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Studies on the Precise Chemical Synthesis of Human Glycoproteins

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Yasuhiro Kajihara received his Ph.D. from Tokyo Institute of Technology in 1993. He spent two years at the Life Science Research Laboratory of Japan Tobacco Inc. as a post doctoral fellow. He studied the synthesis of glycosyltransferase inhibitors and methods for the synthesis of sugar nucleotide. In 1995 he joined Yokohama City University as an Assistant Professor and was then promoted to Associate Professor in 2001 and Full Professor in 2007. At YCU, he developed synthetic methods for oligosaccharides as well as for glycoproteins. In 2009 he moved to the Department of Chemistry at Osaka University.

Abstract

Our research group demonstrated the semi-synthesis of complex-type biantennary sialyloligosaccharide-library by means of branch-specific glycosidase and several sialyltransferase reactions toward complex-type H_2N-Asn-(biantennary compex-type sialyloligosaccharide)-COOH isolated from egg yolk at over a gram scale. This methodology yielded 35 kinds of homogeneous complex-type oligosaccharides. Using this oligosaccharide library, an efficient Boc solid phase peptide synthesis (SPPS) of acid labile sialylglycopeptide-α-thioester was demonstrated. Our research group showed that the sialoside in which the carboxylic acid is protected with a phenacyl group is stable under strong acidic conditions, and this critical finding enabled us to demonstrate the first Boc-SPPS for the synthesis of a sialylglycopeptide-α-thioester. Using both a synthetic method of sialylglycopeptide-α-thioester and native chemical ligation (NCL), our research group synthesized several glycoproteins such as chemokine MCP-3, three kinds of erythropoietin analogues, and interferon-ß. Our research group also demonstrated a unique synthesis that was an intentional synthesis of homogeneous misfolded glycoproteins bearing M9-high-mannose-type oligosaccharide. This unique synthetic misfolded-glycoprotein is useful for the purpose of uncovering the mechanism of molecular recognition in glycoprotein quality control (GQC) in the endoplasmic reticulum (ER). This account discusses the function of the oligosaccharides of glycoproteins based on our research findings.

Introduction

Almost all secreted proteins and membrane bound proteins located on the cell have oligosaccharides for proteins to exhibit their biological activities. Protein oligosaccharides play important roles in a variety of biological events such as cell–cell recognition, acceleration of inflammation, cytokine/antibody activation, and glycoprotein quality control in the endoplasmic reticulum (ER).[1–3] Oligosaccharides are generally linked with asparagine and serine/threonine side-chains, as N-linked and O-linked types, respectively (Figure 1). The N-linked type oligosaccharides are divided into three types: high-mannose **1**, hybrid **2**, and complex-type oligosaccharide **3**, whereas the O-linked oligosaccharides are divided into eight types, core 1 to core 8, which are specified in a manner dependent on the sugar components and linkage modes.

In order to elucidate the functions of these oligosaccharides, methods are needed to vary the structure of oligosaccharides depending on the purpose of the biological study of the relationship between the oligosaccharide structure and the biological activity of the glycoprotein. Initially a homogeneous form of the glycoprotein of interest should be prepared by isolation from natural sources. However, if the target is a glycoprotein such as a cytokine hormone (1 pg mL^{-1} in blood),[4] the subsequent analysis of their oligosaccharides may prove to be difficult due to the tiny amounts available from biological sources.

For the preparation of a glycoprotein, a cell expression system has been proven to be a suitable method. Mammalian cells, especially Chinese hamster ovary (CHO) cells, have the capacity to produce recombinant glycoproteins bearing

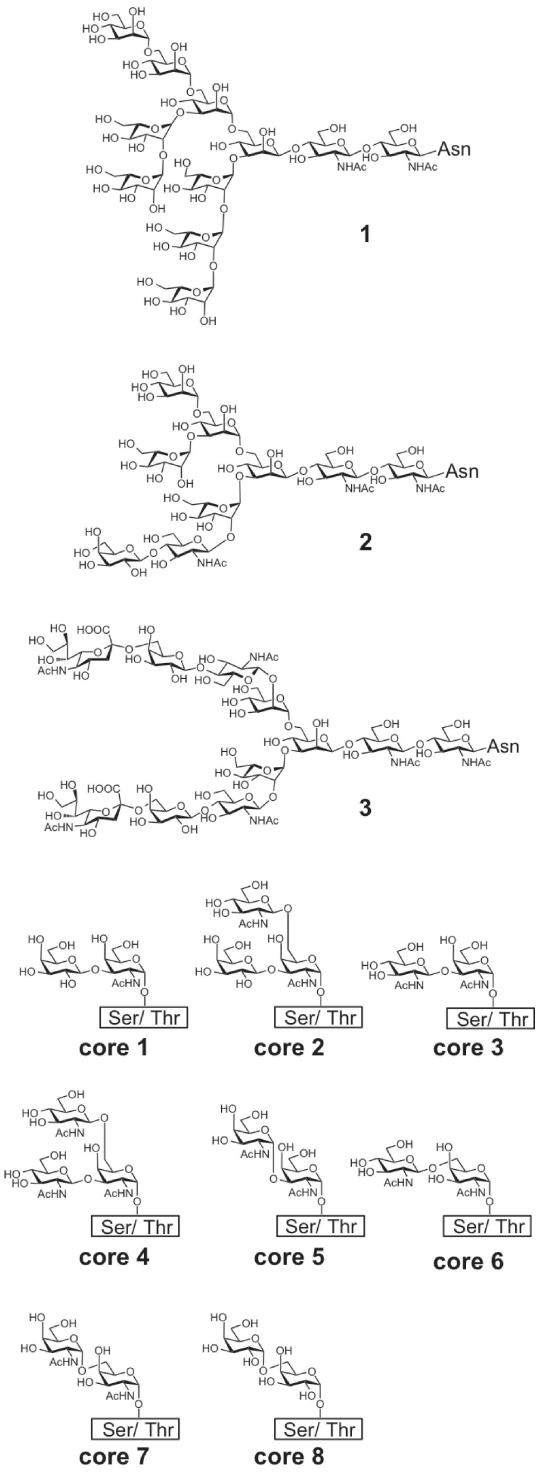


Figure 1. Structure of asparagine-linked oligosaccharides (N-linked) and threonine/serine-linked oligosaccharides (O-linked). N-Linked oligosaccharides are divided into three types, high-mannose-type **1**, hybrid-type **2**, and complex-type **3**. O-Linked oligosaccharide is divided into core 8 types dependent on sugar components and linkage modes.

mammalian-type oligosaccharides, and this system has been developed as a platform for pharmaceutical glycoprotein drugs.[5]

The biosynthesis of glycoproteins in mammalian cells starts to yield glycosylpolypeptides having high-mannose-type oligosaccharide **4** as a co-translational modification in the ER and then glycosylpolypeptide **4** proceeds with the folding process under a glycoprotein quality control (GQC) system (Figure 2).[6–10] The GQC recognizes the structure of high-mannose-type oligosaccharides and regulates the glycoprotein folding process by means of various chaperones, protein disulfide isomerases (PDI), glycosidases and glycoprotein folding sensor enzymes and therefore glycosylpolypeptide **4** forms the correct native protein three-dimensional structure **5**. UDP-Glucose glycoprotein glucosyltransferase (UGGT) is a critical glycoprotein folding sensor enzyme for discriminating the native glycoprotein form **5** and the misfolded glycoprotein form **6**. Once UGGT recognizes the misfolded glycoprotein **6**, UGGT forms the rigid complex **8** to catalyze the transfer of a glucose residue to the terminus of the high-mannose-type oligosaccharide of the misfolded glycoprotein, yielding the glucosylated misfolded glycoprotein **7**. The glucosylation of a glycoprotein in the ER means it is tagged as a misfolded glycoprotein. The monoglucosylated glycoprotein **7** is then passed to the chaperone calnexin or calreticulin, which bears a PDI **8** to repair the misfolded protein structure. After the refolding process, the glucose residue is removed by glucosidase II and then the resulting glycoprotein is inspected using UGGT once again. This refolding process is repeated in order to accumulate the native glycoprotein form **5** in the ER.

After undergoing successful folding processes in the ER, the glycoproteins that have the correct native protein structures **5** are then transported into the Golgi apparatus to modify their oligosaccharide structures, while the misfolded glycoproteins **6** accumulated in the ER and are released in the cytosol to be digested through ER-associated degradation (ERAD) (Figure 2). In the Golgi apparatus, the high-mannose-type oligosaccharides of glycoprotein **9** are further modified by several mannosidases and glycosyltransferases to form glycoprotein **10** bearing acidic oligosaccharides that are complex-type sialyloligosaccharides **3**. In addition to this oligosaccharide modification, certain serines and threonines of glycoproteins are modified with GalNAc residues as an O-linked oligosaccharide, and these GalNAc residues are further modified by several glycosyltransferases to form the eight typical core structures (Figure 1, core 1–8).

It is still unclear why the biosynthesis uses both oligosaccharide patterns, the neutral high-mannose-type **1** in the ER and the acidic sialyloligosaccharide **3** in the Golgi apparatus. In addition to this difference, it is known that the sialyloliogosaccharides exhibit considerable oligosaccharide structural heterogeneity, such as the di-, tri-, and tetra-antennary structures, while the oligosaccharide termini frequently lack sialic acids. This phenomenon of heterogeneity in oligosaccharide structure is recognized to be a hindrance in elucidating oligosaccharide function. In order to elucidate the function of an individual oligosaccharide, methods are needed to yield homogeneous glycoproteins in which the oligosaccharide structure can be varied to the greatest extent possible.

