Chemical Synthesis of Homogeneous Erythropoietin Analogs Bearing High-mannose Type Oligosaccharides for the Elucidation of Endoplasmic Reticulum Glycoprotein Quality Control System

（ハイマンノース型糖鎖を持つエリスロポエチンの精密化学合成とそれらを用いた小胞体糖タンパク質品質管理機構の解明研究）

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Contents

Abbreviation

Ac	acetyl
Acm	acetamidomethyl
Ala	alanine
ANS	1,8-anilinonaphthalene-sulfonate
Asn	asparagine
Arg	arginine
Asp	aspartic acid
BiP	immunoglobulin heavy chain binding protein
Boc	*t*-butoxycarbonyl
Boc_2O	di-tert-butyl dicarbonate
Bn	benzyl
t-Bu	*t*-butyl
Bzl	benzoyl
CD	circular dichroism
CNX	calnexin
CRT	calreticulin
Cys	cysteine
DCM	dichloromethane
DEPBT	3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one
DHFR	dihydrofolate reductase
DIC	*N,N'*-diisopropylcarbodiimide
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNJ	1-deoxynojirimycin
DNP	2,4-dinitrophenyl
DTT	D,L-dithiothreitol
EDEM	ER degradation enhancing α-mannosidase-like protein
EDT	1,2-ethanedithiol
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
ER	endoplasmic reticulum

ERAD	ER-associated degradation
ERGIC-53	ER-Golgi intermediate compartment-53
ESI	electrospray ionization
Fmoc	9-fluorenylmethyloxycarbonyl
Glc	glucose
GlcNAc	*N*-acetyl-D-glucosamine
Gln	glutamine
Glu	glutaminouic acid
Gly	glycine
Gn-HCl	guanidine hydrochloride
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
HMPB	4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid
HOBt	1-hydroxy-benzotriazole
HPLC	high performance liquid chromatography
INF-β	interferon-β
LC	liquid chromatography
Ile	isoleucine
IL-8	interleukin-8
Leu	leucine
Lys	lysine
NCL	native chemical ligation
Man	mannose
MESNa	sodium 2-mercaptoethanesulfonate
Met	methionine
MPAA	4-mercaptophenylacetic acid
MRH	mannose 6-phosphatereceptor homology
MS	mass spectrometry
MSNT	1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-trizole
MTX	methotrexate
NMR	nuclear magnetic resonance
ODS	octa decyl silyl
OS-9	osteosarcoma amplified 9
PAGE	poly-acrylamide gel electrophoresis

Pbf	2,2,4,6,7-pentamethyldihydrobezofuran-5-sulfonyl
PDI	protein disulfide isomerase
PDIR	protein disulfide isomerase-related protein
Phe	phenylalanine
PPI	peptidyl prolyl *cis-trans*-isomerase
Pro	proline
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RNAse B	ribonuclease B
RP	reverse phase
Rpn1	ribophorin I
SBA	soybean agglutinin
SPPS	solid phase peptide synthesis
Ser	serine
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TfOH	trifluoromethanesulfonic acid
Thr	threonine
Thz	thiazolizine
TIPS	triisopropylsilane
Tris	tris(hydroxymethyl)aminomethane
Trt	trityl
Typ	tryptophan
Tyr	tyrosine
UDP	uridine diphosphate
UGGT	UDP-glucose glycoprotein glucosyltransferase
UV	ultraviolet
VA-044	2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride
Val	valine
VIP36	vesicular integral protein of 36 kDa
VIPL	vesicular integral protein of 36 kDa-like
WST-8	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 tetrazolium monosodium salt
Xan	xanthyl
XTP3-B	XTP3-transactivated gene B protein

