin (ca. 10 μmol) was separated, which was used for a coupling of Boc-Asn(diphenacyl-sialyloligosaccharide)-OH **49** as the following manner. Before the coupling reaction, the resin was treated with 10% DIEA in DMF (1 mL) for 30 sec. Boc-Asn(diphenacyl-sialyloligosaccharide)-OH **49** (37.8 mg, 14 μmol) was

pre-activated by the addition of PyBOP (8.3 mg, 16 μmol) and DIEA (6.98 μL, 40 μmol) in DMF-DMSO (1:1, 688 μL), which was poured onto the resin. The suspension was gently shaken at the ambient temperature in the dark for 15 hours. Following coupling steps were performed by using a diluted condition such as Boc- AA (40 μmol, 50 mM), HBTU (38 μmol) and DIPEA (80 μmol) in DMF (800 uL).

Before the segment coupling reaction, side-chain protected Boc-[K^{20}-N^{24}(Glycan)-Gly^{28}] linked on the resin (ca. 10 μmol) was treated with neat TFA for 2 and 5 min to remove Boc group. The resulting resin was treated with 10% DIEA in DMF (1 min) for 30 sec. Coupling of Boc-[Ala^{1}-Ala^{19}]-OH (57.2 mg, 15 μmol) was carried out by using HODhbt (5.0 mg, 30 μmol) and DIC (4.7 uL, 30 μmol) in phenol/chloroform (1:4, 2 mL). The reaction mixture was gently shaken at room temperature in the dark for 23 hours. Then, the resulting resin was washed with DMF and DCM. The resin was treated twice with neat TFA for 1 min to remove Boc group and further treated twice with TFA/DMS/m-cresol/TfOH (1.2 mL/0.72 mL/0.24 mL/0.24 mL) for 20 min on an ice bath. After washing with the resin with TFA, DCM, DMF and DCM thoroughly, the glycopeptide was cleaved from the resin by the treatment with 5% MESNa (w/v) in 0.2 M phosphate buffer containing 6 M Gn-HCl (pH 6.5, 2 mL) for 23 h. The resulting mixture was purified by preparative RP-HPLC (Proteonavi, Φ 10 mm × 250 mm, 0.1% TFA: 90% aq. acetonitrile containing 0.1% TFA = 95:05 to 80:20 over 5 min, then 80:20 to 60:40 over 60 min at 2.5 mL/min) to yield H-[Ala^{1}-Cys^{7}-Asn^{24}(Glycan)-Gly^{28}]-$^{\alpha}$thioester **52** (3.2 mg, 0.56 μmol, ca. 6 %) as a white form; ESI-MS: *m/z* calcd for $C_{239}H_{381}N_{45}O_{109}S_3$: $[M+H]^+$ 5722.5 (monoisotopic mass), found: 5722.7.



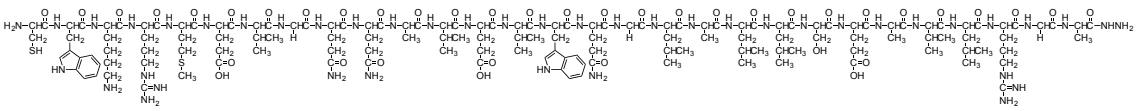
EPO Seg2: **H-[Cys^{29}(Acm)-Ala-Glu-His-Cys(Acm)-Ser-Leu-Asn-Glu-Asn^{38}(Glycan)-Ile-Thr-Val-Pro-Asp-Thr-Lys-Val-Asn-Phe-Tyr^{49}]-$^{\alpha}$thioester (51)**[14]

The glycopeptide-^{a}thioester was synthesized by *in situ neutralization* Boc SPPS. A $HSCH_2CH_2CONH$-PEGA resin (PEGA resin: poly[acryloyl-bisaminopropyl]polyethylene glycol)) (50 μmol) was used. Side chain protection of Boc amino acids was as follows: Boc-Cys(Acm), Boc-Glu(OBzl), Boc-His(CHO), Boc-Ser(Bzl), Boc-Asn(Xan), Boc-Thr(Bzl), Boc-Asp(OBzl), Boc-Lys(Cl-Z), Boc-Tyr(Br-Z). The resin was washed with DMF, DCM, and DMF. *S*-Trityl-3-mercaptopropionic acid (200 μmol) was pre-activated with HBTU (190 μmol) and DIEA (400 μmol) in DMF (1 mL) for 30 sec, and then the solution was poured onto the resin. The resulting suspension was gently shaken at room temperature for 20 min. After washing the resin with DMF and DCM thoroughly, the resin was treated twice with TFA/TIPS (95:5) at room temperature for 1 min to remove trityl group. Next, Boc-Tyr(Br-Z)-OH (200 μmol) was pre-activated with HBTU (190 μmol) and DIEA (400 μmol) in DMF (1 mL) for 30 sec, which was poured onto the resin. The resulting mixture was gently shaken at room temperature for 20 min. After washing the resin with DMF and DCM, the resin was treated with neat TFA at the ambient temperature for 2 min. After filtration of TFA, the resin was treated with TFA again for 5 min to completely remove Boc group. Subsequent peptide elongation was performed in the same manner.

Then the resin (ca. 10 μmol) was separated, which was used for a coupling of *tert*-Boc-Asn(diphenacyl-sialyloligosaccharide)-OH as the following manner. Before the coupling reaction, the resin was treated with 10% DIEA in DMF (1 mL) for 30 sec. Boc-Asn(diphenacyl-sialyloligosaccharide)-OH **49** (40.5 mg, 15 μmol) was pre-activated by the addition of PyBOP (8.4 mg, 16 μmol) and DIEA (6.98 μL, 40 μmol) in DMF-DMSO (1: 1, 688 μL), which was poured onto the resin. The suspension was gently shaken at room temperature in the dark for 11 hours. Following coupling steps were performed by using a diluted condition such as Boc- AA (40 μmol, 50 mM), HBTU (38 μmol) and DIPEA (80 μmol) in DMF (800 μL).

On the completion of the peptide coupling steps, the resin was treated twice with neat TFA for 1 min to remove Boc group and further treated twice with TFA/DMS/m-cresol/TfOH (1.2 mL/0.72 mL/0.24 mL/0.24 mL) for 25 min and 1 hour on an ice bath. After washing the resin with TFA, DCM, DMF and DCM thoroughly, the glycopeptide was cleaved from the resin by the treatment with 5% MESNa (w/v) in 0.2 M phosphate buffer containing 6 M Gn-HCl (pH 6.6, 2 mL) for 13 h. The resulting mixture was purified by preparative RP-HPLC (Proteonavi, Φ 10

mm×250 mm, 0.1% TFA: 90% aq. acetonitrile containing 0.1% TFA = 99:01 to 85:15 over 10 min, then 85:15 to 65:35 over 60 min at 2.5 mL/min) to yield H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Glycan)-Tyr^{49}]-$^{\alpha}$thioester **51** (5.1 mg, 1.0 μmol, ca. 10 %) as a white form; ESI-MS: *m/z* calcd for $C_{211}H_{319}N_{35}O_{102}S_4$: $[M+H]^+$ 5107.3 (average isotope composition), found: 5107.2.

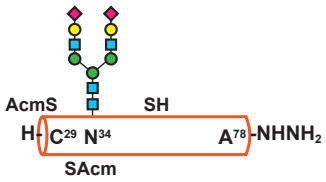


EPO Seg3: **H-[Cys^{50}-Trp-Lys-Arg-Met-Glu-Val-Gly-Gln-Gln-Ala-Val-Glu-Val-Trp-Gln-Gly-Leu-Ala-Leu-Leu-Ser-Glu-Ala-Val-Leu-Arg-Gly-Ala^{78}]-$^{\alpha}$NHNH$_2$ (50)**[14]

The peptide-^{a}hydrazide was synthesized by *in situ neutralization* Boc SPPS. A $HSCH_2CH_2CONH$-PEGA resin (PEGA resin: poly[acryloyl-bis8aminopropyl]polyethylene glycol)) (100 μmol) was used. Side chain protection of Boc amino acids was as follows: Boc-Thz, Boc-Trp(CHO), Boc-Lys(Cl-Z), Boc-Arg $(Z)_2$, Boc-Glu (OBzl), Boc-Ser(Bzl). The resin was washed with DMF, DCM, and DMF. *S*-Trityl-3-mercaptopropionic acid (400 μmol) was pre-activated with HBTU (380 μmol) and DIEA (800 μmol) in DMF (2 mL) for 30 sec, and then the solution was poured onto the resin. The resulting suspension was gently shaken at room temperature for 20 min. After washing the resin with DMF and DCM thoroughly, the resin was treated twice with TFA/TIPS (95:5) at room temperature for 1 min to remove trityl group. Next, Boc-Ala-OH (400 μmol) was pre-activated with HBTU (380 μmol) and DIEA (800 μmol) in DMF (2 mL) for 30 sec, which was poured onto the resin. The resulting mixture was gently shaken at room temperature for 20 min. After washing the resin with DMF and DCM, the resin was treated with neat TFA at the ambient temperature for 2 min. After filtration of TFA, the resin was treated with TFA again for 5 min to completely remove Boc group. Subsequent peptide elongation was performed in the same manner.

On the completion of the peptide coupling steps, the resin (ca. 20 μmol) was separated and subjected to deprotection steps to remove Boc and side chain-protecting groups. The deprotection was performed as follows; i) neat TFA for 1 min at rt, ii) TFA/DMS/m-cresol/TfOH (1.2 mL/0.72 mL/0.24 mL/0.24 mL) for 30 min at 0^{o}C, iii) TFA/thioanisole/EDT/TfOH/ (1.6 mL/0.16 mL/0.08 mL/0.16 mL) for 1 h at 0 ^{o}C, iv) TFA/thioanisole/EDT/TfOH/ (1.6 mL/0.16 mL/0.08 mL/0.16 mL) for 1 h at 0 ^{o}C. After all

deprotection steps, the resin was washed with TFA, DCM, and DMF thoroughly. Then, the peptide was cleaved from the resin by the treatment with 5% MESNa (w/v) in 0.2 M phosphate buffer containing 6 M Gn-HCl (pH 6.5, 4 mL) with agitation at room temperature for 12 hours. The resulting mixture was filtered, and then hydrazine monohydrate (80 μL, 1.6 mmol) was added to the resulting filtrate, which was gently shaken at room temperature for 30 min. The pH value of the mixture was adjusted to 6.03 by the addition of 5 M HCl and then further adjusted to 4.3 by the addition of *O*-methylhydroxylamine hydrochloride (solid), which was gently shaken at room temperature for 4 hours. The resulting mixture was purified by preparative RP-HPLC (Proteonavi, Φ10 mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 95:05 to 65:35 over 10 min, then 65:35 to 10:90 over 30 min at 2.5 mL/min) to yield H-[Cys^{50}-Ala^{78}]-$^{\alpha}$hydrazide **50** (9.5 mg, 2.9 μmol, ca. 15%) as a white form. ESI-MS: *m/z* calcd for $C_{143}H_{233}N_{43}O_{39}S_2$: $[M+H]^+$ 3243.8 (average isotope composition), found: 3243.6.

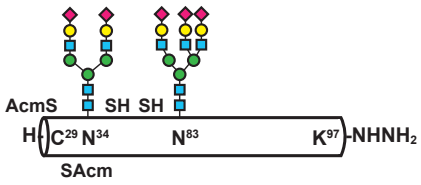


Seg23 (**54**)

Seg23 (**54**) by NCL

Seg2: H-[Cys^{29}(Acm)-Asn^{38}(Glycan)-Tyr^{49}]-$^{\alpha}$thioester **51** (4.1 mg, 0.8 μmol) and Seg3: H-[Cys^{50}-Ala^{78}]-$^{\alpha}$NHNH$_2$ **50** (3.9 mg, 1.2 μmol) were dissolved in freshly prepared 50 mM MPAA buffer containing 6 M Gn-HCl, 0.2 M phosphate, 40 mM TCEP (pH 6.7, 266 μL), which was gently agitated at room temperature for 4 hours. The pH value of the mixture was then adjusted to 9.9 by the addition of 5 M NaOH, which was gently shaken at room temperature for 3 hours. On the completion of removal of phenacyl ester, 200 mM TCEP buffer containing 6 M Gn-HCl and 0.2 M phosphate (pH 7.0, 400 μL) was added to the mixture to remove undesired disulfide bonds. And the pH value of the mixture was adjusted to 5.3 by the addition of 5 M HCl, which was purified by preparative RP-HPLC (Proteonavi, Φ10 mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 70:30 over 10 min, then 70:30 to 40:60 over 60 min at 2.5 mL/min) to yield Seg23: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-

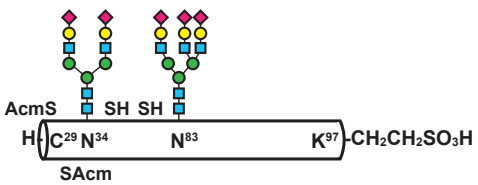
Asn^{38}(Glycan)-Cys^{50}-Ala^{78}]-$^{\alpha}$hydrazide **54** (3.2 mg, 0.40 μmol, 50%) as a white form; HRMS: *m/z* calcd for $C_{336}H_{534}N_{78}O_{136}S_4$: $[M+H]^+$ 7966.6223 (monoisotopic mass), found: 7966.6000.