Recently a sophisticated form of protein chemical synthesis has emerged, because peptide coupling reactions creating large polypeptides and the folding of the polypeptide obtained have

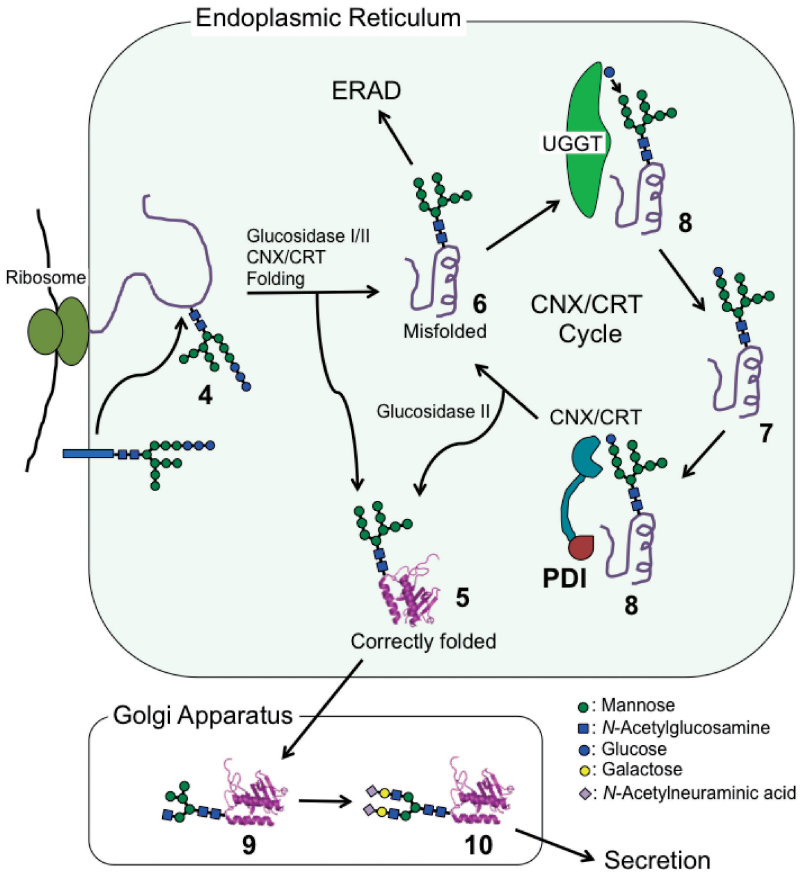


Figure 2. Biosynthesis of glycoprotein in the endoplasmic reticulum and Golgi apparatus. A nascent polypeptide made from ribose was glycosylated **4** and then starts folding process. Misfolded glycoprotein **6** was found by UGGT and modified with a glucose as a tag of misfolded one in the ER and passed to CNX/CRT/chaperone in order to perform refolding (**8** → **5**). Accumulated correctly folded glycoprotein **5** is transported in the Golgi and then the high-mannose-type oligosaccharide **5** or **9** is converted into complex type oligosaccharide **10**. UGGT: UDP-glucose glycosyltransferase, CNX/CRT: calnexin/caleticulin chaperones.

been successfully developed.[11] We have applied these findings to glycoprotein synthesis (Figure 3A). A representative peptide coupling reaction is native chemical ligation (NCL)[12] that can couple two peptides, the peptide-α-thioester **11** and the peptide **12** bearing a cysteine at the N-terminus through a thiol exchange reaction between the thioester and the cysteine-thiol, with subsequent S–N intramolecular migration to yield the native amide bond **13**. The development of a method of protein synthesis employing repetitive sequential NCL can yield large polypeptides. However, this NCL needs to use a cysteine at the ligation site and therefore NCL cannot be applied to proteins that do not have any cysteines in their peptide backbones. Under these circumstances, an alanine site has been used as a ligation site because the cysteine in a peptide can be converted into the alanine **14** through a desulfurization reaction after NCL (**13** → **14**). This ligation–desulfurization concept was developed to use with another amino acid, phenylalanine bearing a thiol at the β position, and now many amino acid derivatives bearing a thiol at the β position have been synthesized as a cysteine surrogate (Figure 3C).[11,13–15]

For the synthesis of glycoproteins, the coupling of both a homogeneous glycopeptide-α-thioester **15** (R = sialyloligosaccharide, e.g. **3** in Figure 1) and a polypeptide **14** bearing a cysteine at the N-terminus under the NCL condition would be suitable. The glycopolypeptide thus obtained is then subjected to folding processes under thermodynamically controlled disulfide bond formation to yield the correctly folded protein **16**. However an efficient method of synthesizing glycopeptide-α-thioester **15** was not established (Figure 3).

In addition to this difficulty, there were two other major problems to solve in order to achieve the chemical synthesis of glycoproteins. 1) There is no suitable method for incorporating acid-labile sialyloligosacharides with a peptide under the solid phase peptide synthetic conditions employing strong acid conditions in the final side-chain-deprotection and the detaching of the glycopeptide from solid supports. 2) The isolation or synthesis of a suitable amount of the sialyloligosaccharide building blocks.

Under these circumstances, our research group has examined solving these several problems and have achieved the first chemical synthesis of a glycoprotein bearing an intact human-type biantennary sialyloligosaccharide. This account describes the chemical synthesis of a variety of glycoproteins bearing complex-type biantennary sialyloligosaccharides or high-mannose-type oligosaccharides.

The Semisynthesis of Human-Type Oligosaccharides

As mentioned, a suitable amount of oligosaccharides such as

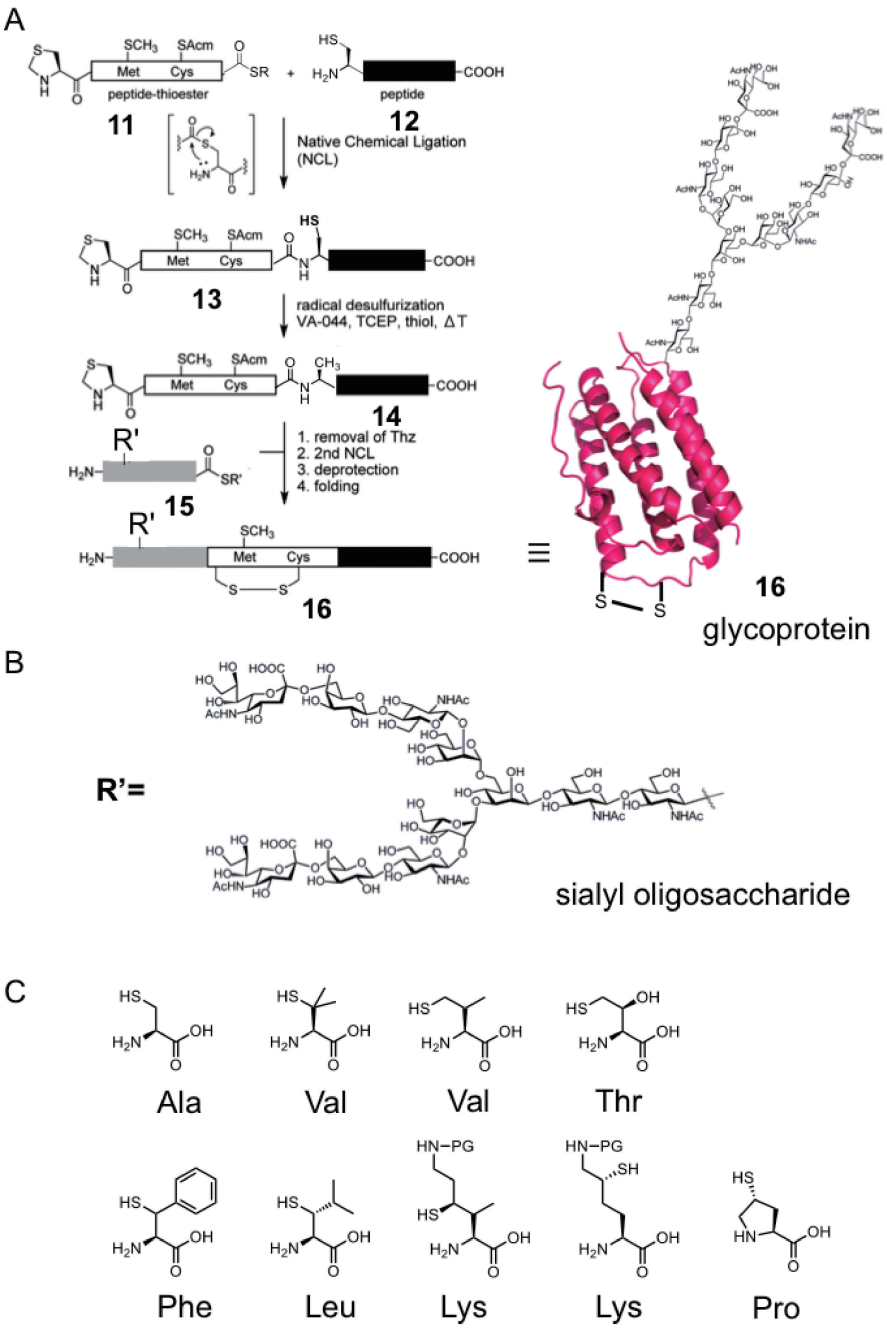


Figure 3. Chemical synthesis of glycoprotein by native chemical ligation (NCL) and subsequent desulfurization concept. A) Reaction mechanism of NCL and general synthetic strategy of glycoprotein. R′ = sialyloligosaccharide. B) Sialyloligosaccharide structure. C) Amino acid derivative bearing a thiol at the β position. These derivatives have been used for NCL as a cysteine surrogate.

high-mannose **1**, hybrid **2**, and complex type **3**, as well as O-linked oligosaccharide,[1,2] are essential for the synthesis of glycoproteins, and a current promising strategy to have such oligosaccharides is still chemical synthesis by repetitive glycosylation reactions from a monosugar. However, chemical synthesis needs the multistep preparation of glycosyl donors and acceptors via the selective protection and deprotection of sugar hydroxy groups, and it is necessary to regulate the newly formed anomeric configuration that is α- and β-selectivity during glycosylation. Such time consuming and multistep syntheses result in only a small amount of final product. In particular, complex-type biantennary oligosaccharide requires over 30 steps of chemical conversion from the starting monosugar.[16–18]

It is known that hen egg yolk includes diverse mammalian type N-linked oligosaccharides, and peptidyl biantennary complex-type disialyloligosaccharide **17** was found in suitable amount for several synthetic applications.[19] This peptidyl biantennary sialyloligosaccharide **17** can be isolated in a soluble glycopeptide form, but this fraction includes certain other glycopeptides due to the heterogeneity of the oligosaccharide isomers. It is well known that the purification of these oligosaccharide isomers is difficult, because these oligo-

saccharides have similar characteristics, such as similar hydrophilicity, which makes it difficult to purify them even with high-performance liquid chromatography (HPLC).