Abstract

The folding of glycoprotein is rigorously controlled by a quality control system in the endoplasmic reticulum (ER). A nascent proteins generated in the ER are transferred G3M9-high-mannose type oligosaccharide, which consists of three glucoses, nine mannoses and two *N*-acetyl glucosamine (Fig. 1, ①). Subsequent glucose trimming by glucosidase I and glucosidase II yields G1M9-high-mannose type oligosaccharyl- glycoprotein (Fig. 1, ②). Molecular chaperone, calnexin (CNX) and calreticulin (CRT) interact with this G1M9-oligosaccharyl-glycoprotein specifically and conduct a folding of the protein moiety (Fig. 1, ③). After the folding, glucosidase II trims the upper most glucose of glycoproteins and produces M9-oligosaccharyl- glycoproteins (Fig. 1, ④). Although correctly folded glycoproteins are transported to Golgi apparatus (Fig. 1, ⑤), misfolded glycoproteins are recognized specifically by folding sensor enzyme, UDP-glucose: glycoprotein glucosyltransferase (UGGT) and transferred a glucose as a tag of immature glycoprotein (Fig. 1, ⑥). This G1M9-oligosaccharyl-glycoprotein interacts with CNX/CRT again and folds into the correct structure (Fig. 1, ⑦). Hence, this quality control system prevents the accumulation of misfolded glycoproteins and keeps the efficiency of the production of glycoproteins. Although each function of these chaperones and enzymes have been studied to some extent, the sequence of interactions are still ambiguous, such as when and how these molecules interact with glycoprotein substrates.

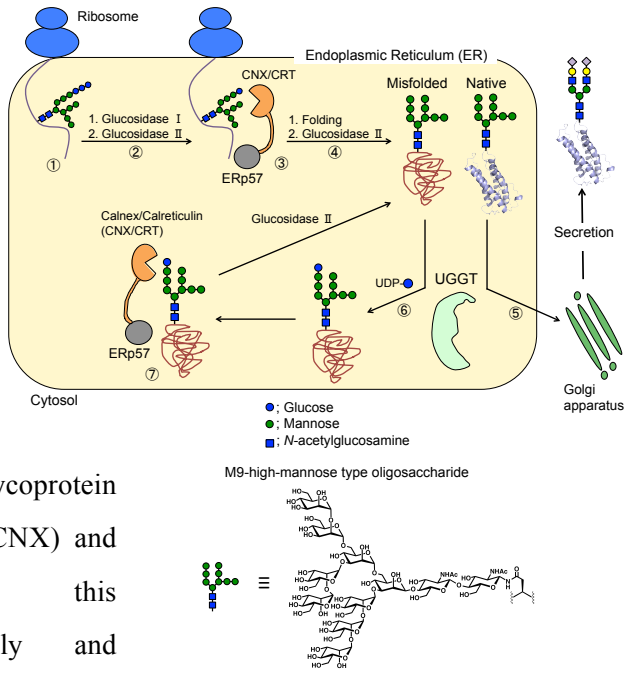

Figure 1. Glycoprotein quality control system

In my study, I synthesized erythropoietin (EPO) analogs that have M9-high-mannose type oligosaccharides as probes to investigate the interactions of these molecules. EPO is a hormone involved in the maturation of red blood cells and has three *N*-linked oligosaccharides. In order to investigate how the number and the position of oligosaccharide of glycoproteins affect the interactions, I synthesized eight kinds of EPO analogs bearing one or three M9-oligosaccharides (Fig. 2). I divided the whole sequence, which consist of 166 amino acid residues, into six peptide and glycopeptide segments. The key glycopeptide thioester segments were prepared by Boc solid phase peptide synthesis (SPPS) using M9-oligosaccharide isolated from egg yolk. The other peptide thioester and

peptide segments are prepared by Boc or Fmoc SPPS. The repetitive native chemical ligations of these segments yielded the four kinds of glycosylated polypeptides of EPO. The folding of each glycosylated polypeptide under redox conditions yielded four kinds of correctly folded EPO analogs along with four kinds of misfolded ones successfully.