Seg234 (**56**)

Seg234 (**56**) by hydrazide ligation

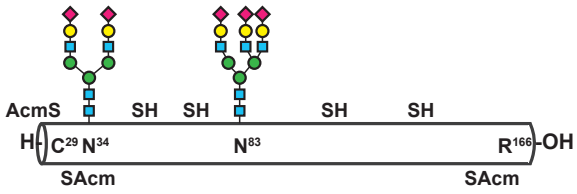
Seg23: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Glycan)-Cys^{50}-Ala^{78}]-$^{\alpha}$hydrazide **54** (1.5 mg, 0.20 μmol) was dissolved in freshly prepared 0.2 M phosphate buffer containing 6 M Gn-HCl (pH 3.5, 30 μL), then the temperature of the reaction mixture was cooled to -15^{o}C. 0.5 M aq. $NaNO_2$ (3.9 uL, 2.0 μmol) was added to the reaction mixture, which was kept at the same temperature. After 20 min, ca. 1.3 M MPAA in 0.2 M phosphate buffer containing 6 M Gn-HCl (7.5 μL) was added, and then the pH value of the mixture was adjusted to 6.6 and kept at rt for 30 min. The reaction mixture was added to Seg4: H-[Cys^{79}-Asn^{83}(Triantennary sialyltetradecasaccharide)-Lys^{97}]-$^{\alpha}$$NHNH_2$ **33** (0.5 mg, 0.098 μmol), which was kept at rt 3 h. The solution was treated with 100 mM TCEP buffer (40 μL) for 30 min to remove undesired disulfide bonds and purified by preparative RP-HPLC (Proteonavi, Φ10mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 70:30 over 10 min, then 70:30 to 20:80 over 50 min at 2.5 mL/min). Fractions containing the desired product were combined, which was lyophilized to give Seg234: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Biantennary oligosaccharide)-Cys^{50}-Cys^{79}-Asn^{83}(Triantennary oligosaccharide)-Lys^{97}]-$^{\alpha}$$NHNH_2$ **56** (0.8 mg, 68%) as a white form. ESI-MS: *m/z* calcd for $C_{544}H_{864}N_{112}O_{243}S_5$: $[M+H]^+$ 13022.7 (average isotope composition), found: 13024.9.



Seg234 (**63**)

Seg234 thioester (**63**) converted from Seg234 hydrazide (**56**)

Seg 234: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Biantennary oligosaccharide)-Cys^{50}-Cys^{79}-Asn^{83}(Triantennary oligosaccharide)-Lys^{97}]-$^{\alpha}$NHNH$_2$ **56** (0.8 mg, 0.061 μmol) was dissolved in freshly prepared 0.2 M phosphate buffer containing 6 M Gn-HCl (pH3.5, 30 μL), and the mixture was cooled at -15 ºC. Then, 0.5 M $NaNO_2$ (1.23 uL, 0.62 μmol) was added to the mixture, which was kept at -15 ºC. After 20 min, ca. 2 M MESNa in 0.2 M phosphate buffer (6.1 μL, ca. 12 μmol) was added, and the mixture was kept at rt for 1 h. The resulting solution was treated with 100 mM TCEP (100 μL) to remove undesired disulfide bonds and purified by preparative RP-HPLC (Proteonavi, Φ10 mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 70:30 over 10 min, then 70:30 to 20:80 over 50 min at 2.5 mL/min). Fractions containing the desired product were combined, which was lyophilized to give Seg234: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Biantennary oligosaccharide)-Cys^{50}-Cys^{79}-Asn^{83}(Triantennary oligosaccharide)-Lys^{97}]-$^{\alpha}$thioester **63** (0.6 mg, 74%) as a white form. ESI-MS: *m/z* calcd for $C_{546}H_{866}N_{110}O_{246}S_7$: $[M+H]^+$ 13132.9 (average isotope composition), found: 13134.5.

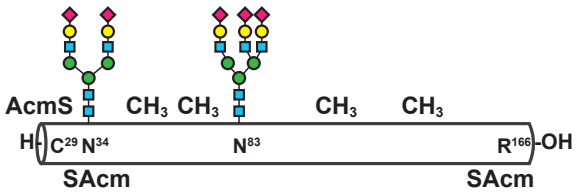


Seg 23456 (**65**)

Seg23456 (**65**) by NCL of Seg234 (**63**) and Seg56 (**64**)

Seg56: H-[Cys^{98}-Cys^{128}-Cys^{50}(Acm)-Arg^{166}]-$^{\alpha}$OH **64**[14] (0.7 mg, 0.092 μmol) in 50 mM MPAA buffer containing 6 M Gn-HCl, 0.2 M phosphate, and 40 mM TCEP (pH6.6, 23 μL) was added to Seg 234: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Biantennary oligosaccharide)-Cys^{50}-Cys^{79}-Asn^{83}(Triantennary oligosaccharide)-Lys^{97}]-$^{\alpha}$thioester **63** (0.6 mg, 0.046 μmol), which was kept at rt. After 2 h, the reaction mixture was treated with 100 mM TCEP buffer (pH 6.6, 200 μL) for 20 min to remove undesired disulfide bonds and purified by preparative RP-HPLC (Proteonavi, Φ10 mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 70:30 over 10 min, then 70:30 to 20:80 over 50 min at 2.5 mL/min). Fractions containing the desired product were combined, which was lyophilized to give Seg23456: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Biantennary oligosaccharide)-Cys^{50}-Cys^{79}-Asn^{83}

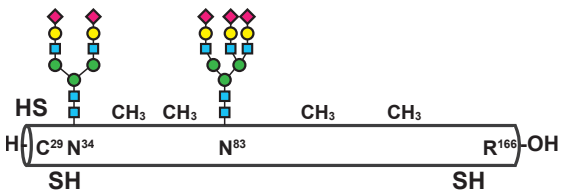
(Triantennary oligosaccharide)-Cys^{98}-Cys^{128}-Cys^{50}(Acm)-Arg^{166}]-$^{\alpha}$OH **65** (0.2 mg, 25%) as a white form. ESI-MS: *m/z* calcd for $C_{878}H_{1414}N_{210}O_{340}S_8$: $[M+H]^+$ 20609.6 (average isotope composition), found: 20613.3.



Seg 23456 (**66**)

Desulfurization of Seg23456 (**65**)

Seg23456: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Biantennary oligosaccharide)-Cys^{50}-Cys^{79}-Asn^{83}(Triantennary oligosaccharide)-Cys^{98}-Cys^{128}-Cys^{50}(Acm)-Arg^{166}]-$^{\alpha}$OH **65** (0.2 mg, 0.001 μmol) was dissolved in 250 mM TCEP buffer (pH 7.0, 20 μL) containing 6 M Gn-HCl, 0.2 M phosphate, and VA-044 (0.1 mg, 0.3 μmol), and then 2-methyl-2-propanethiol (1.44 μL, 12.8 μmol) was added to the reaction mixture, which was kept at 37^{o}C for 3 h. The reaction solution was treated with 250 mM TCEP buffer containing 6 M Gn-HCl, 0.2 M phosphate (pH 7.0, 50 μL) to remove undesired disulfide bonds and then purified by preparative RP-HPLC (Proteonavi, Φ10 mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 70:30 over 10 min, then 70:30 to 20:80 over 50 min at 2.5 mL/min). Fractions containing the desired product were combined, which was lyophilized to give Seg23456: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Biantennary oligosaccharide)-Asn^{83}(Triantennary oligosaccharide)-Cys^{50}(Acm)-Arg^{166}]-$^{\alpha}$OH **66** as a white form; ESI-MS: *m/z* calcd for $C_{878}H_{1414}N_{210}O_{340}S_4$: $[M+H]^+$ 20481.3 (average isotope composition), found: 20485.4.

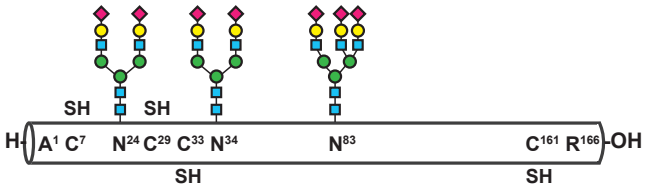


Seg 23456 (**67**)

Acm deprotection of Seg23456 (**66**)

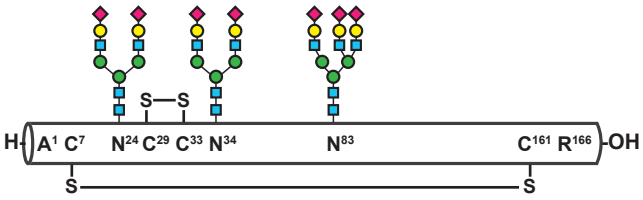
Seg23456: H-[Cys^{29}(Acm)-Cys^{33}(Acm)-Asn^{38}(Biantennary oligosaccharide)- Asn^{83}

(Triantennary oligosaccharide)-Cys^{50}(Acm)-Arg^{166}]-$^{\alpha}$OH **66** was dissolved in 90% AcOH (30 UL) containing AgOAc (ca. 0.3 mg) and the reaction mixture was kept at rt. After 3 h, DTT (9.4 mg, 81 Umol) in 90% AcOH (60 UL) was added to the reaction mixture to remove undesired disulfide bonds. The resulting mixture was purified by preparative RP-HPLC (Proteonavi, Φ10 mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 70:30 over 10 min, then 70:30 to 20:80 over 50 min at 2.5 mL/min). Fractions containing the desired product were combined, which was lyophilized to give Seg23456: H-[Cys^{29}-Cys^{33}-Asn^{38} (Biantennary oligosaccharide)-Asn^{83}(Triantennary oligosaccharide)-Cys^{50}-Arg^{166}]-$^{\alpha}$OH **67** as a white form; ESI-MS: *m/z* calcd for $C_{869}H_{1399}N_{207}O_{337}S_4$: $[M+H]^+$ 20268.1 (average isotope composition), found: 20271.4.



Seg123456 (**68**) by NCL of Seg1 (**51**) and Seg23456 (**66**)

Seg1: H-[Ala^{1}-Asn^{24}(Phenacyl esterified biantennary oligosaccharide)-Gly^{28}]-$^{\alpha}$thioester **52** (0.1 mg, 0.017 μmol) in 50 mM MPAA buffer containing 6 M Gn-HCl, 0.2 M phosphate, and 40 mM TCEP (pH6.5, 5.0 μL) was added to Seg23456: H-[Cys^{29}-Cys^{33}-Asn^{38}(Biantennary oligosaccharide)-Asn^{83}(Triantennary oligosaccharide)-Cys^{50}-Arg^{166}]-$^{\alpha}$OH **67**, which was kept at rt. After 3 h, the reaction mixture was diluted with 0.2 M phosphate buffer containing 6 M Gn-HCl (90 μL) and the pH value of the reaction mixture was adjusted to 10.0 to remove phenacyl ester. After 2 h, the solution was treated with 20 mM TCEP buffer containing 6 M Gn-HCl and 0.2 M phosphate (pH 6.6, 100 μL) for 10 min to remove undesired disulfide bonds and purified by RP-HPLC (Proteonavi, Φ4.6 mm×250 mm, 0.1% TFA:90% aq. acetonitrile containing 0.1% TFA = 99:01 to 70:30 over 5 min, then 70:30 to 15:85 over 30 min at 1.0 mL/min). Fractions containing the desired product were combined, which was lyophilized to give a full length of the EPO: H-[Ala^{1}-Cys^{7}-Asn^{24}(Biantennary oligosaccharide)-Cys^{29}-Cys^{33}-Asn^{38}(Biantennary oligosaccharide)-Asn^{83}(Triantennary oligosaccharide)-Cys^{50}-Arg^{166}]-$^{\alpha}$OH **68**. ESI-MS: *m/z* calcd for $C_{1090}H_{1762}N_{252}O_{441}S_5$: $[M+H]^+$ 25614.7 (average isotope composition), found: 25618.6.



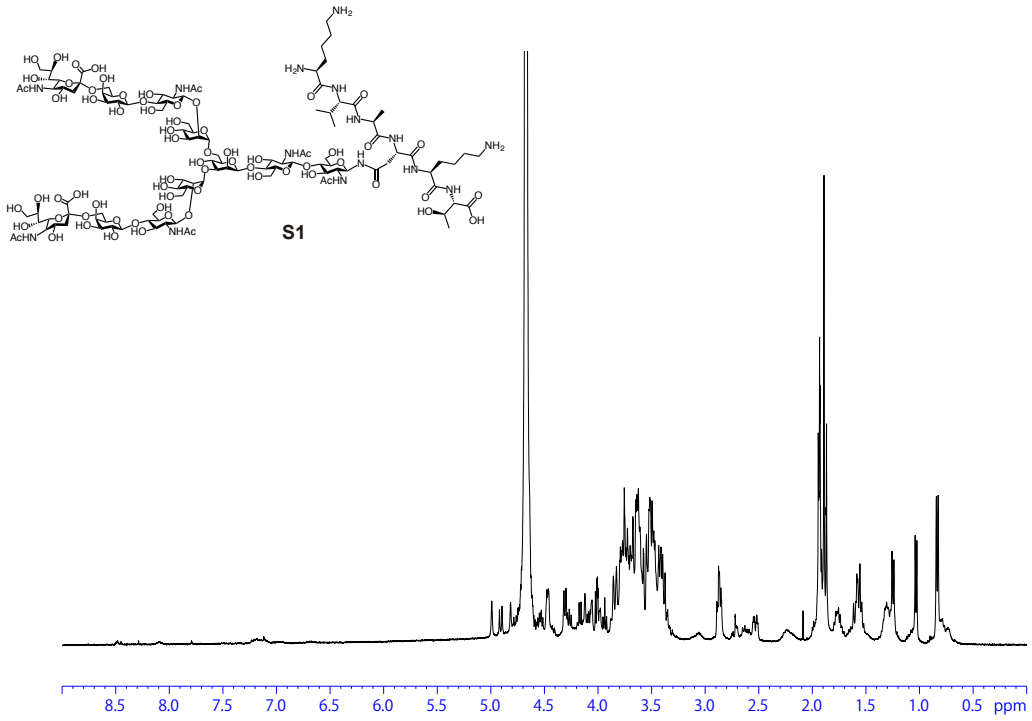
EPO (**69**)

Stepwise dialysis of Seg123456 (**68**)