In order to isolate a suitable amount of biantennary sialyloligosaccharide, our research group examined peptidase digestion toward the sialylglycopeptides **17** isolated from egg yolk and subsequent selective protection of the resulting asparaginyl sialyloliogsaccharide **18** with a hydrophobic 9-fluorenylmethyl group (Fmoc) (Figure 4).[20] This hydrophobic protecting group enhances the interaction between the oligosaccharide and the reverse phase column used for HPLC purification, and this protocol enabled us to isolate homogeneous Fmoc-Asn-biantennary sialyloligosaccharide **19** at over a gram scale (Figure 4).

Using this idea, a high-mannose-type oligosaccharide consisting of nine mannose residues was also isolated from egg yolk[21] and our research group has used it for the synthesis of glycoproteins bearing high-mannose-type oligosaccharides.[21] However, high-mannose-type oligosaccharides are not contained in a soluble glycopeptide form in egg yolk, other proteins are incorporated. Therefore our research group optimized the isolation protocols of Fmoc-Asn-high-mannose-type oligosaccharide **26** by the peptidase digestion of egg yolk proteins, Fmoc protection of asparagine was linked with the reducing terminal of the oligosaccharide, and the purification of Fmoc-Asn-high-mannose-type oligosaccharide was performed by HPLC.

In terms of a structural analysis of the oligosaccharides isolated by nuclear magnetic resonance (NMR), oligosaccharides exhibit heavily overlapping signal patterns,[22] so the analysis of glycosyl bond positions is known to be laborious work. Our research group sought to establish efficient assignment methods that were comprised of selective 1D-TOCSY-DQFCOSY, selective 1D-TOCSY-NOESY, and selective 1D-TOCSY-HSQC (Figure 5). These NMR experiments may be used to abstract a spectrum of individual monosugar unit in oligosaccharides based on the selective 1D-based TOCSY method by exciting a well separated anomeric proton signal and subsequent second pulse set such as COSY, NOESY, and HSQC. It shows the corresponding 2D spectrum of the desired monosugar unit. These experiments enabled us to assign all of the proton and carbon signals.[23–25]

Because oligosaccharides display diverse structural patterns on the cell surface as well as secreted proteins, homogeneous oligosaccharide repertoires are useful for synthetic and biological experiments to elucidate what kinds of oligosaccharide structures are critical for a biological event of interest. In order to prepare such an oligosaccharide library, a combined chemical and enzymatic conversion of Fmoc-Asn-(biantennary sialyloligosaccharide)-OH **19** into several structural isomers was performed. Our research group examined the non-selective acid hydrolysis of one of the disialyl linkages of Fmoc-Asn-(biantennary sialyloligosaccharide)-OH **19** followed by galactosidase digestion. After this treatment, Fmoc-Asn-(biantennary monosialyloligosaccharide)-OH **20** was found to be able to purify from another isomeric Fmoc-Asn-(biantennary monosialyloligosaccharide)-OH. This strategy of protection of the oligosaccharide with hydrophobic Fmoc groups successfully enabled easy purification of each Fmoc-Asn-(biantennary

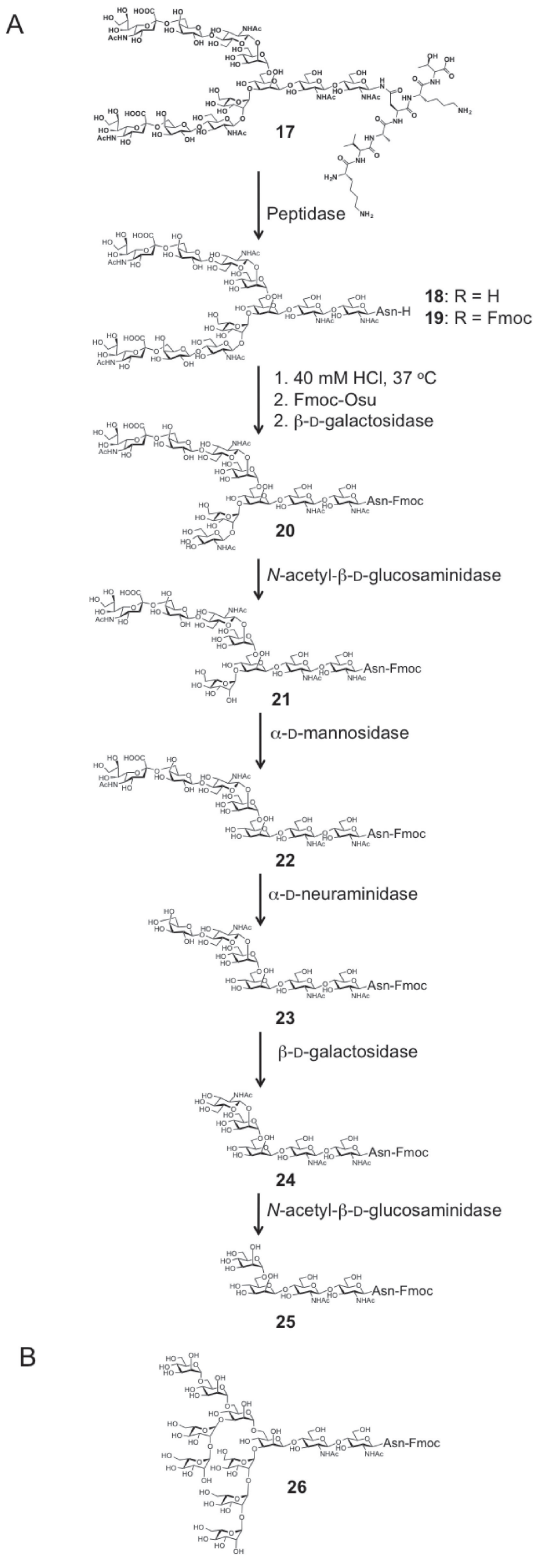


Figure 4. Isolation of asparaginyl oligosaccharide and a concept for conversion into diverse oligosaccharide with sequential glycosidase digestions. A) Conversion of sialyl-oligosaccharyl peptide **17** isolated from egg yolk and subsequent conversion into several oligosaccharide structure. B) The structure of high-mannose-type oligosaccharyl-asparagine.

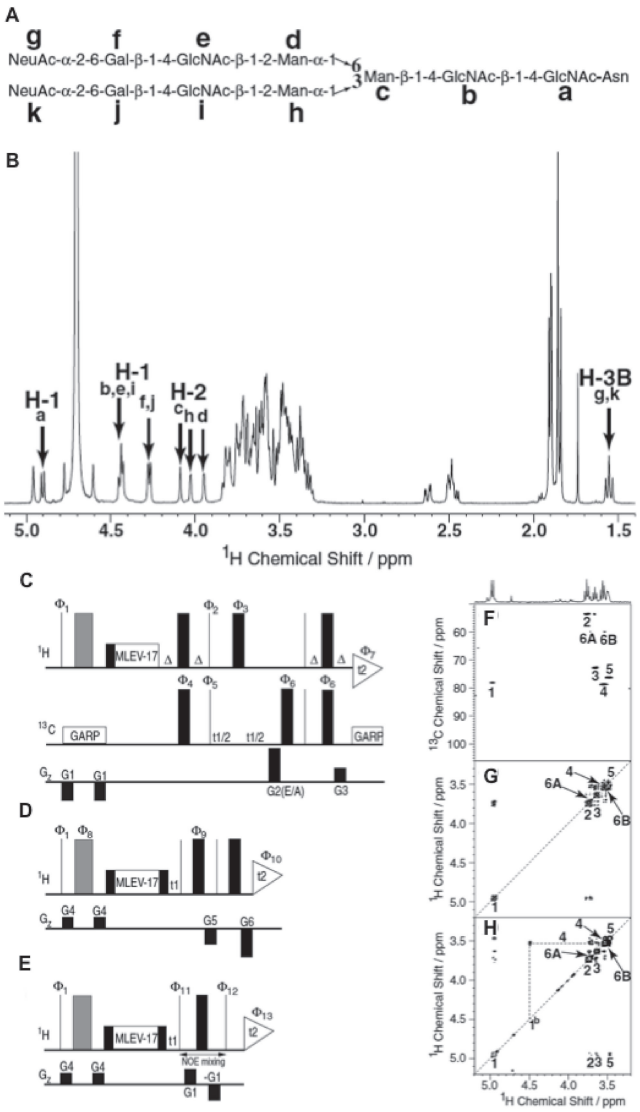


Figure 5. Structural analysis of sialyloligosaccharide by newly developed NMR analytical methods. A) A structure of oligosaccharide. B) ^{1}H NMR spectrum of oligosaccharide. C) Pulse sequence of selective TOCSY-HSQC. D) Pulse sequence of selective TOCSY-NOESY. E) Pulse sequence of selective TOCSY-DQFCOSY. F) 2D spectrum of selective TOCSY-HSQC. G) 2D spectrum selective TOCSY-DQFCOSY. H) 2D spectrum of selective TOCSY-NOESY.