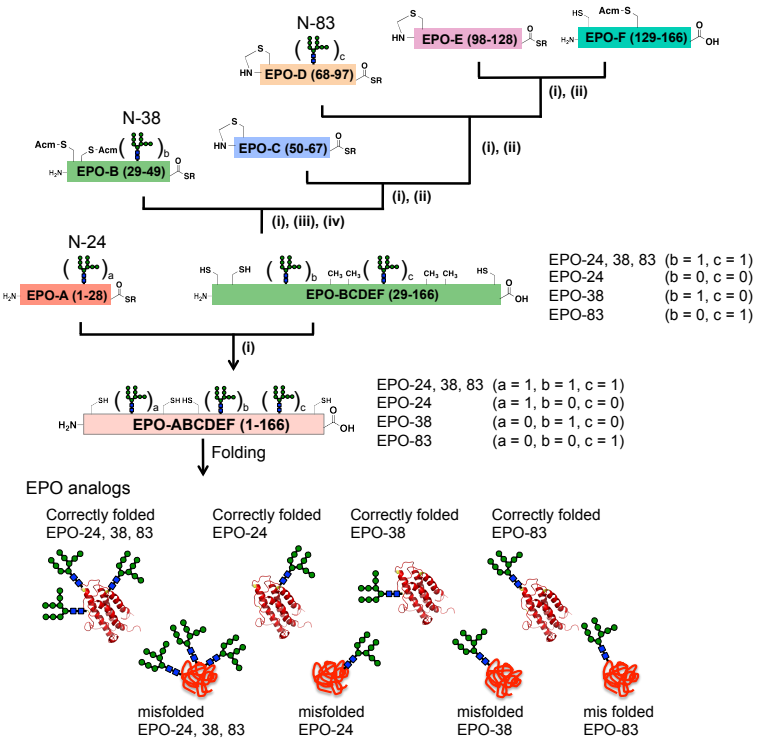

Figure 2. Synthetic strategy of EPO analogs. (i) Native chemical ligation; (ii) Removal of thiazolidine; (iii) Dsulfurization; (iv) Removal of the Acm groups

Next, I conducted the assay with recombinant UGGT and synthetic eight kinds of EPO analogs (Fig. 3(a)). As a result, UGGT transferred a glucose to not only misfolded EPO analogs but also correctly folded EPO analogs unexpectedly. In terms of EPO analogs bearing an oligosaccharide, I assumed that UGGT recognize hydrophobic surface exposed by the lack of two oligosaccharides. However, the EPO analog bearing three oligosaccharides is a definitely native glycoprotein form in ER. Thus the glucosylation to this EPO analogs is more surprising result. I assumed that because EPO is hydrophobic glycoprotein, UGGT recognized hydrophobic surface that cannot be covered by even the three oligosaccharides.

I also conducted the assay with ER lysate isolated from rat liver and synthetic EPO analogs (Fig. 3(b)). This ER lysate contains all the enzymes and chaperones in glycoprotein quality control system. As a result, a reversible glucosylation/deglucosylation reaction by the action of UGGT and glucosidase II respectively was observed at the first time. Additionally, I investigated when and how CNX/CRT interact with G1M9-oligosaccharyl EPO during the exchange of the substrate between UGGT and glucosidase II by using antibodies that can block CNX/CRT. Consequently, I found out that UGGT, CNX/CRT and glucosidase II seems to be working in ordered sequence. In my thesis, I discussed the synthesis of eight kinds of EPO analogs and the refolding process of glycoproteins regulated by chaperones as well as enzymes.

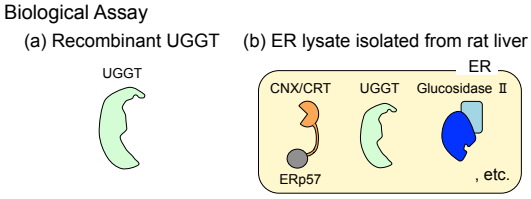

Figure 3. Biological assay with synthetic EPO analogs

Chapter 1

General Introduction and Purpose of My Study

Doctoral thesis

This thesis describes chemical syntheses of erythropoietin (EPO) analogs having a M9-high-mannose type oligosaccharide at the individual native glycosylation position and three M9-high-mannose type oligosaccharides at the three native glycosylation positions. Elucidation of glycoprotein folding process in the endoplasmic reticulum using these EPO analogs will be also described.

Oligosaccharides of glycoprotein

Glycoproteins, a protein that have oligosaccharides, concern various biological events, such as cell recognition, immune response and signal transduction[1]. A half of proteins in mammalian body are glycoproteins. Oligosaccharide moieties contribute to the structural stability and biological activity of glycoprotein.

Oligosaccharides on protein are classified into two groups, one is *O*-linked oligosaccharide, which is attached to the side chain alcohol of serine or threonine residue and the other is *N*-linked oligosaccharide attached to the side chain nitrogen of asparagine residue (Fig. 1-1). *O*-linked oligosaccharides are divided into 8 groups from Core 1 to Core 8 dependent on the second residue following the first residue, *N*-acethylgalactosamine. On the other hand, *N*-linked oligosaccharides are divided into high-mannose type, hybrid type and complex type (Fig. 1-2). Secretory proteins, such as erythropoietin, have complex type oligosaccharides and sialic acids incorporated at the non-reducing end. These sialic acids relate to the half-life of glycoproteins in blood. High-mannose type oligosaccharide concerns with the initial step of the biosynthesis of glycoprotein in endoplasmic reticulum (ER)[2].