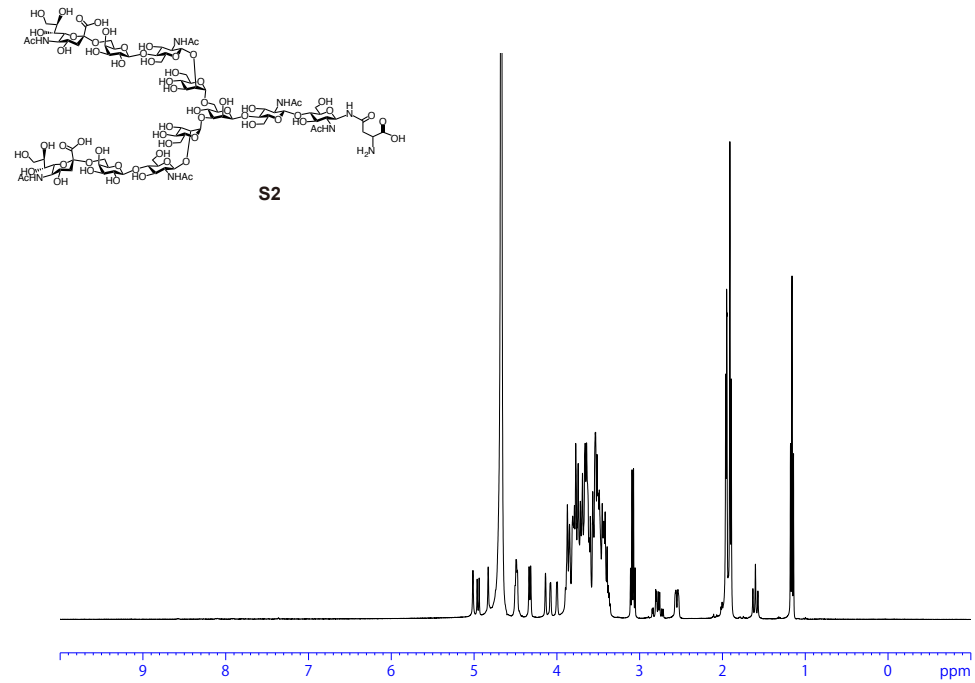
The obtained polypeptide H-[Ala^{1}-Cys^{7}-Asn^{24}(Biantennary oligosaccharide)-Cys^{29}-Cys^{33}-Asn^{38}(Biantennary oligosaccharide)-Asn^{83}(Triantennary oligosaccharide)-Cys^{50}-Arg^{166}]-$^{\alpha}$OH **68** was dissolved in 6 M Gn-HCl buffer containing 100 mM Tris-HCl (pH 7.5, 1 mL), which was poured into a dialysis tube (molecular weight cut off at 8000, Spectra/Por). The resulting mixture was subjected to dialysis against 3 M Gn-HCl buffer containing 100 mM Tris-HCl, 4 mM cysteine, and 0.5 mM cystine (pH 8.5, 200 mL), which was kept at 4 ^{o}C for 24 h. then, the external solution was replaced with 1 M Gn-HCl buffer containing 100 mM Trs-HCl (pH 8.0, 200 mL), which was further kept at 4 ^{o}C for 11 h. Finally, the dialysis tube was subjected to 10 mM Tris-HCl buffer (pH 7.0, 200 mL), which was kept at 4 oC for 22 h. An aliquot of the mixture was used for LC-FTICR analysis to show the ESI-MS of folded EPO **69**; ESI-MS: *m/z* calcd for $C_{1090}H_{1758}N_{252}O_{441}S_5$: $[M+H]^+$ 25610.7 (average isotope composition), found: 25611.8.