monosialyloligosaccharide)-OH bearing a sialic acid at either end of the two branch-terminus (Figure 4). After the isolation of monosialyloligosaccharide **20**, sequential glycosidase digestion was performed with galactosidase, glucosaminidase, and mannosidase to give the corresponding oligosaccharides **20**–**25**. Varying the sequence of the glycosidase digestions yielded 24 kinds of homogeneous oligosaccharides.[20] In addition to these experiments, this semisynthetic approach also yielded oligosaccharides bearing α-2,3-sialoside using α-2,3-sialyltransferase. Using several different glycosidases and α-2,3-sialyltransferase enabled us to obtain an additional 11 oligosaccharide repertoire.[26]

Synthesis of Glycopeptide and Their Thioester Form

Because a suitable amount of N-linked oligosaccharides is obtainable from egg yolk, our research group then examined the synthesis of glycopeptides as well as the glycopeptide-α-thioesters **15** (Figure 3A), which is essential for NCL peptide coupling reactions. Sialic acid is an essential sugar required for glycoprotein activity,[27] but chemical synthesis of sialylglycopeptide was difficult due to the lability of the sialyl linkages under acid treatment. This chemical characteristic has been understood to be due to 3-deoxy sugar and therefore utilizing sialyloligosaccharide was recognized to be difficult in solid phase peptide synthesis (SPPS) that needs an acid treatment, such as trifluoroacetic acid (TFA), in the final step. Fmoc-SPPS is a typical protocol for the synthesis of glycopeptides, but it is not efficient for the synthesis of sialylglycopeptide-α-thioesters (e.g. **15**:Figure 3A). An efficient protocol for the synthesis of peptide-α-thioesters is to use the thioester linkage between the solid support and the C-terminal amino acid.[11] The piperidine that is used to remove Fmoc groups in each amino acid coupling during SPPS easily cleaves the thioester linkage, however. Under these circumstances, efficient thioesterification protocols have been developed utilizing functional linkers that can convert the ester linkage between solid support and the C-terminal amino acid into a thioester after the Fmoc-SPPS protocol.[11,28,29]

t-Boc-SPPS is known to be a suitable protocol for the synthesis of peptide-α-thioester **11** (Figure 3A),[30] but needs a strong acidic treatment that cleaves acid labile glycosides, especially sialoside. In both Fmoc- and Boc-SPPS, the synthesis of sialyloligosaccharypeptide-α-thioester **15** is known to be a difficult task. Our research group undertook studies on how to improve the lability of sialyl linkage.

Capon reported the very interesting experimental result that the k_{cat} of the acid hydrolysis of 2-carboxy-phenyl-β-D-glucoside is 1000 times faster than that of simple phenyl-β-D-glucoside, indicating the *ortho*-carboxylic acid seems to act as an intramolecular acidic catalyst.[31] Looking at the structure of sialoside reveals the same situation, i.e. that the carboxylic acid of sialic acid **27** can interact with sialoside oxygen in a five-membered cyclic form (Figure 6A), and this finding enabled us to re-examine the reason why sialoside is labile under acidic conditions. In order to investigate this hypothesis, we synthesized Fmoc-Asn-(sialyloligosaccharide)-OH **28** in which the carboxylic acid of sialic acid was protected with a phenacyl group that exhibits considerable stability under strong acidic conditions, but is easily removed by nucleophilic cleavage. A hydrolysis reaction using 40 mM HCl indeed hydrolyzed the non-protected sialoside **19** (Figure 4), but did not affect the sialoside **28** protected with the phenacyl group. These results indicate that the carboxylic acid of sialoside acts as an intramolecular acid catalyst to accelerate acid hydrolysis,[32] rather than the electron donation by the 3-deoxyl structure.

The finding that sialoside acts as a self-intramolecular acidic catalyst encouraged us to examine *t*-Boc-SPPS for the synthesis of sialylglycopeptide-α-thioester **31** (Figure 6). However, our research group did not employ an HF deprotection protocol requiring a special instrument. Our research group employed the in situ neutralization conditions established by the Kent and Alewood groups[30,33] while examining deprotection with tri-

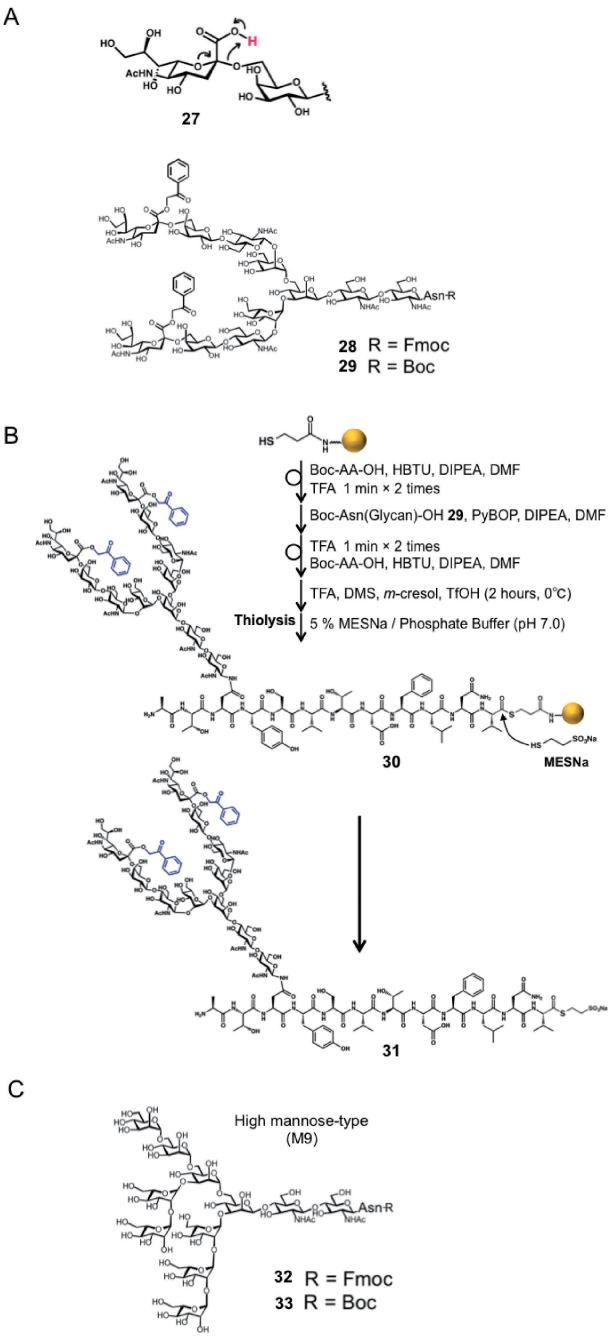


Figure 6. Boc-solid phase synthesis (Boc-SPPS) of acid labile sialylglycopeptide-α-thioester. A) A proposed mechanism of intramolecular acid catalyst of sialoside. Phenacyl esterified oligosaccharide **28** showed stability in 40 mM HCl and **29** was used for Boc-SPPS. B) Conventional in situ neutralization Boc-SPPS and thiolysis condition yield phenacyl esterified sialyglycopeptide-α-thioester of which phenacyl group interferes the acceleration of intramolecular acid hydrolysis of sialoside. C) High-mannose-type **33** was also used for Boc-SPPS.

fluoromethane sulfonic acid (TfOH) in TFA.[32] The linker was selected in order to use a simple mercaptopropionic acid in which the carboxylic acid and thiol were designed so as to link with amino-resins and to form a thioester with the C-terminal amino acid, respectively (Figure 6B). Therefore, since the peptide linked with the resin through a thioester linker was not cleaved by TfOH/TFA, we can examine the deprotection of the side-chain protecting groups repeatedly until all of the protecting groups are removed. This condition is easily performed, because TfOH/TFA are removed to wash out the resin-bearing peptide through filtration. After this deprotection, thiolysis toward the glycopeptide **30** on the resin yields the target peptide-α-thioester as well as sialylglycopeptide-α-thioester **31**.[32] As expected, the protection of the carboxylic acid of sialic acid enabled sialoside to be resistant toward a strongly acidic condition (Figure 6B).

In addition to the synthesis of the sialylglycopeptide-α-thioester, other glycopeptides, such as glycopeptide-α-thioester bearing M9-high-mannose-type oligosaccharide or asialooligosaccharide, were also prepared by using the Boc-Asn-(high-mannose-type oligosaccharide)-OH **33**.[20] However moisture in the deprotection steps seems to accelerate hydrolysis of the glycosyl bond, so a drying procedure before acid hydrolysis appears to be critical.