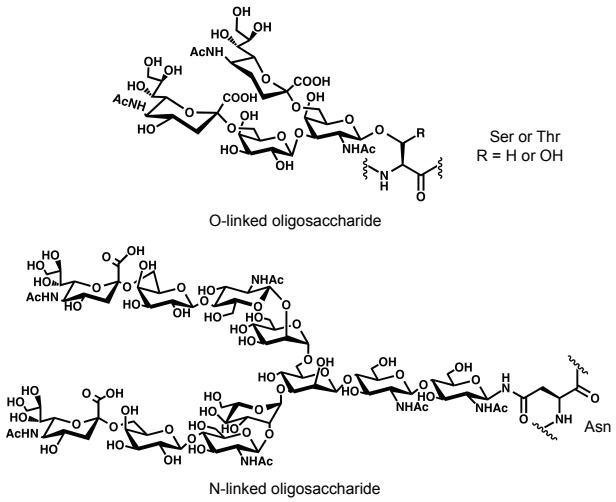

Figure 1-1. Typical structure of *O*- and *N*-linked oligosaccharide

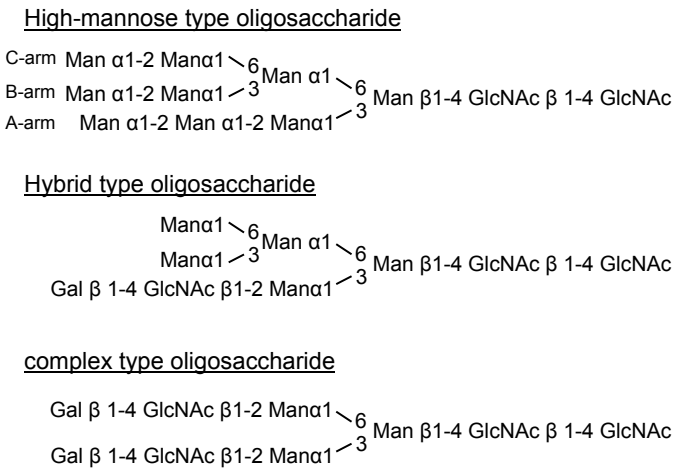

Figure 1-2. Structure of three kinds of *N*-linked oligosaccharides

Biosynthetic pathway of glycoproteins

In the biosynthetic pathway of glycoprotein, high-mannose type oligosaccharide aids the folding of protein in ER and it is converted to complex-type oligosaccharide in Golgi apparatus (Fig. 1-3). The function of high-mannose type oligosaccharide is still under investigation. High-mannose type oligosaccharide seems to play important role in ER specifically. High-mannose type oligosaccharide transiently emerges in the ER and then alters their structure to acidic oligosaccharide that is a complex type sialyl oligosaccharide.