3. NMR Data

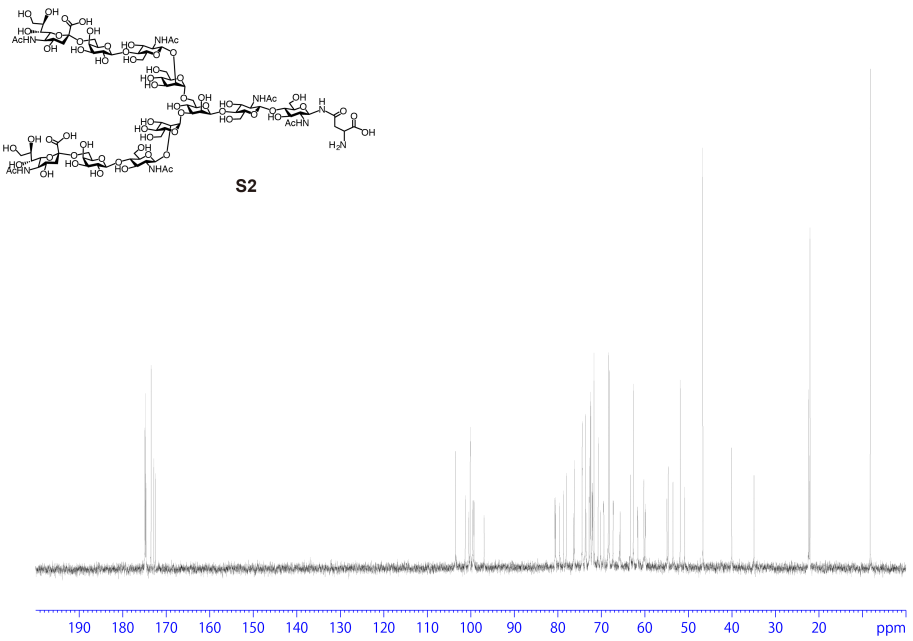
^{1}H NMR of **S1**



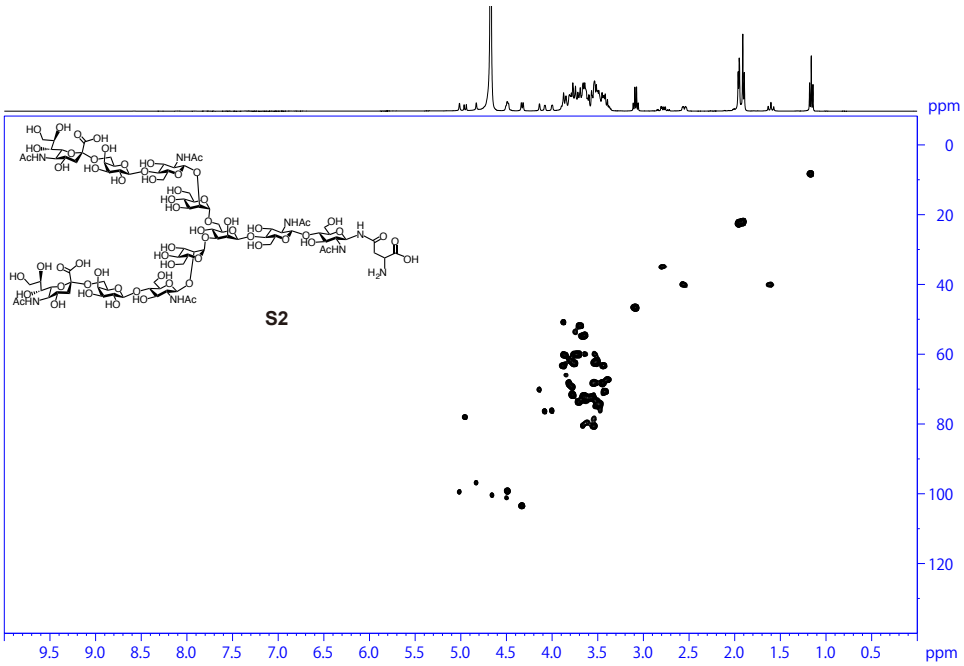
^{1}H NMR of **S2**



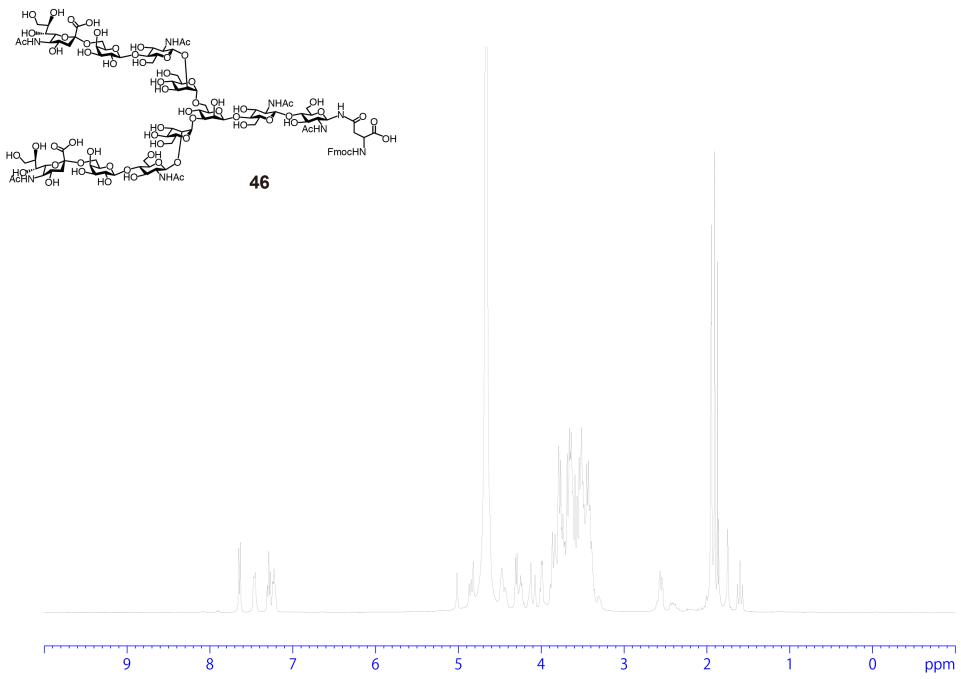
^{13}C NMR of **S2**



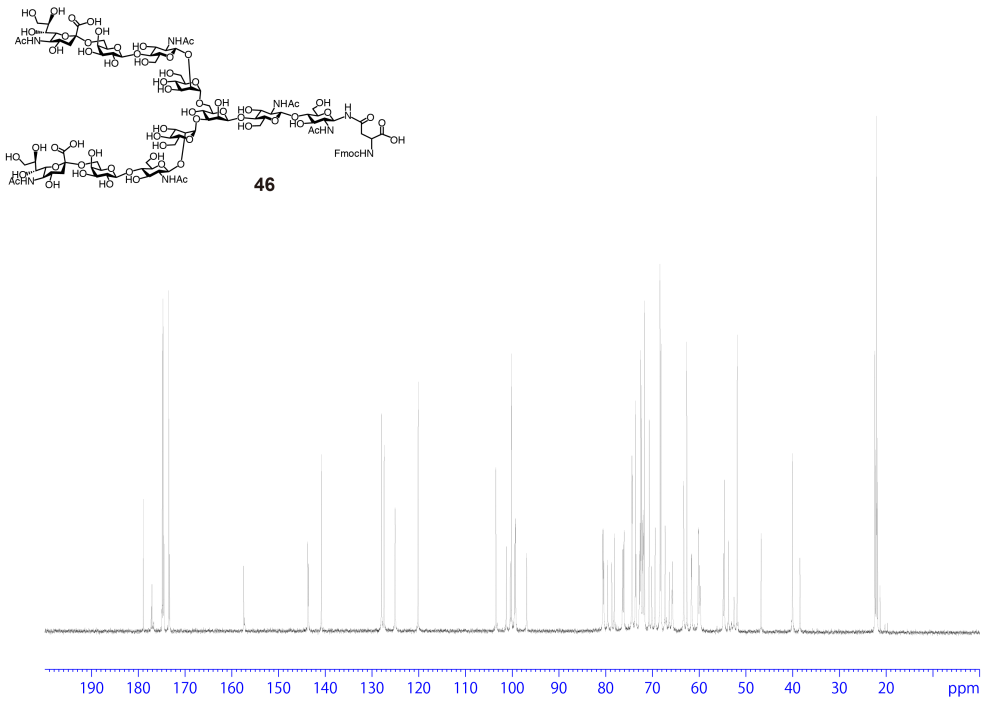
HSQC of **S2**



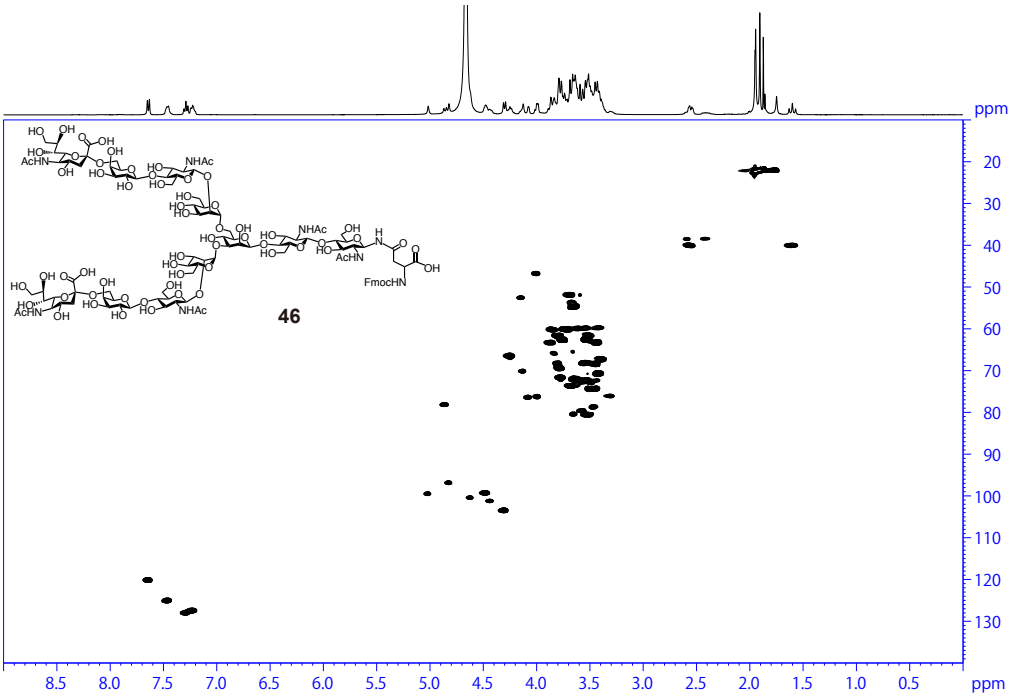
^{1}H NMR of **46**



^{13}C NMR of **46**



HSQC of **46**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50
60
70
80
90
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110
120
130
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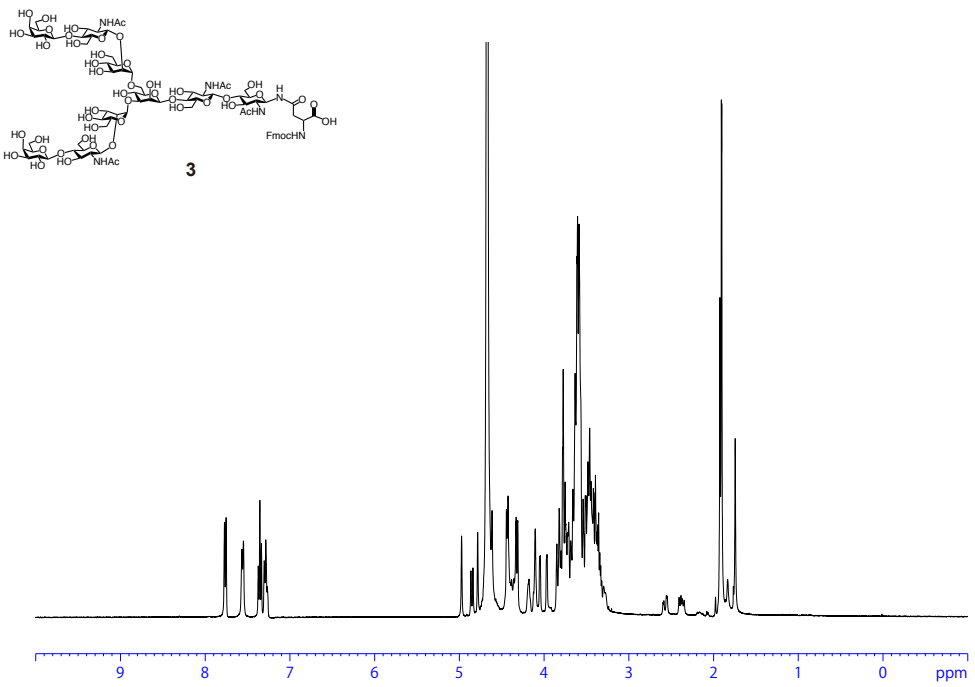
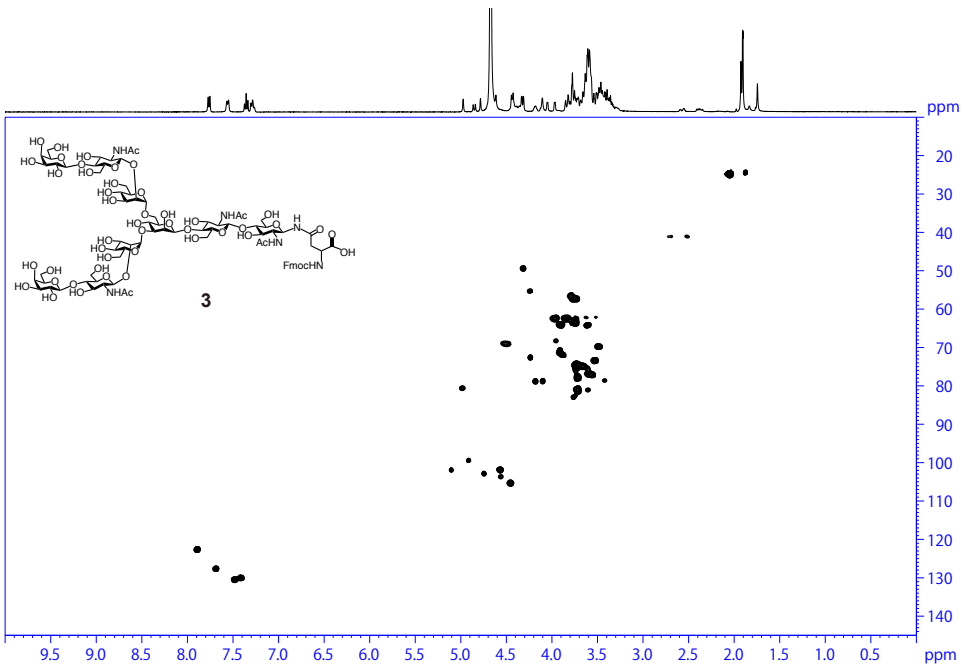
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
46