Chemical Synthesis of Glycoproteins

Utilizing the three major methods: 1) semisynthesis of diverse oligosaccharides, 2) synthesis of glycopeptide-α-thioester,[28] and 3) NCL, our research group in collaboration with the Dawson group examined the first chemical synthesis of a glycoprotein, the chemokine MCP-3 **38**, bearing an intact complex-type biantennary disialyloligosaccharide.[34] The strategy employed three-segment coupling by NCL at the 11 and 36 cysteine sites. In terms of oligosaccharide, biantennary sialyloligosaccharide **39** or **40** was used and the sialylglycopeptide-α-thioester **37** was prepared by both the Fmoc and Boc conditions. For the Boc condition, none of the amino acids in glycopeptide **37** needed any side chain protection due to the alkyl type amino acids in the peptide backbone. Therefore, a final acidic deprotection step was not required and thiolysis yielded the sialylglycopeptide-α-thioester **37**. Protection of the carboxylic acid of sialoside used a benzyl group[35] for both compound **39** and **40**. Segment-2 **34** and segment-3 **35** were prepared as peptide-α-thioesters using a conventional Boc condition (Figure 7).

As shown in Figure 7, the two peptides **34** and **35** as well as the glycopeptide **37** were sequentially coupled by NCL reactions. Subsequent in vitro folding experiments employing 6 M guanidine–HCl and cysteine–cystine under dialysis successfully yielded MCP-3 **38** in the folded form. All the synthetic intermediates and final product were analyzed by electrospray ionization (ESI) mass spectrometry, and peptidase digestion and subsequent mass/mass analysis supported the formation of the two disulfide bonds at suitable positions. Enzyme-linked immuno sorbent assay (ELISA) using anti-MCP-3 antibody-sandwich conditions exhibited a positive response. As shown here, our research group demonstrated the first synthesis of a glycoprotein bearing the homogeneous biantennary complex-type sialyloligosaccharide **38**.[34]

Using our chemical strategy of glycoprotein synthesis, our research group also synthesized other unique glycoproteins with a variety of oligosaccharide structures, glycosylation positions, and disulfide bond positions. In the following sections,

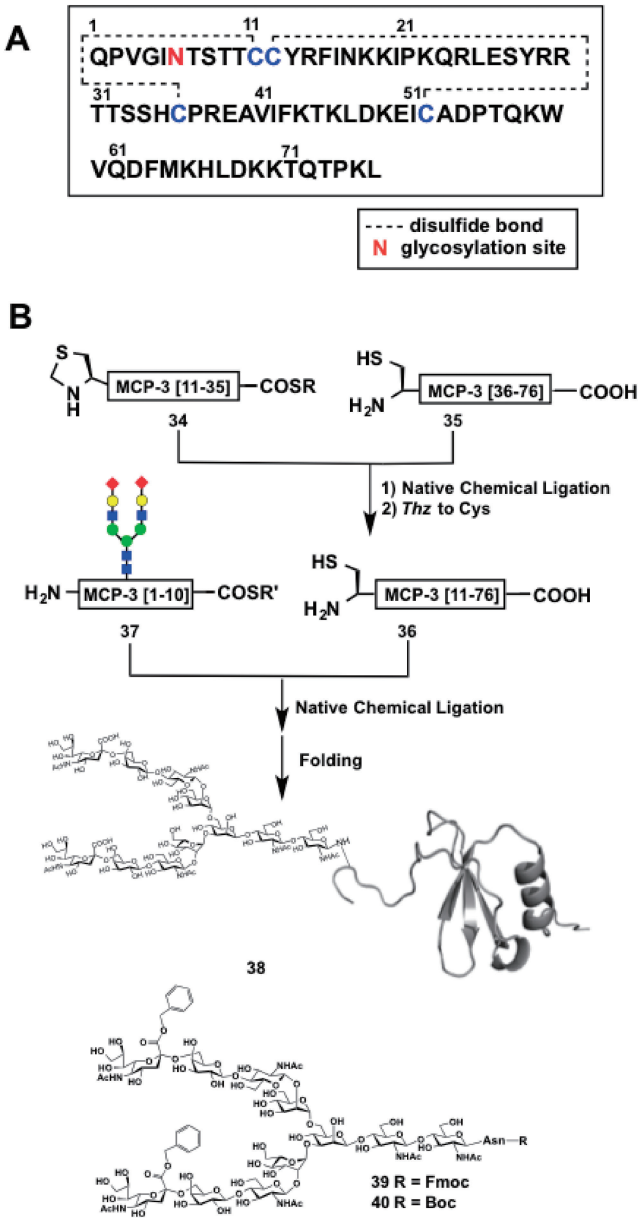


Figure 7. Chemical synthesis of glycoprotein having an intact human sialyloligosaccharide. A) Amino acid sequence of monocite chemotactic protein-3. B) Three-segment coupling strategy by NCL and folding protocol to yield a folded glycoprotein **38**. Oligosaccharide **39** and **40** were used for Fmoc-SPPS and Boc-SPPS, respectively.

this account describes the chemical synthesis of intentionally misfolded glycoproteins, including cytokines such as erythropoietin and interferon-β.

As mentioned in the introduction, the glycoprotein quality control system in the ER regulates the accumulation of native glycoproteins forming correct secondary and tertiary protein structures. UGGT is a key folding sensor enzyme that senses the native glycoprotein form and misfolded form (Figure 2). It is known that UGGT binds high-mannose-type oligosaccharide and then recognizes protein surfaces. If UGGT identifies a misfolded protein surface, UGGT catalyzes the transfer of a glucose residue to the terminus of the high-mannose oligosaccharide in order to make a tag of misfolded glucosyl-glycoprotein **7**. During this molecular recognition process to find misfolded glycoprotein **6**, UGGT seems to recognize hydrophobic protein surfaces; these consist of hydrophobic amino acids that were originally located on the inside of protein molecule and interact with each other through hydrophobic interaction to sustain protein molecules. However, it is not well understood why this unique molecular recognition event is sustained by UGGT. In order to understand this molecular recognition event, our research group and the Ito group synthesized both the intentionally misfolded **6** and correctly folded **5** glycoproteins in a homogeneous form and then investigated how UGGT recognizes both of these glycoproteins.

Our first synthesis undertaken to understand the ability of the folding sensor enzyme UGGT was interleukin 8 (IL8), which is originally a non-glycosylated protein, because IL8 consists of 72 amino acid residues, including two disulfide bonds (Figure 8B).[36,37] The structure of IL8 has been well studied by NMR and X-ray analytical methods indicating that IL8 has both an α-helix and β-sheet, and chemical synthesis using NCL was already examined.[12]

The concept for obtaining both native and misfolded homogeneous glycoproteins bearing high-mannose type oligosaccharide consisting of nine mannose residues (M9) was to shuffle the disulfide bond networks during the in vitro folding process. In terms of intentional glycosylation, the Asn 36 position was selected, because this region has no typical secondary structure and our research group believed that intentional glycosylation at this position would not have a negative effect on the original native IL8 structure (Figure 8B). Our research group employed a two peptide coupling strategy at the Cys 36 position by NCL and the subsequent kinetic in vitro folding process without any cysteine–cystine condition in order to synthesize both misfolded and native glycosylated forms of IL8. Segment 1 (1–33 position), the peptide-α-thioester, and segment 2 (34–72 position), a glycopeptide bearing a high-mannose-type oligosaccharide at the 36 position, were successfully synthesized by Boc-SPPS and Fmoc-SPPS, respectively, and subsequent NCL yielded the target glycosylpolypeptide **42** (Figure 8A).[38] All of the synthetic intermediate and the target glycosylpolypeptide **42** were analyzed by ESI-mass spectrometry.

For the folding experiments of glycosylpolypeptide, our research group employed Anfinzen's protocol using 1.0 M guanidine–HCl and cysteine–cystine redox conditions to obtain native IL8 under thermodynamically controlled folding processes (data not shown),[38] while a kinetic folding process (Figure 8C) in the absence of the redox condition yielded the misfolded IL8 **43**, **44**, and **45** due to the shuffling of the disulfide bond formation along with the correctly folded IL8 **41**.[38] All of the products were isolated and analyzed by mass spectrometry. In order to determine the disulfide bond positions, peptidase digestion with the Lys-C enzyme was performed and the resultant peptide fragments, including the disulfide bonds, were analyzed by mass/mass analysis. The circular dichroism (CD) spectra of individual IL8s were also examined to characterize the protein secondary structure (Figure 8E). Using these data, our research group determined that **41** is a native form, **43** and **44** are misfolded monomers due to disulfide bond shuffling, and