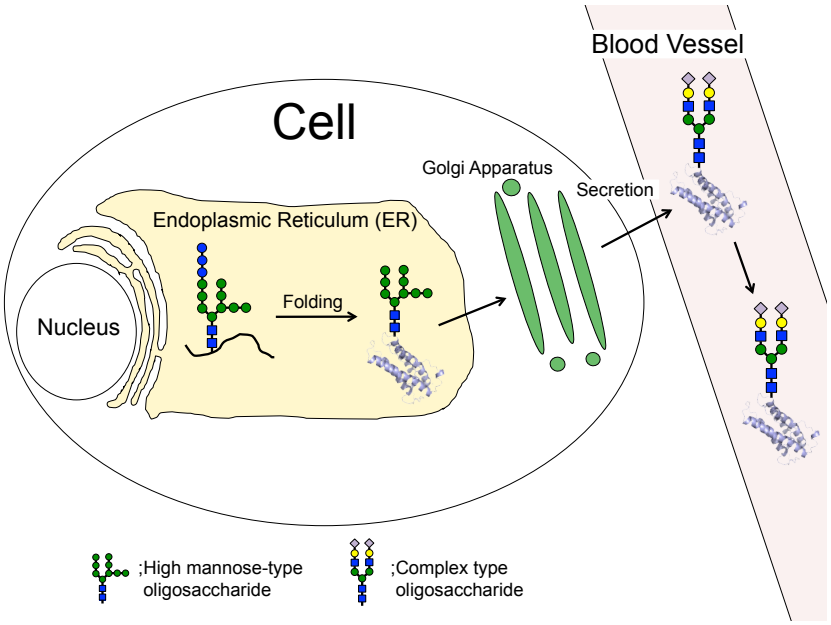

Figure 1-3. Outline of biosynthetic pathway of glycoproteins

Glycoprotein quality control system in endoplasmic reticulum

Nascent peptides translated by ribosomes are attached $Glc_3Man_9GlcNAc_2$ (G3M9) oligosaccharide at the consensus sequence (NXT/S, X except Proline) and then fold into three-dimensional structure with the aid of chaperones and enzymes (Fig. 1-4). Glucosidase I trims the terminal glucose to yield G2M9-oligosaccharide. Malectin, carbohydrate binding protein, recognizes G2M9-oligosaccharide and make a complex consist of malectin-G2M9-oligosacchary-protein. However the function of malectin is still unclear. Glucosidase II trims another glucose at the terminal to yield G1M9-oligosaccharide. Calnexin and Calreticulin (CNX/CRT) bind with the G1M9-oligosaccharide and promote the folding of protein moiety. After the folding, glucosidase II remove the last glucose to yield M9-oligosaccharide.

Depends on the folding state of glycoproteins such as correctly folded glycoprotein, folding intermediates and misfolded glycoprotein, these are transported to different biosynthetic pathways from ER. Correctly folded glycoproteins are transported to the Golgi apparatus by a cargo receptor, ER-Golgi intermediate compartment-53 (ERGIC-53), or vesicular integral protein of 36 kDa-like (VIPL) and then ultimately secreted to the outside of cell or embedded into cell membrane. Misfolded glycoproteins in the ER are recognized by folding sensor enzyme, UDP-glucose; glycoprotein glucosyltransferase (UGGT) and transferred a glucose to the terminal of A-arm of M9-high-mannose type oligosaccharide as a tag of immature glycoproteins. G1M9-high-mannose type oligosaccharyl misfolded glycoproteins interact with CNX/CRT again to refold protein part. Terminally misfolded glycoproteins in the ER are degraded through ER-associated degradation (ERAD) process. These systems combined accelerate the efficient production of glycoproteins, which is called as a glycoprotein quality control system.