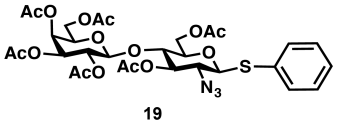
^{1}H NMR of **3**



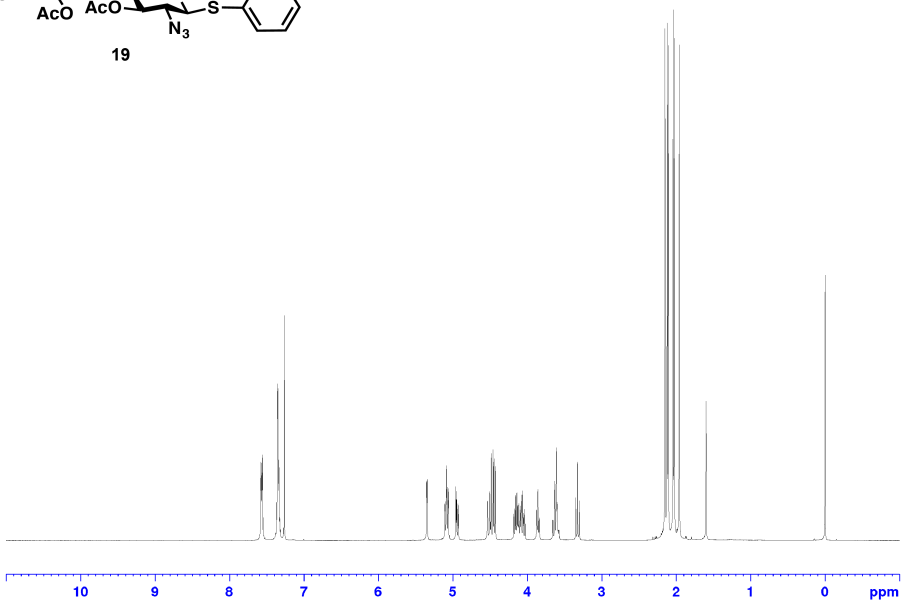
HSQC of **3**



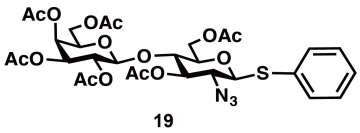
^{1}H NMR of **19**



19

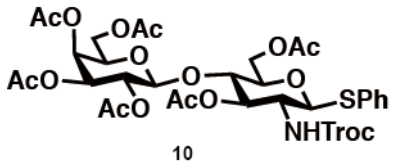


^{13}C NMR of **19**

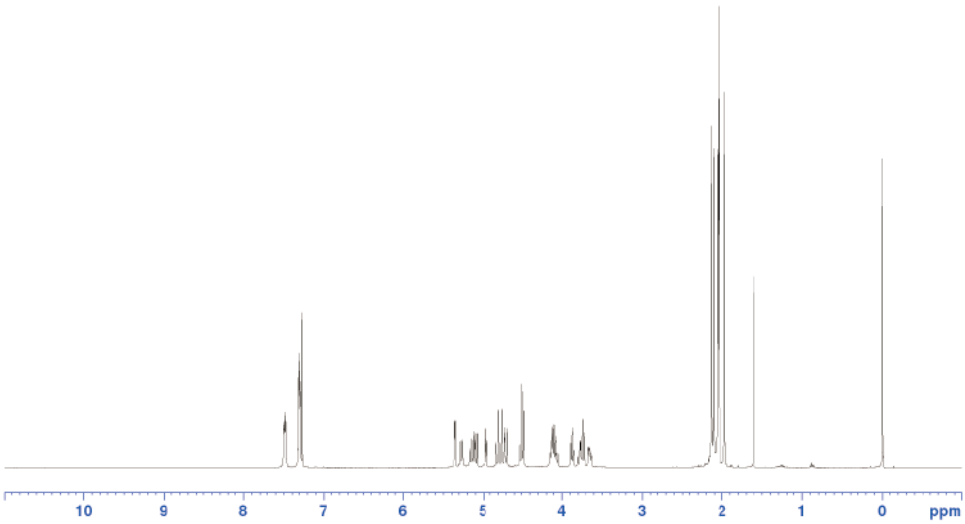


19

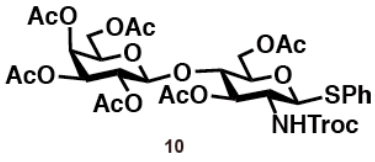
^{1}H NMR of **10**



10

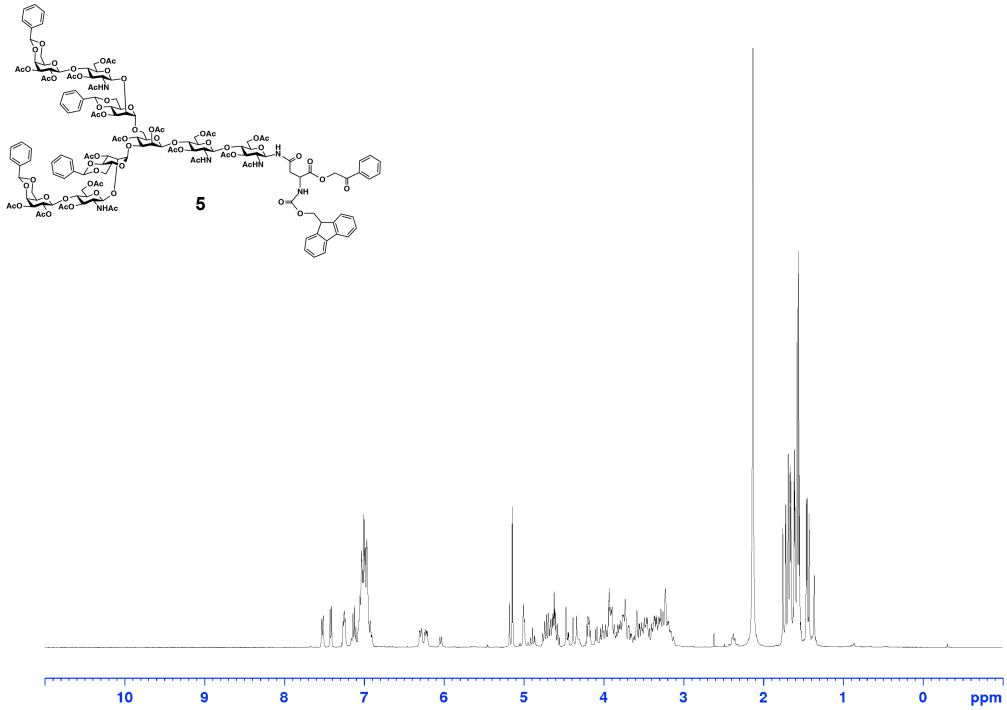


^{13}C NMR of **10**

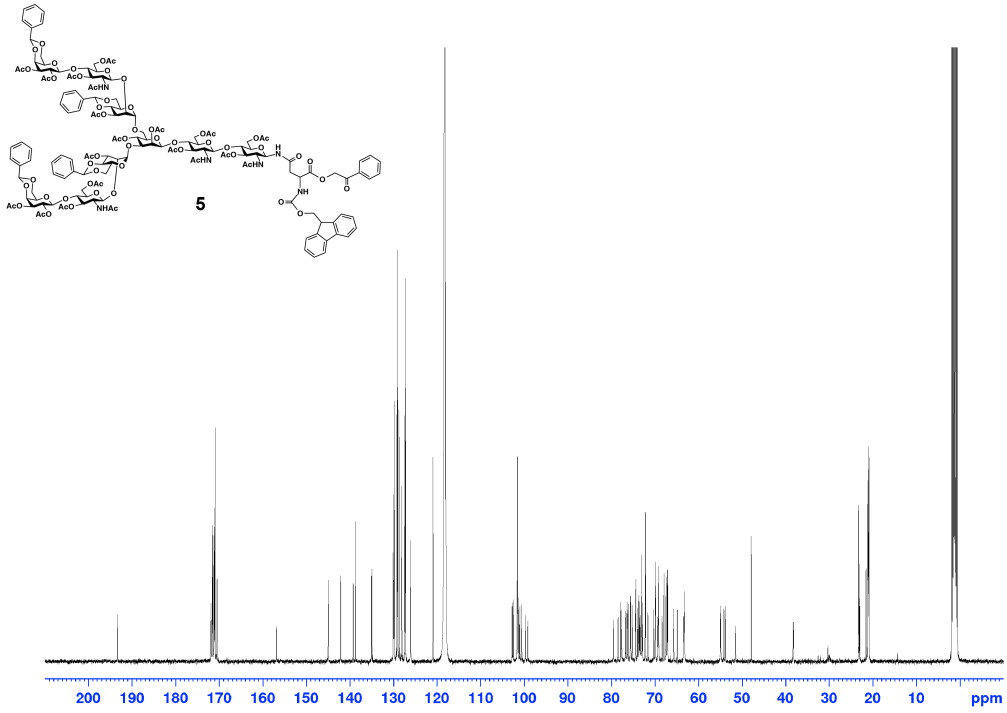


10

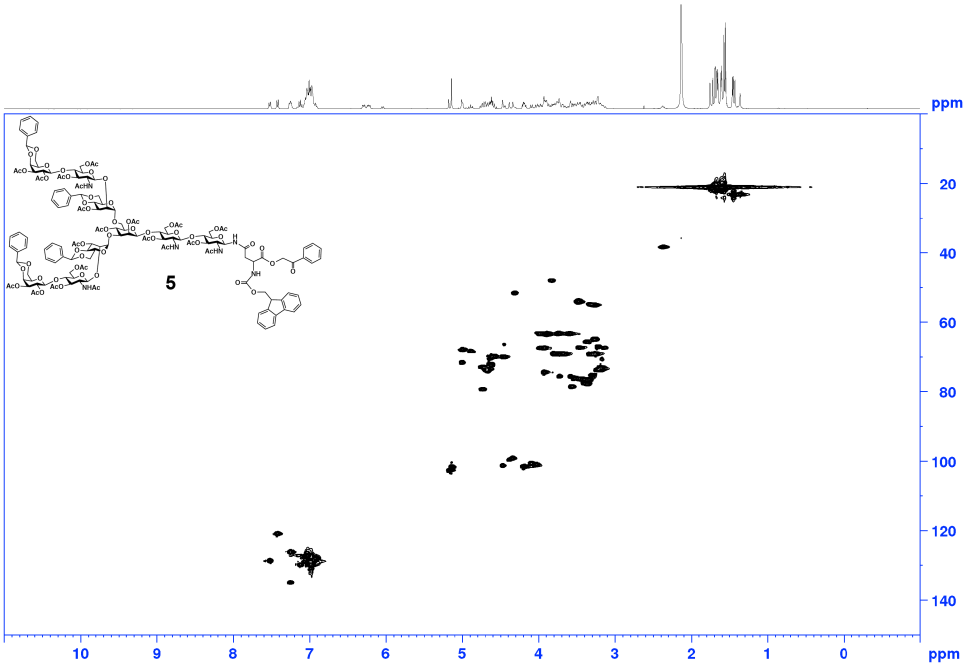
^{1}H NMR of **5**