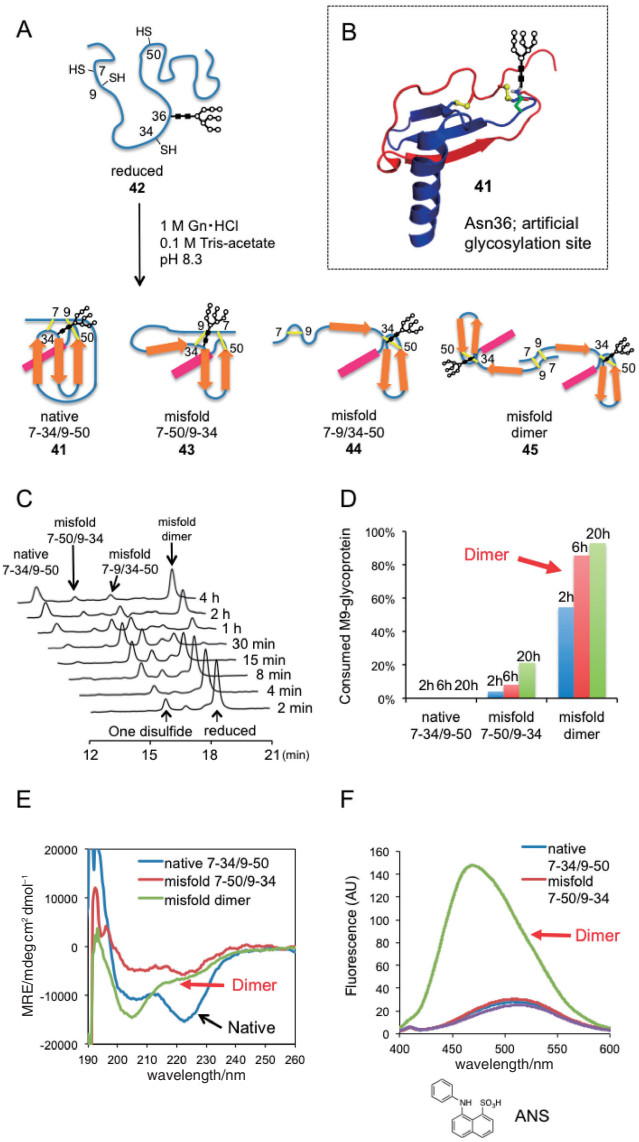


Figure 8. Chemical synthesis of both correctly folded and misfolded glycoprotein. A) Folding experiment without redox conditions to yield four kinds of glycoprotein due to the scrambling of the disulfide bond network. B) A structure of glycosylated IL8. Protein structure is based on PDB 3IL8. C) Folding process without redox condition. D) Glucosyltransfer rate with UGGT toward native **41**, misfolded **43**, and dimer **45**. E) Circular dichroism of native **41** (blue), misfolded **43** (red), and dimer **45** (green). F) ANS binding assay.

45 is a dimer form of **44** through intermolecular two disulfide bonds between Cys 7 and 9.

Both native and misfolded glycosylated forms of IL8 were obtained, UGGT assays were performed in order to determine whether UGGT discriminates the glycoprotein tertiary structure and then catalyzes glucosyl transfer to only the misfolded glycoproteins in order to tag the misfolded protein. Human-type UGGT was prepared by a mammalian cell expression system, and the molecular weight is 150 KDa. The three dimensional structure has not been solved yet. UGGT assay with UDP-Glucose toward individual glycosyl IL8 indeed showed that UGGT transferred a glucose residue toward only the misfolded forms of glucosylated (Figure 8D). Increasing the amount of UGGT in the assay mixture did not result in the transfer of a glucose residue toward the native glycosyl IL8 **41**. These results clearly indicated UGGT can discriminate between the structure of the native glycoprotein **41** and the misfolded glycoproteins **43**, **44**, and the dimer **45**.[38]

Figure 8 provides analytical data on the synthetic glycosyl IL8s and the results of the UGGT assay. According to those CD data, the misfolded IL8 lost the β-sheet structure consisting of the hydrophobic amino acids (data not shown). It is known that 8-anilino-1-naphthalenesulfonic acid (ANS) binds the hydrophobic protein surface and this may be evaluated with fluorescent spectroscopy (Figure 8F). Our research group examined the ANS assay to determine whether the synthesized glycoproteins exhibited a hydrophobic protein surface. Indeed the dimeric glycosyl IL8 **45** displayed considerable glucosyl transfer and potent fluorescence in the ANS assay, indicating the formation of hydrophobic patches on the surface of the dimer that had lost the β-sheet form. Therefore, our research group concluded that UGGT recognizes these hydrophobic surfaces formed on the misfolded glycoprotein surface.[38]

Our chemical approach confirmed that UGGT indeed recognizes the characteristic nature of misfolded glycoproteins, and this finding enabled us to consider how UGGT recognizes the glycoprotein folding intermediates in which hydrophobic amino acids may be exposed on the glycoprotein. There are many folding intermediates, such as the random coil structure **46**, native form folded **48**, misfolded form **50**, and heavily aggregated **51** in the course of the folding process (Figure 9A), and some of these folding intermediates are in equilibrium with each other. If UGGT binds with the folding intermediate **47** that would be certainly going to become the correct native form (Figure 9A; **46** → **47** → **48**), the folding velocity of correctly folded glycoproteins accumulating in the ER may slow down, because UGGT disturbs the folding process. On the other hand, if UGGT binds the intermediate **49** that is falling into the misfolded **50**, the yield of the folding process may improve due to the opportunity for a refolding of the misfolded glycoprotein by the UGGT-chaperone refolding process (for example, **49** → **48**).

In order to study this subject, our research group designed productive and unproductive folding processes in which both of the intermediates are exclusively converted into only the native glycoprotein and only the misfolded glycoprotein, respectively. For this, crambin was selected as a model.[39] Crambin is a small protein consisting of 42 amino acid residues and three disulfide bonds. Its random coil polypeptide smoothly yields a native folded form as a sole product in in vitro folding experiments without any redox reagent or guanidine–HCl. This crambin is not a glycoprotein, but intentional glycosylation did not change its protein conformation that had already been confirmed by ^{1}H TOCSY.[40] For the making of the unproductive folding process, two cysteines were removed (Figure 9C) in order to interfere with the formation of the three native disulfide bonds so this mutant cannot assume its native form (Figure 9A: **52** → **49** → **50**; Figure 9B: **52** → **53**, **54**, and **55**). Based on this concept, the glycosylated crambin **46** (Figure 9B) and its mutant **52** (Figure 9C) were considered to give suitable folding intermediates of the productive and unproductive fold-

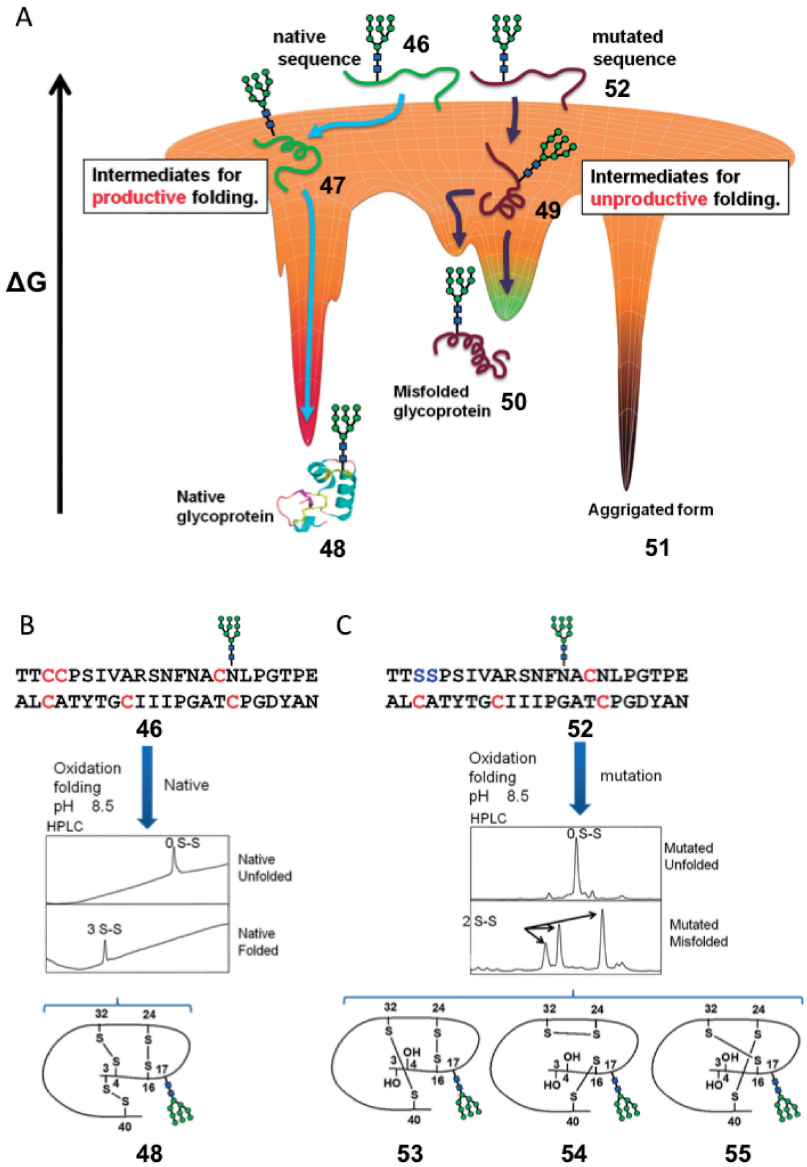


Figure 9. Dynamic folding processes. A) Productive folding and unproductive folding process. A native sequence **46** yields a correctly folded glycoprotein and mutated sequence of **52** yields misfolded glycoprotein **50**. The folding processes are called productive folding and unproductive folding, respectively. B) Native sequence of model glycoprotein, crambin and their folding process monitored by HPLC. C) A mutated sequence of model glycosylated crambin and their folding process monitored by HPLC. This mutant yielded three kinds of misfolded glycosylated crambin **53**, **54**, and **55**.

ing process, respectively. A glycosylated crambin polypeptide bearing the M9-high-mannose-type oligosaccharide **46** and its mutant **52** with the two cysteines at the 3 and 4 positions removed were synthesized by a two-segment coupling strategy under NCL (data not shown).[39]

The UGGT assay for the folding intermediates was designed to use a mixture of two kinds of glycosylpolypeptides that pursue the corresponding productive folding and unproductive folding, because the folding events in the ER include both of these folding processes, and they are inspected by UGGT. This artificial folding process was monitored by reverse phase HPLC/mass spectrometry and this experiment clearly showed all of the glycoprotein folding intermediates were glycosylated. This result indicated that UGGT recognizes even the intermediates in productive folding. According to these results, our research group concluded that even productive folding process intermediates accumulated in the rate-determining steps are recognized as misfolded glycoproteins by UGGT.[39] The research is still in progress in an effort to reveal the function of high-mannose-type oligosaccharides in glycoprotein quality control.