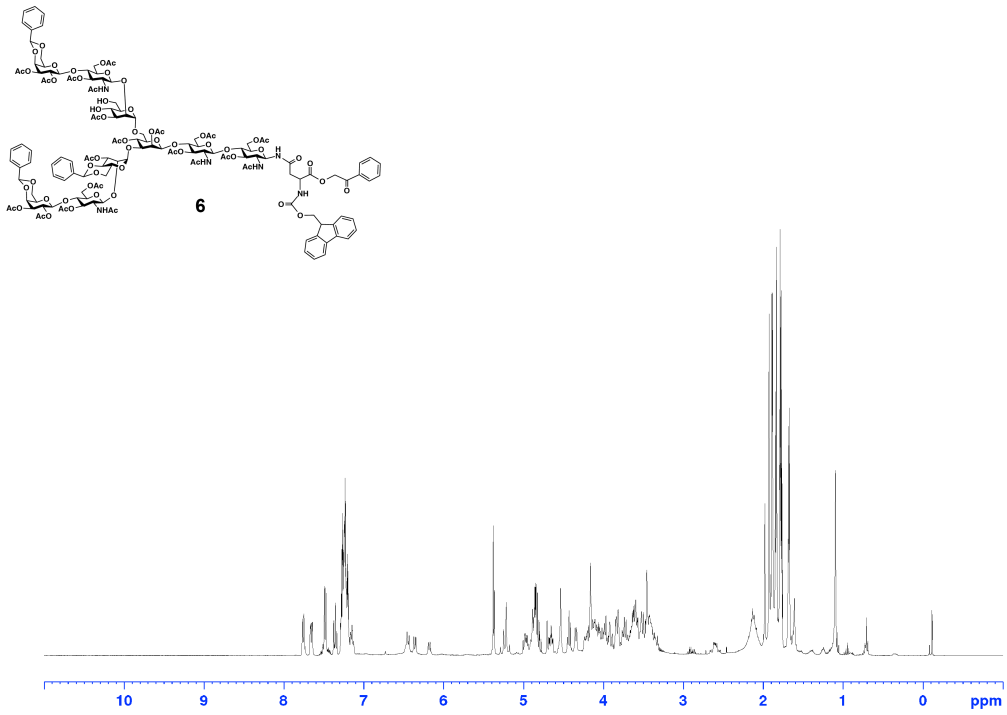
^{13}C NMR of **5**



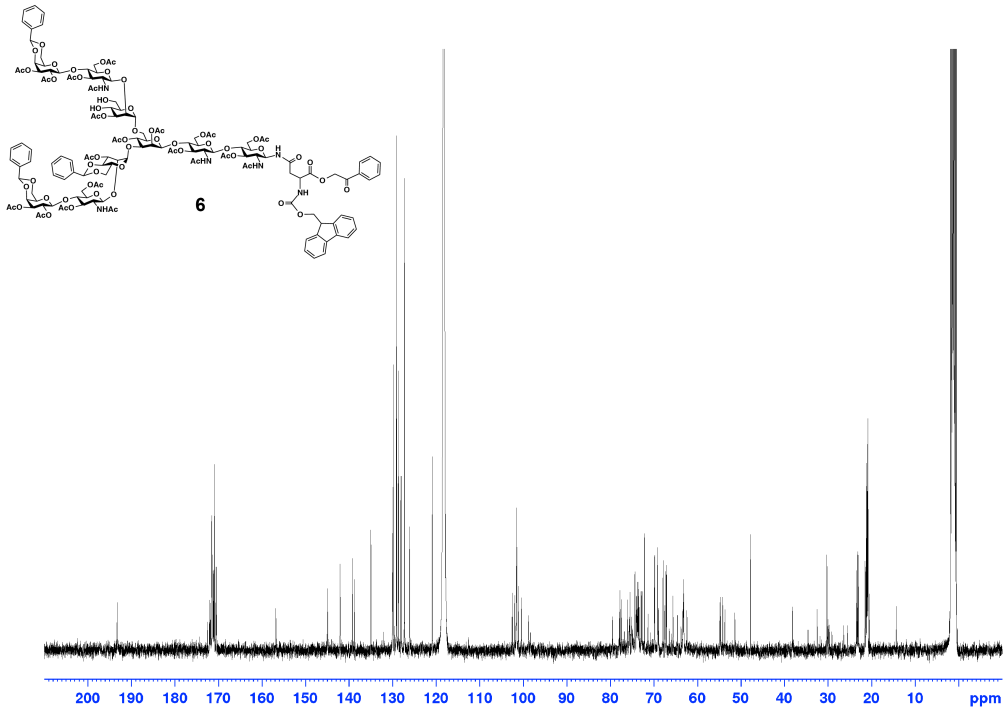
HSQC of **5**



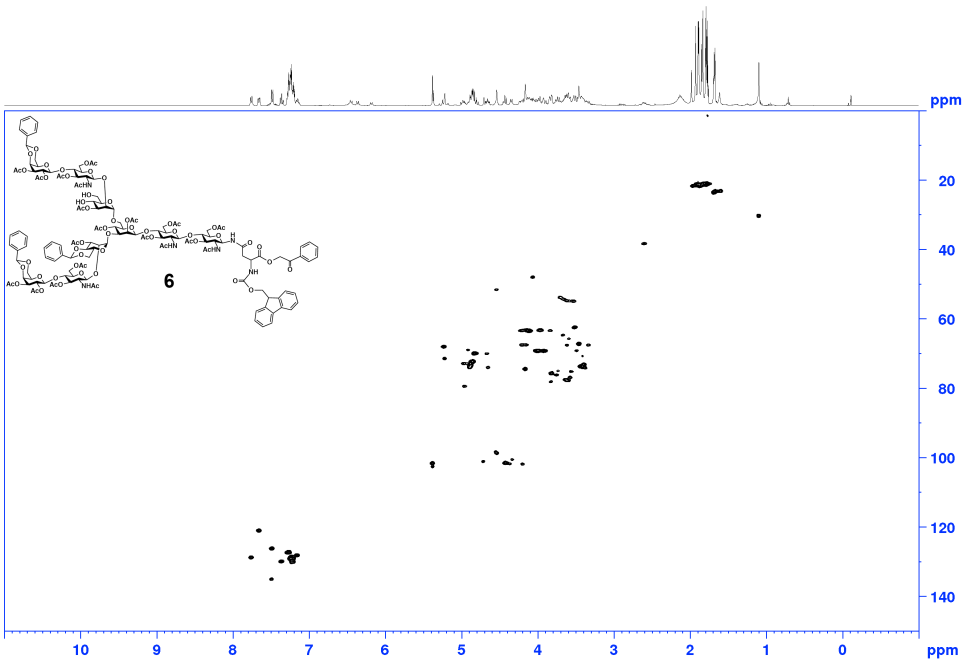
^{1}H NMR of **6**



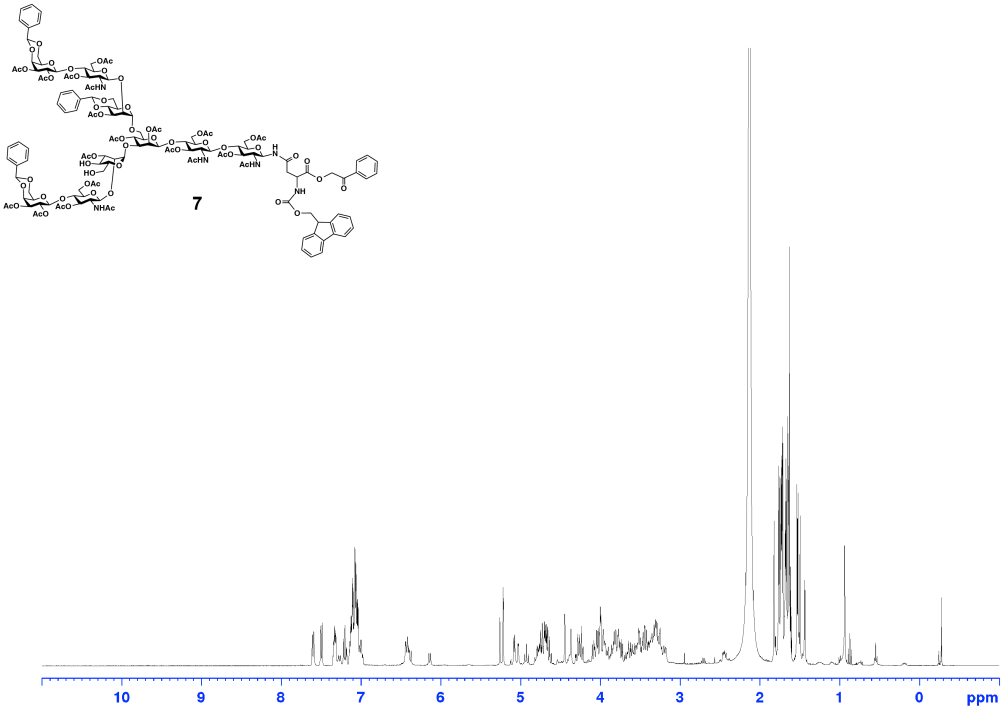
^{13}C NMR of **6**



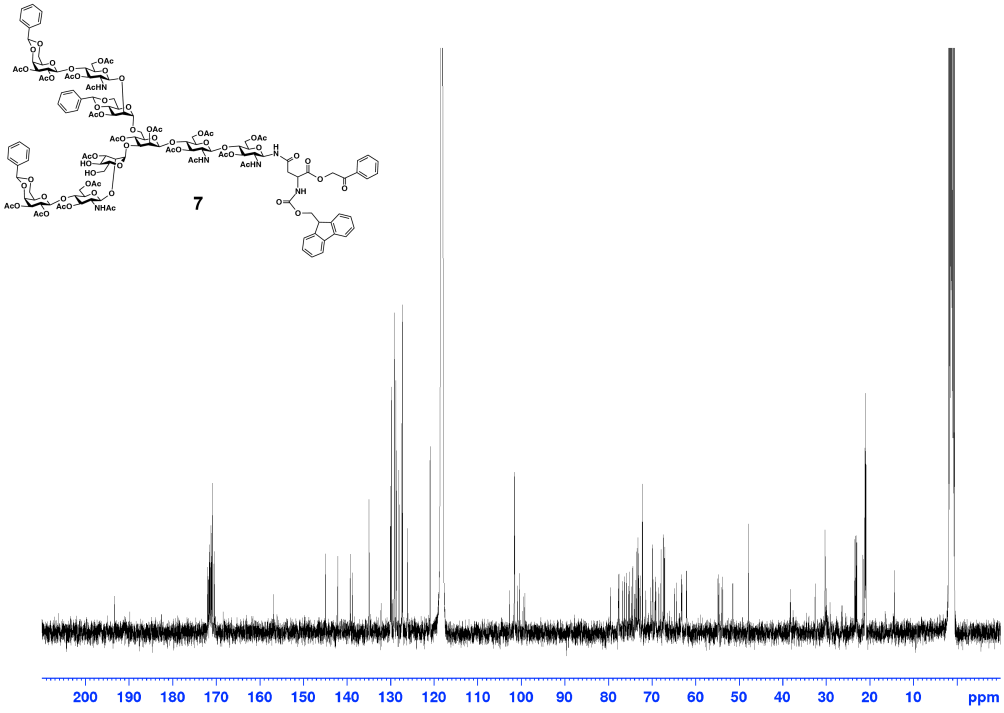
HSQC of **6**



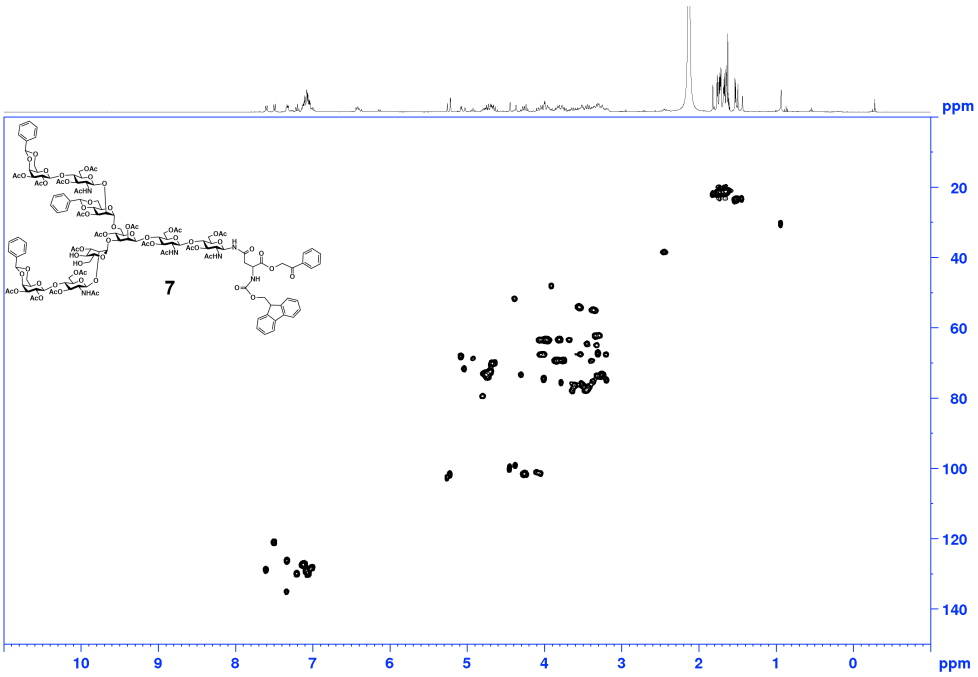
^{1}H NMR of **7**



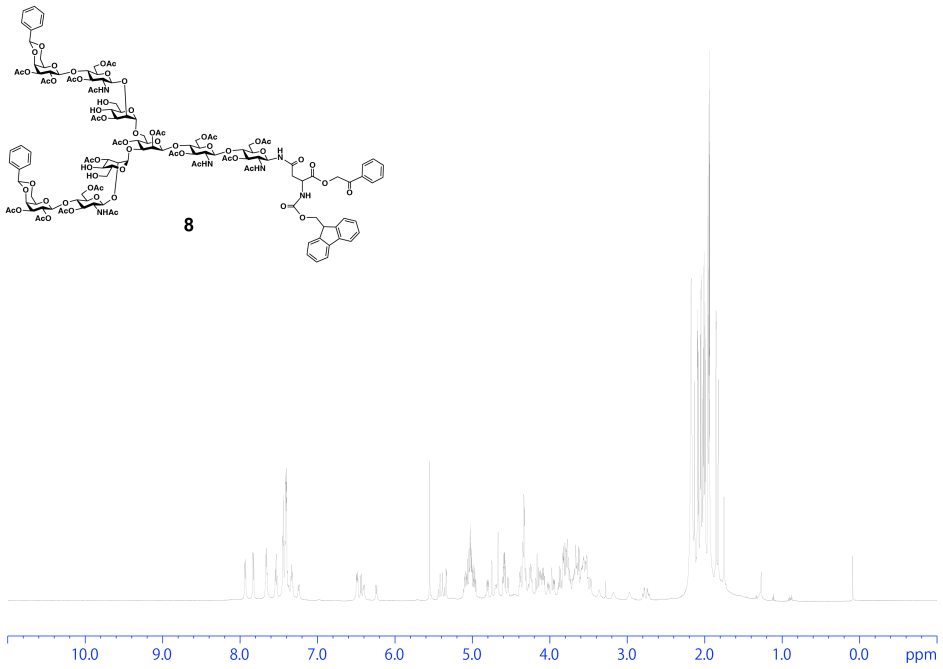
^{13}C NMR of **7**



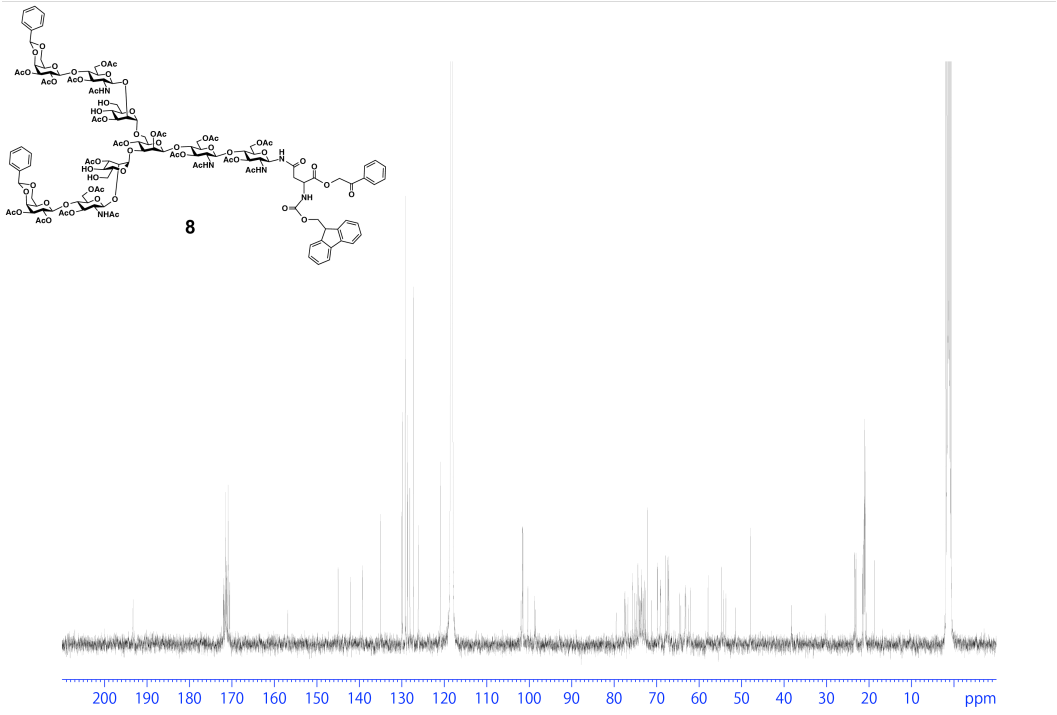
HSQC of **7**



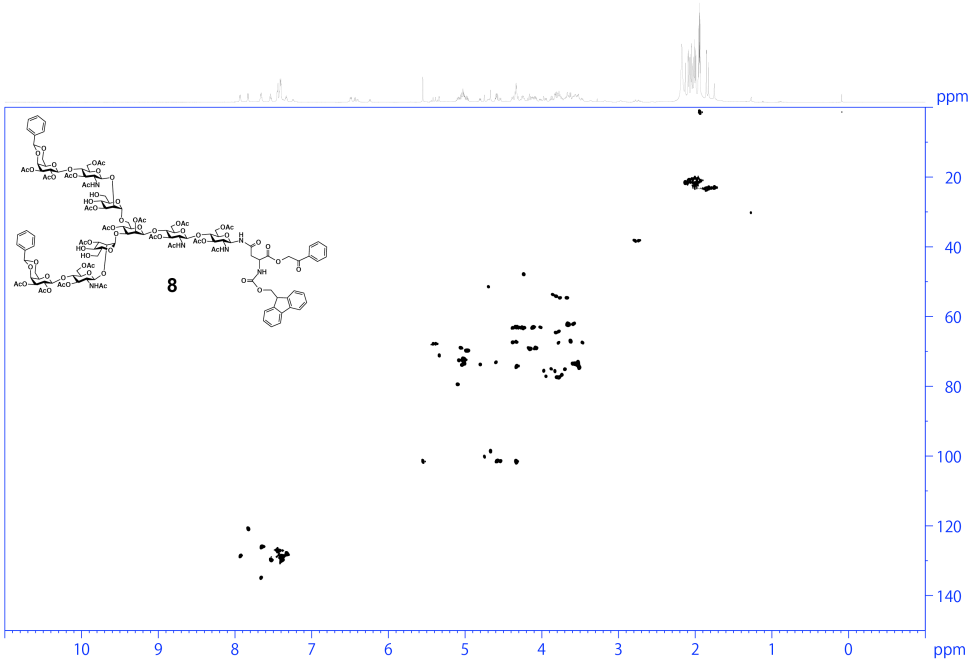
^{1}H NMR of **8**



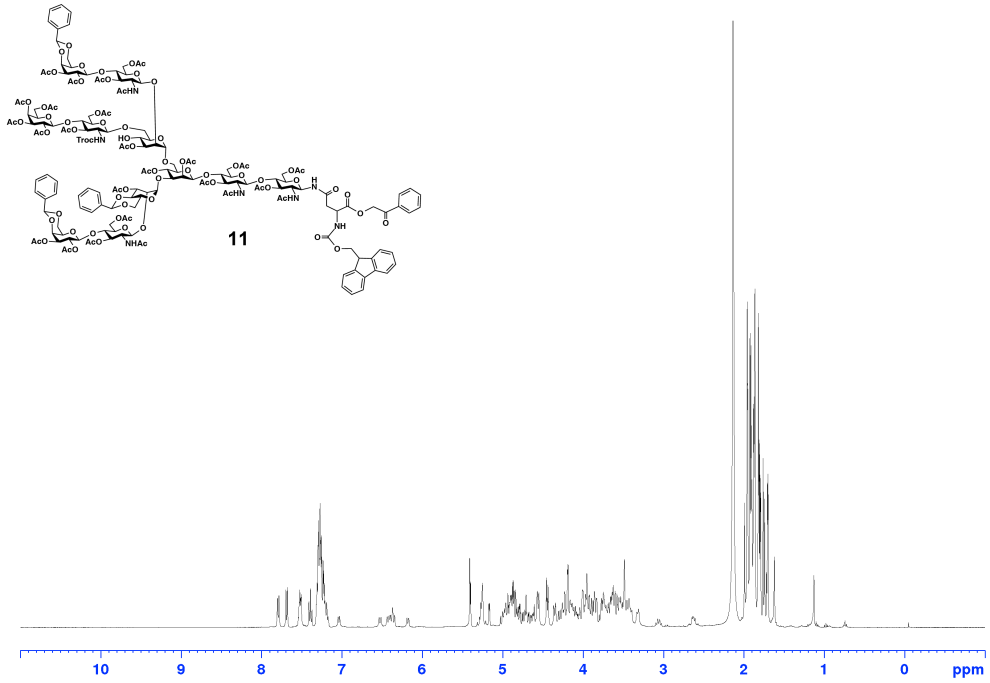
^{13}C NMR of **8**



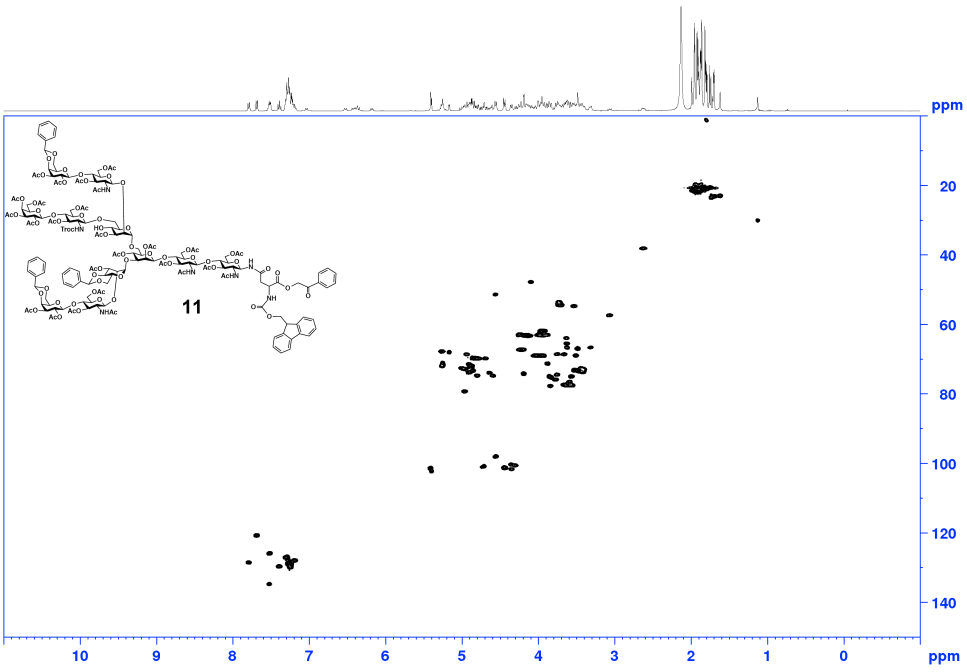
HSQC of **8**



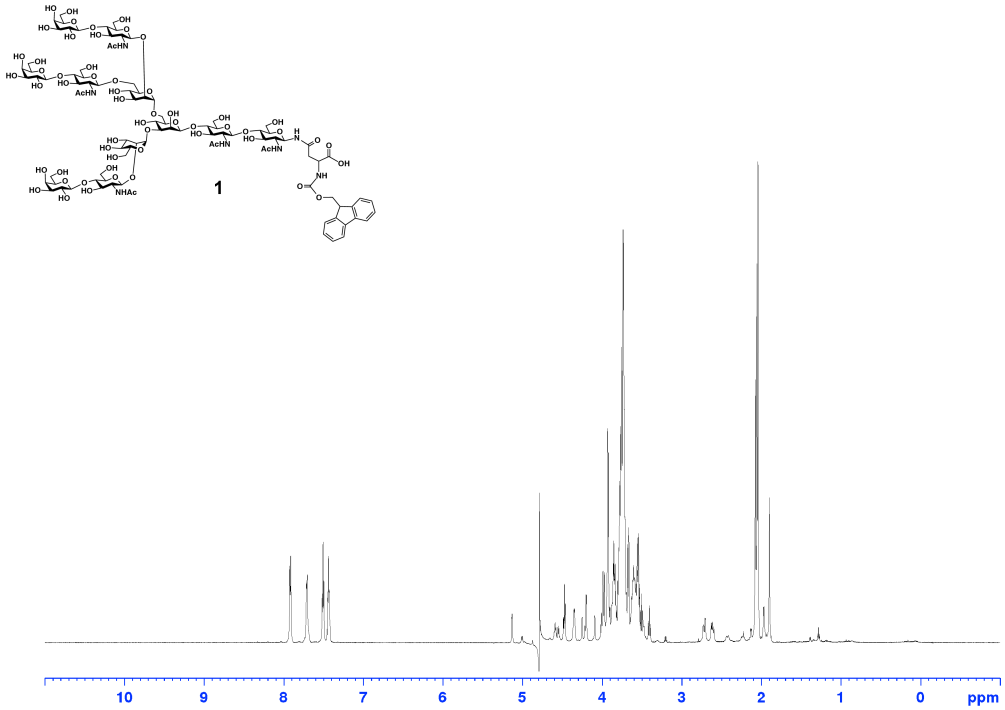
^{1}H NMR of **11**



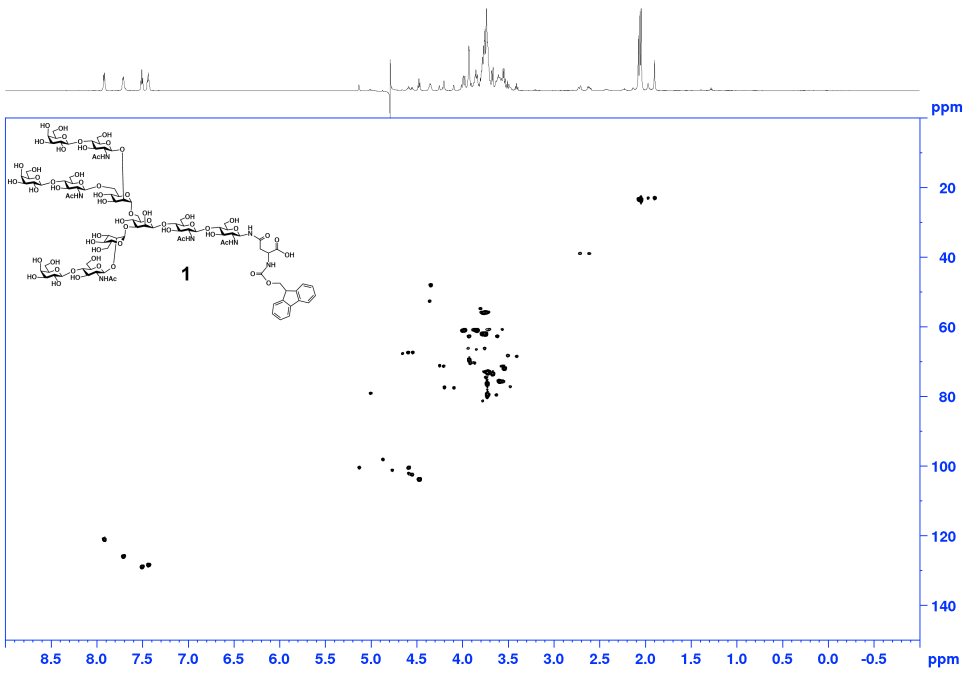
HSQC of **11**



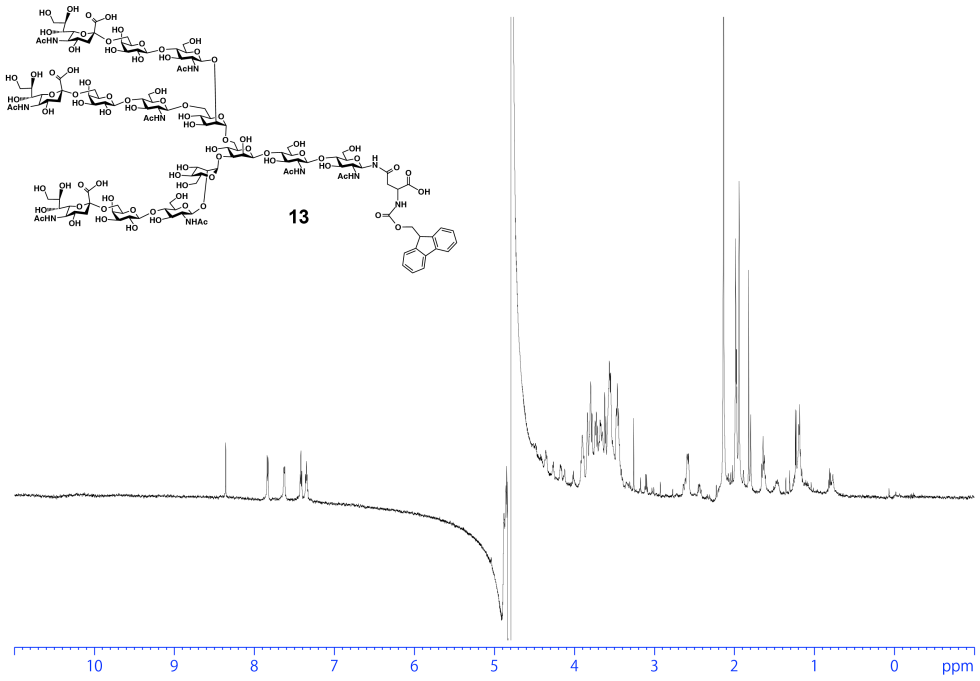
^{1}H NMR of **1**



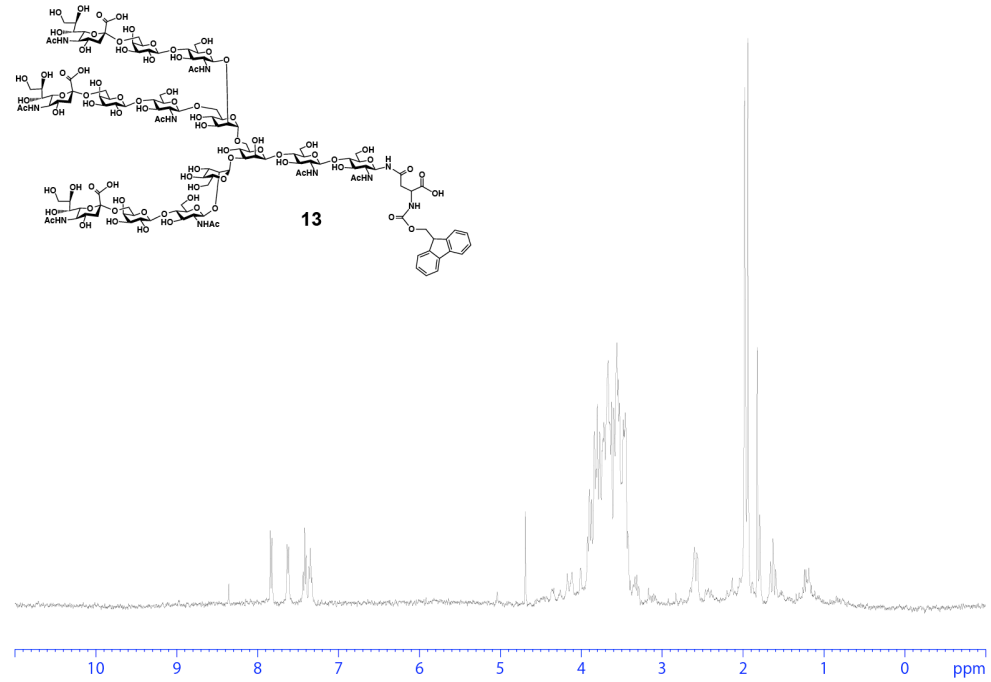
HSQC of **1**



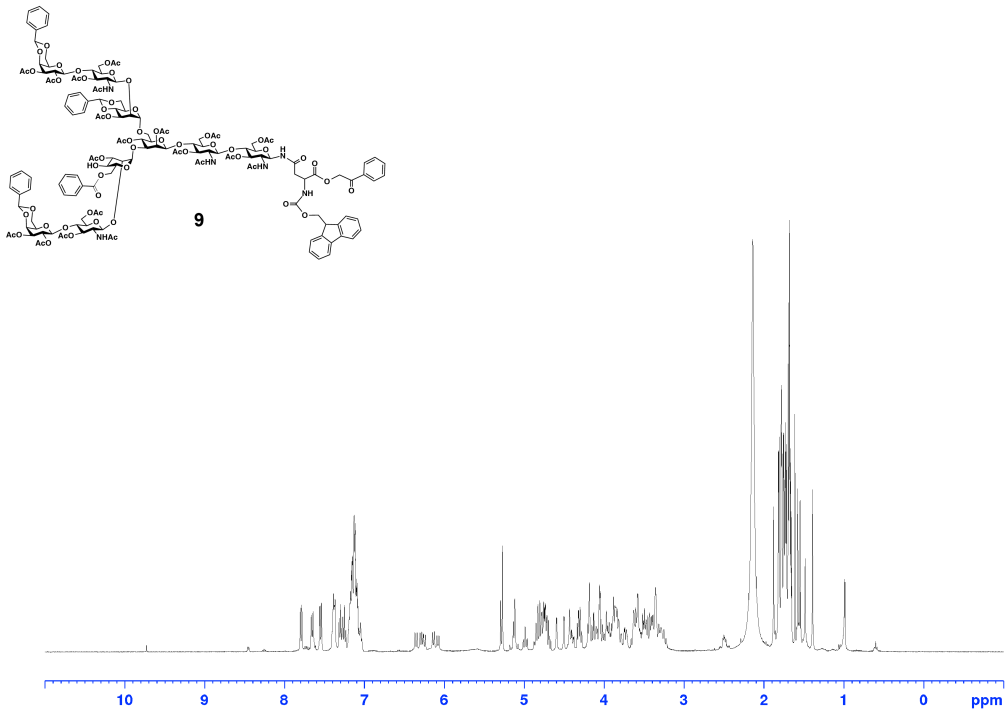
^{1}H NMR of **13** (presaturation)



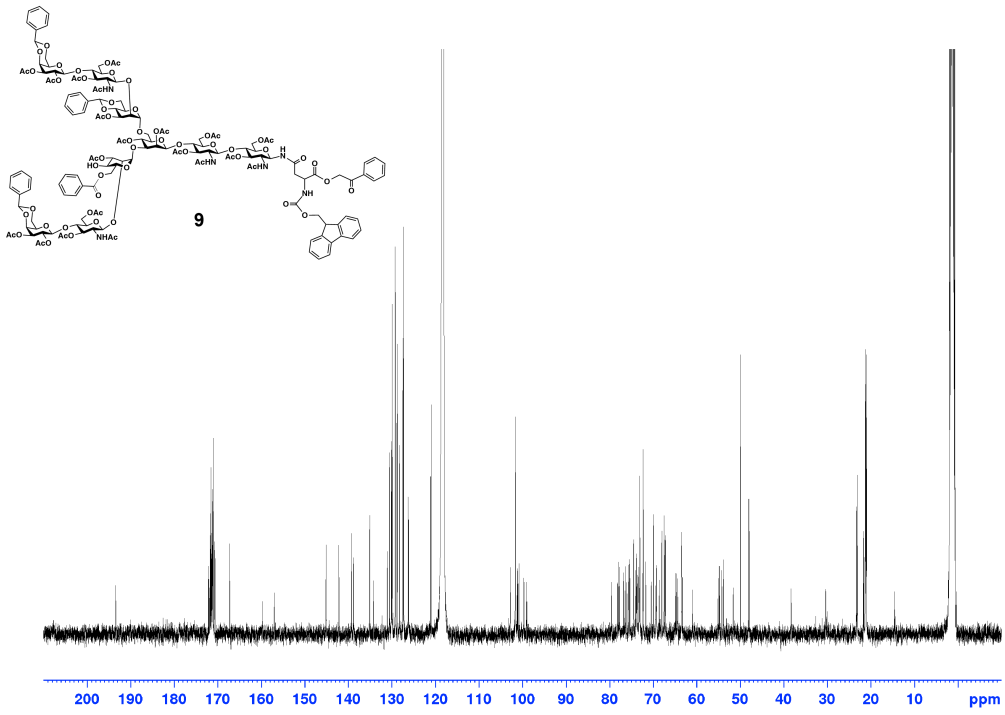
^{1}H NMR of **13** (watergate)