After glycoproteins are synthesized in the ER, they are subsequently transported into the Golgi apparatus that contains various mannosidases and the membrane glycosyltransferases that are responsible for converting high-mannose-type oligosaccharides into acidic complex-type sialyloligosaccharides.[1–3] This conversion is performed for almost all secreted glycoproteins, including cytokines, hormones, and antibodies, but cell membrane-bound glycoproteins seem to have complex as well as the high-mannose type, which are not modified in the

Golgi. Complex-type oligosaccharides have a wide diversity of types, such as biantennary, triantennary, and tetraantennary structures, and almost all of the termini are modified with a sialic acid through either an α-2,3- or 2,6-sialyl linkage. Sialic acid exhibits an acidic nature on the protein surface and is an essential sugar for the bioactivity of glycoprotein hormones as well as cytokines. Galactose residues positioned at the non-reducing terminus of oligosaccharide is known to be trapped by the liver cells through galactose binding lectin, so sialic acid may mask galactose residues and thus interfere with galactose binding lectin. This system contributes to regulate the lifetime of glycoproteins in the blood.

Erythropoietin (EPO) is a well studied glycoprotein hormone bearing three N-linked complex-type sialyloligosaccharides at the 24, 38, and 83 positions and an O-linked oligosaccharide at the 126 serine.[41] EPO activates the production of red blood cells and therefore has been utilized as a biologic for the treatment of both acute and chronic anemia. However, it is still unclear why EPO needs so many oligosaccharides compared with other cytokines such as the interleukins,[2,41,42] because almost all cytokines and EPO have the same helical bundle structure.

EPO also exhibits considerable heterogeneity in the structure of complex-type sialyloligosaccharides and this has been a hindrance in elucidating the oligosaccharide structure that enhances EPO bioactivity.[43] Moreover, there is no method available for obtaining homogeneous EPO in which the oligosaccharides can be sufficiently modified.

In order to obtain homogeneous EPO and to investigate the function of oligosaccharides, our research group has examined both semi-chemical (Figure 10A) and chemical synthesis (Figure 11) of EPO derivatives bearing homogeneous complex-type biantennary sialyloliogsaccharides.[44]

EPO consists of 166 amino acids and was divided into two segments that were a glycopeptide-(1–32)-α-thioester prepared by chemical synthesis and a C-terminal long peptide-(33–166) having a cysteine at the N-terminus prepared by *E. Coli* expression (Figure 10A). In order to vary the glycosylation position easily, a haloacetamide coupling method[45] (Figure 10B) was employed for artificial glycosylation rather than the preparation of the native amide linkage between the reducing terminal of the oligosaccharide and the asparagine side chain, because the cysteine positions are easily varied during SPPS, so this strategy is useful for investigating the oligosaccharide function that is dependent on the position of the polypeptide. During synthesis, the cysteine residues that form disulfide bonds were protected with an acetamide methyl (Acm) group. In terms of *E. coli* expression, the N-terminus **57** was modified as the His (×10)-Met-Cys-peptide in which the His-Met was designed to be removed by CNBr treatment to obtain a target peptide bearing a cysteine at the N-terminus. The coupling of these two peptides **56** and **57** by NCL and subsequent deprotection of the Acm group of the cysteine followed by folding yielded the suitable EPO derivative **58**. Using this strategy, our research group synthesized the two types of EPO derivatives **58** and **59**[46] bearing two and three sialyloligosaccharides, respectively (Figure 10).

The first target of a total chemical synthesis was EPO bearing a biantennary disialyloligosaccharide at the 83 position

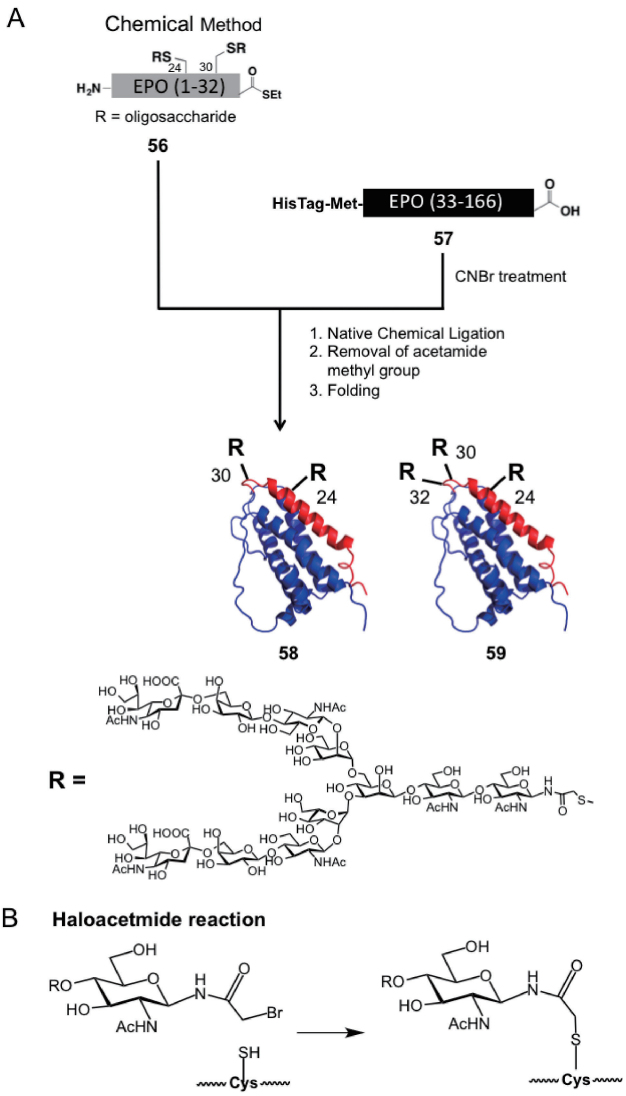


Figure 10. Semisynthesis of erythropoietin analogues. A) Glycopeptide analogue **56** was prepared by chemical synthesis based on solid phase peptide synthesis and subsequent glycosylation with a haloacetamide method (Figure 10B) between cysteine of peptide and oligosaccharyl-*N*-bromoacetamide. The oligosaccharyl-*N*-bromoacetamide was prepared by chemical modification toward oligosaccharyl peptide **17** (Figure 4) isolated from natural source. HisTag-Met-EPO (33–166) segment **57** was prepared by *E. Coli* expression method and subsequent CNBr treatment yielded EPO (33–166) segment having cysteine at the N-terminal. NCL between **56** and **57** and subsequent folding protocol yielded EPO derivative **58**. In terms of EPO having three glycans, peptide (1–32) segment having three glycans was prepared and then used it for the same synthetic protocol.

(Figure 11).[47] Our chemical synthetic strategy employed 6 segment-coupling by NCL (Figure 11A). However, EPO does not have an adequate number of cysteines for the repetitive NCL reactions required to have a whole polypeptide. This difficulty was met by employing NCL at the alanine sites with a cysteine followed by the desulfurization of the cysteine so as to convert the cysteine used for NCL into alanine (Figure 3A:

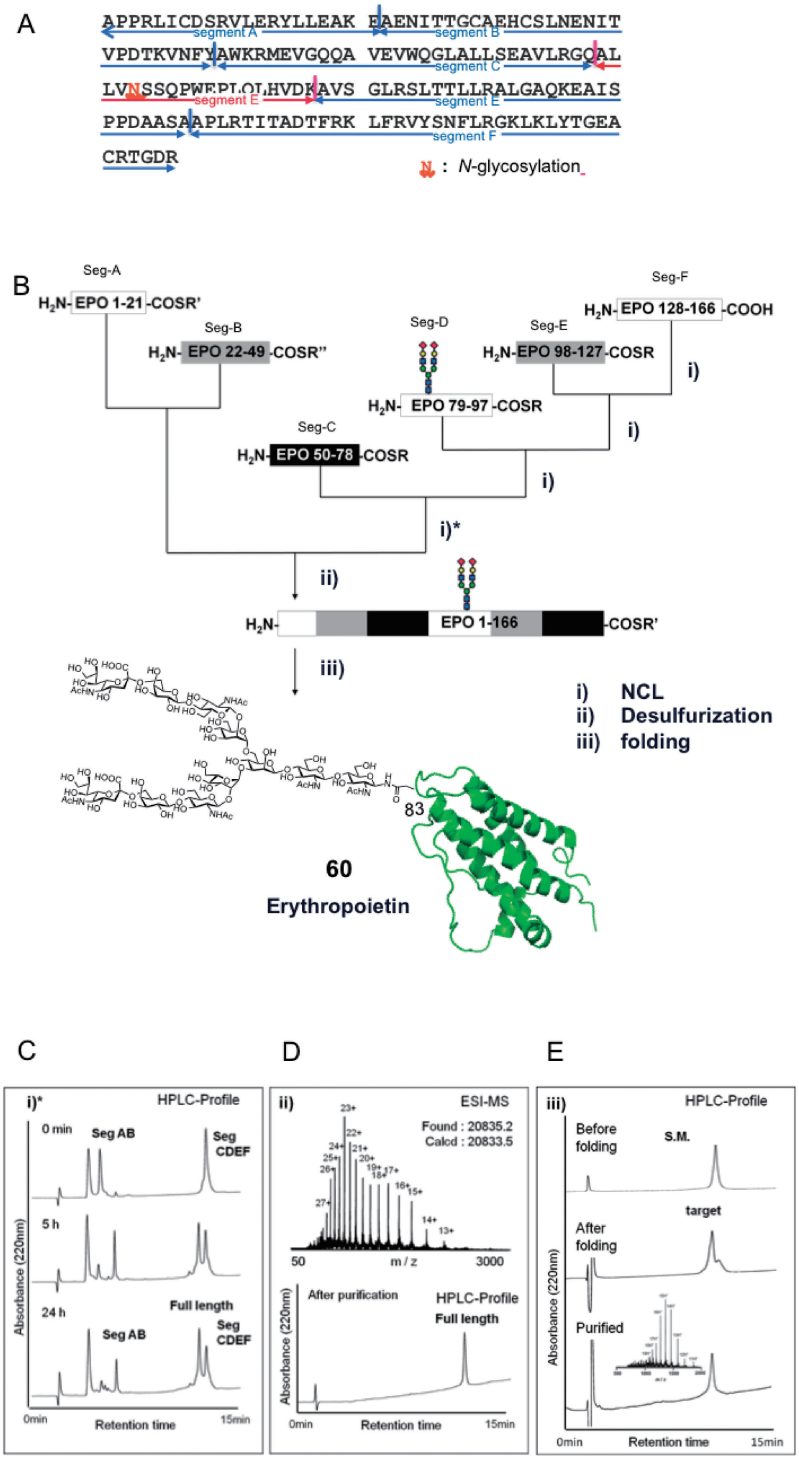


Figure 11. Chemical synthesis of erythropoietin (EPO) bearing a sialyloligosaccharide at the 83 position. A) Amino acid sequence of EPO. B) Synthetic strategy of EPO by NCL. C–E) HPLC profiles and ESI mass spectrometry corresponding conversion steps i), ii), and iii) shown in B).

13 → **14**). All of the peptide-α-thioesters and sialylglycopeptide-α-thioesters in which the glycosylation position was at the 83 position were prepared by a *t*-Boc method (Figure 11B). The N-terminal hydrophobic peptide-α-thioesters could be prepared by an Fmoc method. It is well known that sialic acid is labile and therefore SPPS employing a strong acid treatment is an unsuitable method for the synthesis of an acid labile glycopeptide. In this experiment, the improved *t*-Boc condition using a phenacyl-protected sialyl oligosaccharide yielded a sialylglycopeptide-α-thioester (segment D). This procedure is described in Figure 6. The four cysteine residues that form disulfide bonds were protected with Acm group.

Sequential NCLs were performed under the conventional conditions, and subsequent desulfurization and deprotection of

the Acm group with AgOAc were performed to obtain an EPO-glycosylpolypeptide (Figure 11B). In terms of the phenacyl ester, deprotection with piperidine was performed after NCL between the segment-D and segment-FG. A folding experiment was also performed with the same Gn–HCl and cysteine–cystine redox condition. The folding experiments successfully yielded a correctly folded glycosyl-EPO along with a tiny amount of misfolded glycosyl-EPO. After purification, the correctly folded glycosyl-EPO **60** was characterized by ESI-mass spectrometry (Figure 11E), CD spectrometry and disulfide bond mapping.[47]

Because the homogeneous EPO derivatives were obtained by chemical synthesis and semisynthesis, bioassays for cell proliferation were examined using both in vitro and in vivo conditions (Figure 12). In the in vitro experiments, the synthesized EPO exhibited suitable cell proliferation activity, but very weak or no activity was observed in in vivo experiments.[44,46] Even EPO analogues bearing three biantennary sialyloligosaccharides did not exhibit suitable bioactivity in vivo.[46] In general, it has been suggested that glycoproteins need tri- or tetraantennary sialyloligosaccharides in order to exert potent bioactivity of glycoprotein in vivo, because multiantennary oligosaccharide enables to extend the half-life of glycoprotein in vivo.[41] Our EPO derivatives are indeed biantennary sialyloligosaccharides and our findings indicated a weak capacity of biantennary sialyloligosaccharides for glycoprotein activity.

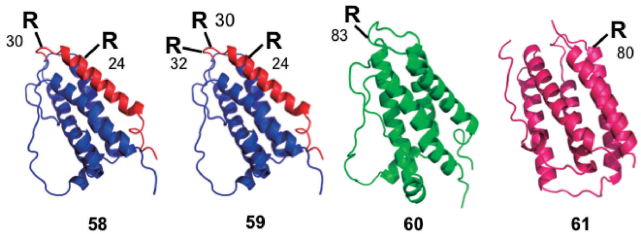
In order to study the ability of biantennary sialyloligosaccharides, our research group examined another glycoprotein synthesis of interferon-β (IFN-β) bearing a biantennary sialyloligosaccharide.[48]

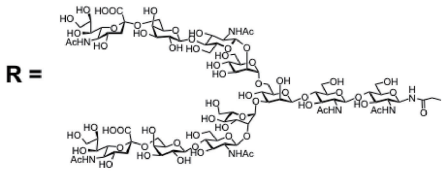
Native IFN-β also consists of 166 amino acids and has a biantennary sialyloligosaccharide at the 80 position along with a tiny amount of triantennary sialyloligosaccharide. The major oligosaccharide is a biantennary sialyloligosaccharide and this population comprises over 90%.[42,49] For this synthesis, our research group employed a three segment coupling strategy and the peptide-α-thioester and glycopeptide-α-thioester were prepared by an Fmoc strategy.[35] Sequential NCL and a subsequent folding experiment were performed according to our procedure for the preparation of EPO (Figure 11). Purification by a reverse phase HPLC yielded native IFN-β **61** in good yield (Figure 12).[48]

Bioassay of IFN-β was performed both the in vitro and in vivo experiments (Figure 12). As expected, IFN-β bearing a homogeneous biantennary sialyloligosaccharide exhibited potent anticancer activity in in vivo experiments. In particular, this IFN-β bearing a biantennary sialyloligosaccharide exhibited more potent anticancer activity in vivo compared with asialooligosaccharide (the oligosaccharide terminal is galactose, data not shown here).[48] It is known that a glycoprotein bearing asialooligosaccharide is easily trapped in the liver through galactose binding lectin. This might be the reason that IFN-β bearing a biantennary asialooligosaccharide displayed a low level of anticancer activity in vivo.

According to in vivo assay as an anticancer drug, IFN-β indeed exhibited activity, indicating that tri- or tetraantennary sialyloligosaccharides are not critical for the bioactivity of all glycoproteins in vivo. Indeed, only EPO is a heavily glycosylated cytokine. However, our synthetic EPO does not have oligosaccharides at the native 24, 38, and 83 positions. This may be the reason why these synthetic EPOs did not exhibit adequate bioactivity in vivo. Very recently our research group probed this by chemical synthesis of homogeneous EPO bearing three biantennary silayloligosaccharides at the three native positions, with potent bioactivity in vivo.[50] Some non-glycosylated cytokines and O-glycosylated cytokines are also known to exhibit suitable bioactivity. According to the biological data reported to date and our data from the chemical point of view (Figure 12), the glycosylation position and number are critical for biological activity in vivo, and this glycosylation must apparently be regulated in order for a cytokine to exhibit bioactivity in vivo during the evolution of glycoprotein biosynthesis.

In conclusion, glycoproteins have previously been prepared by a cell expression system as a sole method, but our research





Synthetic compounds	erythropoietin			INF-β
	tetraantenary x 3 Asn 24, Asn 30, Asn 32			biantenary x 1 Asn 80
	59	**58**	**60**	**61**
Branch style	biantenary	biantenary	biantenary	biantenary
Glycan number	3	2	1	1
Position	**24**, 30, 32	**24**, 30	**83**	**80**
in vitro	o	o	o	o
in vivo	×	×	×	o

Figure 12. Bioactivity of synthetic glycoproteins. Erythropoietin having an oligosaccharide **60**, two oligosaccharides **58**, three oligosaccharides **59**, and interferon-β (INF-β) having an oligosaccharide **61** are shown in the table. In terms of EPO derivatives, cell proliferation activity (in vitro) and hematocrit (in vivo) were evaluated. In terms of INF-β, inhibition of cell proliferation (in vitro) and anticancer activity (in vivo) were evaluated. Although all glycoproteins show bioactivity in vitro, EPO having oligosaccharide at the unnatural position did not show activity in vivo. Only INF-β **61** having an oligosaccharide at the natural position showed potent bioactivity in vivo. EPO derivatives **58** and **59** having oligosaccharides was synthesized by haloacetamide method. EPO **60** and INF-β **61** having an oligosaccharide at the 83 position and 80 position, respectively, through natural N-glycosyl linkage.

group has demonstrated the successful preparation of homogeneous biantennary sialyloligosaccharide and high-mannose-type oligosaccharide at over a gram scale, then utilized these oligosaccharides for chemical and semi-chemical synthesis of homogeneous glycoproteins.

Recently, the enzymatic synthesis of glycoproteins has also been demonstrated by means of an oxazoline oligosaccharide donor.[51] This method also improved the synthesis of homogeneous glycoproteins.[52]

These synthetic methods complement the biological preparation method and open an avenue to synthesizing homogeneous glycoproteins, such as a total synthesis of natural products of small or middle molecular size. Our synthesis method was applied in several biological experiments and demonstrated how a glycoprotein folding sensor enzyme (UGGT) is able to discriminate between native and misfolded glycoproteins during glycoprotein quality control in the ER, and also how EPO and interferon-β exert bioactivity in vivo in a manner dependent on the glycosylation position, number, and oligosaccharide structure. Research is in progress to develop a more efficient preparation method of homogeneous glycoproteins in order to elucidate the relationship between oligosaccharide function and protein bioactivity.

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