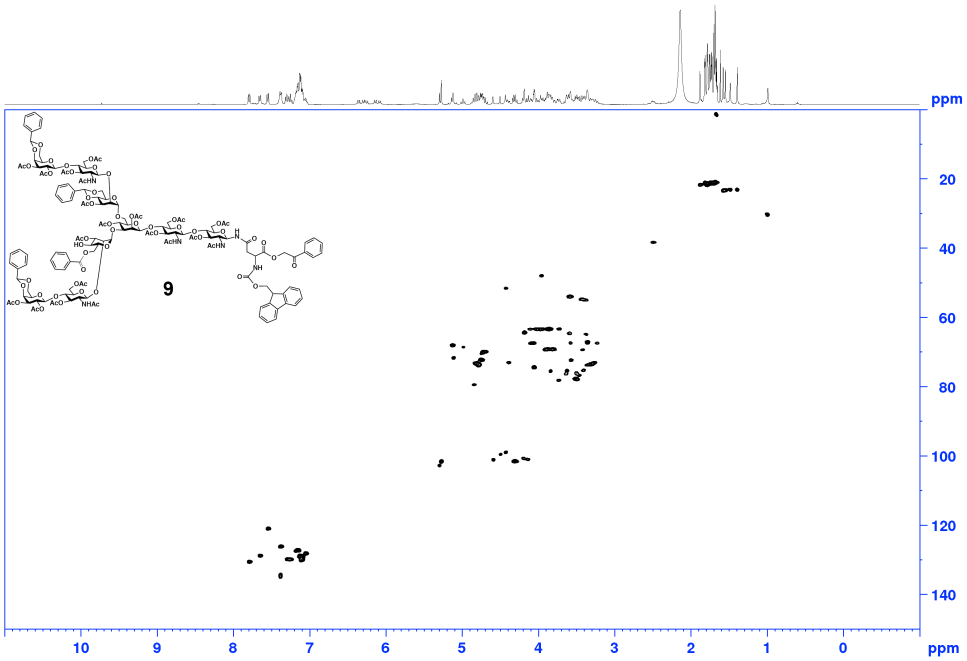
^{1}H NMR of **9**



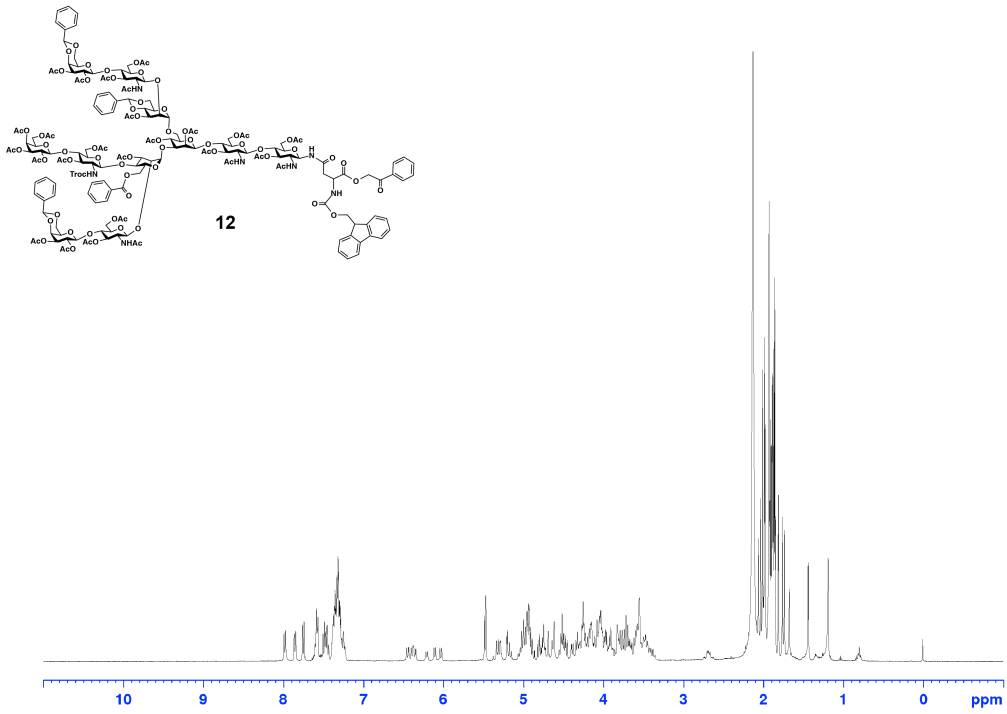
^{13}C NMR of **9**



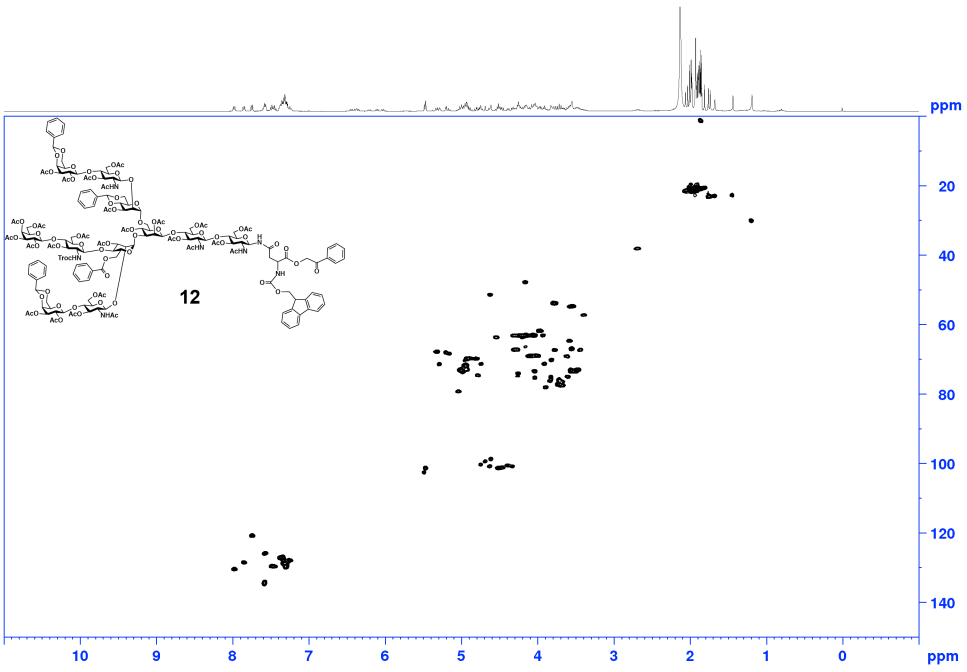
HSQC of **9**



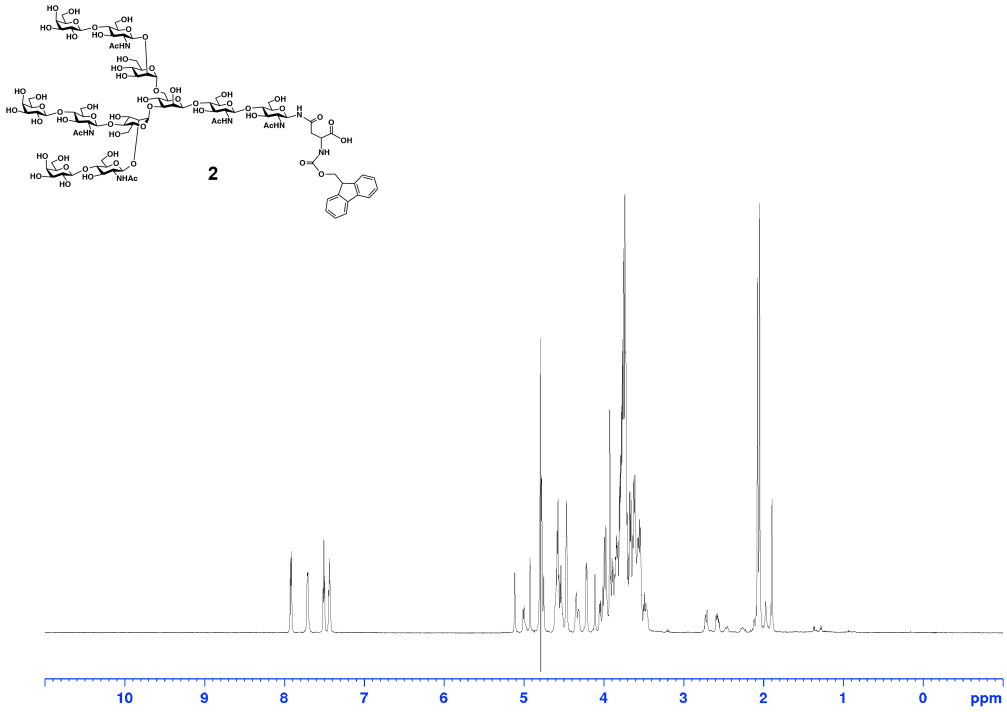
^{1}H NMR of **12**



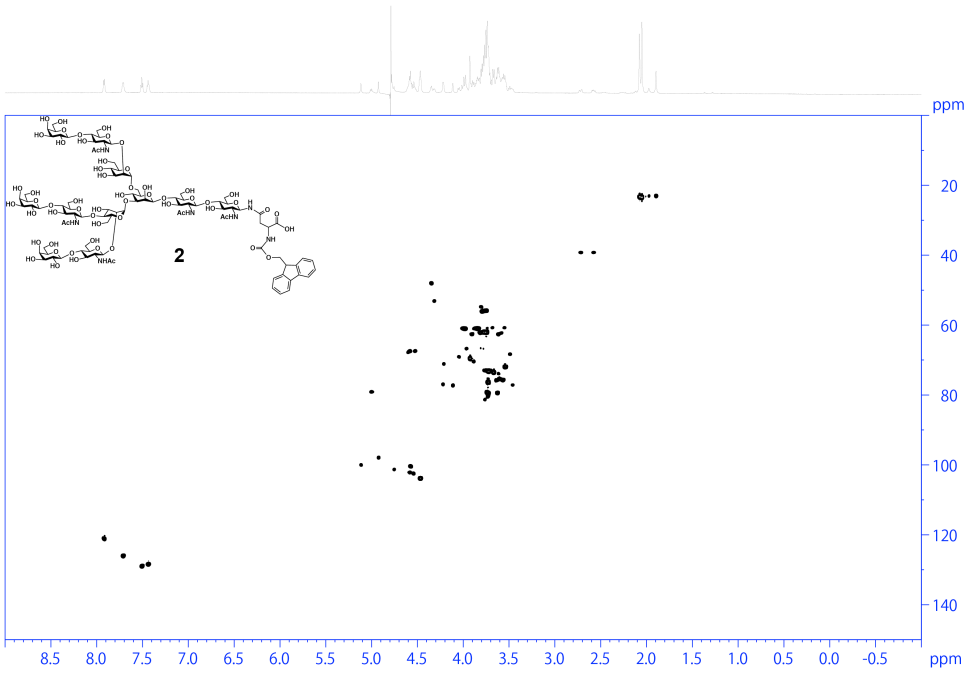
HSQC of **12**



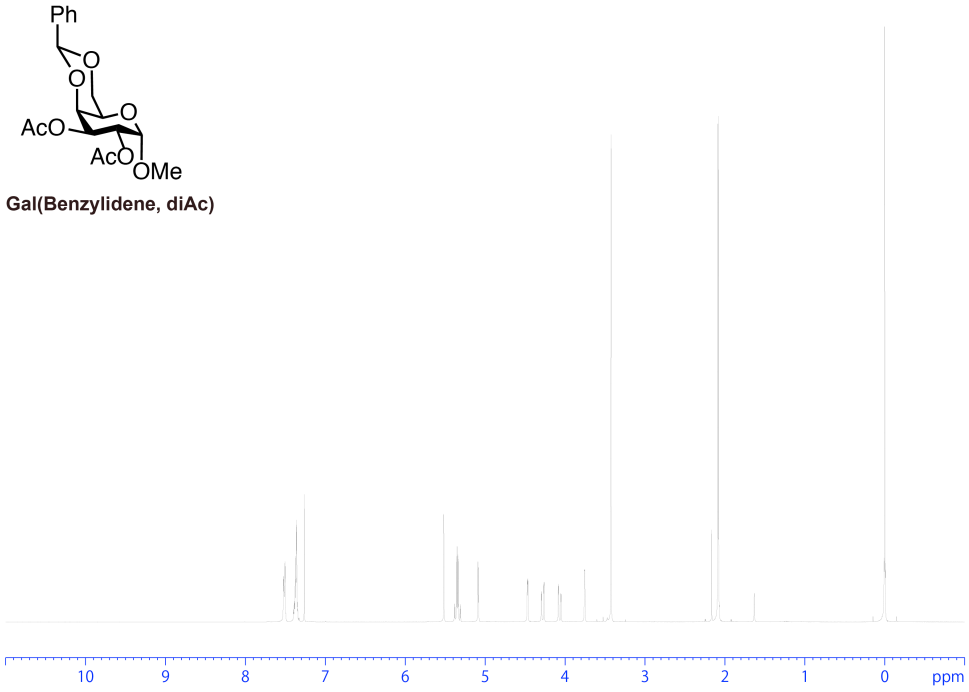
^{1}H NMR of **2**



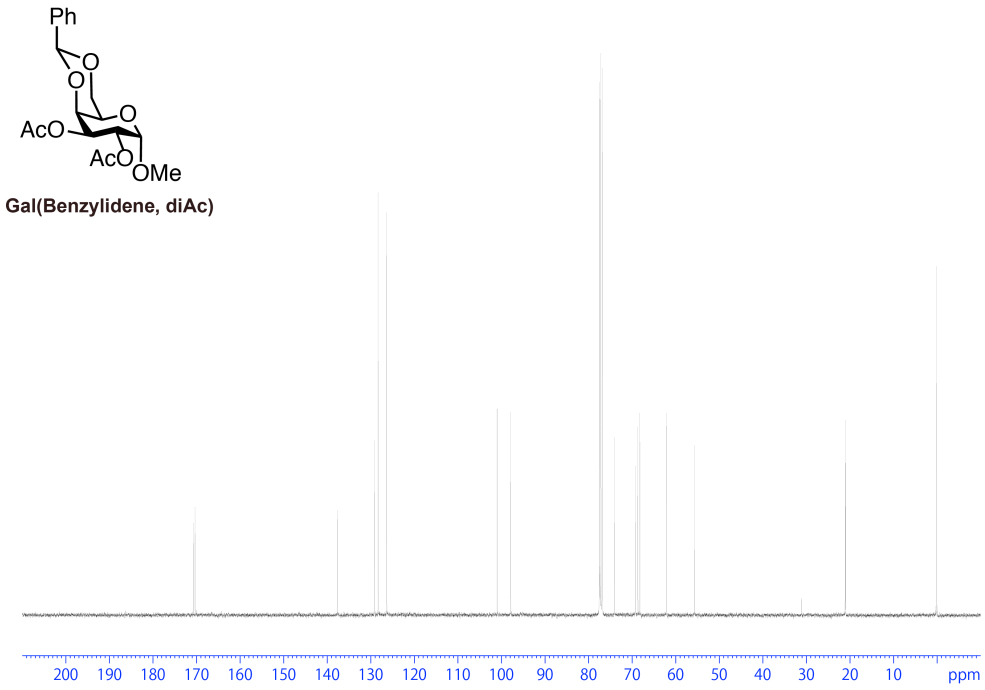
HSQC of **2**



^{1}H NMR of **29**



^{13}C NMR of **29**



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