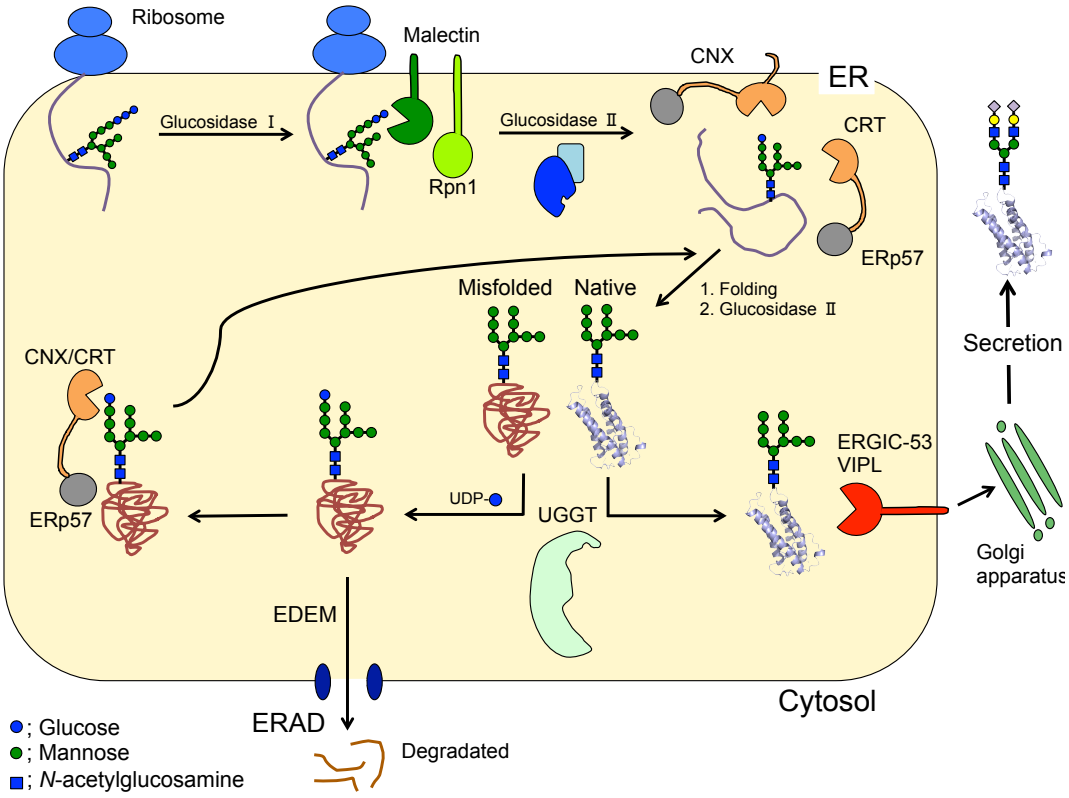

Figure 1-4. Glycoprotein quality system in endoplasmic reticulum (ER). Rpn1: ribophorin I, CNX: calnexin, CRT: calreticulin, ERGIC-53: ER-Golgi intermediate compartment-53, VIPL: vesicular integral protein of 36 kDa-like, UGGT: UDP-glucose: glycoprotein glucosyltransferase, EDEM: ER degradation enhancing α-mannosidase-like protein, ERAD: ER-associated degradation

Malectin

Malectin recognize G2M9 oligosaccharide and especially associate with misfolded glycoproteins by forming a complex with ribophorin I (Rpn 1)[3,4]. Overexpression of malectin showed that malectin promotes the degradation of misfolded glycoproteins through ERAD pathway[5]. Carmela et al showed that malectin is induced by ER stress[6]. They suggested that malectin capture and inhibit the secretion of misfolded glycoproteins that cause ER stress and is working as support of glycoprotein quality control system.

Glucosidase II

Glucosidase II is responsible for the removal of two glucose residues from G2M9 oligosaccharide of glycoprotein. Glucosidase II is heterodimer consist of catalytic α–subunit and regulatory β-subunit containing mannose 6-phosphatereceptor homology (MRH) domain[7]. β-subunit binds the C-arm of high-mannose type oligosaccharide and this supplementary binding is essential for the catalytic activity of α–subunit[8].

Calnexin (CNX) and Calreticulin (CRT)

CNX and CRT are homolog proteins, which recognize G1M9-oligosaccharide of glycoprotein and aid the folding of glycoprotein with a protein disulfide isomerase, ERp57[9]. CNX is a membrane protein and CRT is a soluble protein. Both CNX/CRT require calcium ion and consist of a globular domain, where bind G1M9-oligosaccharide. In order to recognize the surface of misfolded protein, CNX/CRT have an extended arm-like domain called the P-domain, which is proline rich domain. ERp57 bind CNX/CRT through P-domain. Recent study showed that the peptidyl prolyl *cis-trans*-isomerase (PPI) cyclophilin B also makes a complex with CNX/CRT through P-domain[10]. This study indicates that PPI is also a sub-player in the part of the CNX/CRT cycle.

Several studies showed that CNX/CRT can bind the protein moiety of glycoprotein as well as G1M9 oligosaccharide. Ihara et al showed that CNX can suppress the thermal denaturation of not only glycosylated proteins but also non-glycosylated ones[11]. They also showed that CNX interacts with only denatured proteins but not correctly folded ones. Brockmeier et al showed that CNX binds with substrate protein moieties through globular domain[12]. Molinari et al suggested that CNX and CRT work independently in vivo for the support of the retention of misfolded glycoproteins in ER and the folding of unfolded glycoprotein substrates[13]. Dependent on the glycoprotein substrates, CNX and CRT rescue their function each other in order to keep glycoprotein quality control system.

UDP-glucose: glycoprotein glucosyltransferase (UGGT)

UGGT recognizes the exposed hydrophobic surface of misfolded glycoprotein as well as the innermost GlcNAc residue of the oligosaccharide[14]. UGGT consists of about 1500 amino acid residues and have two domains[15]. The C-terminal domain, which corresponds with 20% amino acid residues of UGGT, is a catalytic domain of glucosylation and has similarity to the member of glycosyltransferase family 8. The N-terminal domain, which consist of rest ca. 80% amino acid residues, is thought to recognize protein moieties of misfolded glycoprotein, but their amino acid sequence have no similarity to other known proteins. Although partial structure of UGGT was recently solved, the whole structure is still unknown[16].

Thus the mechanism how UGGT recognizes a variety of substrates and distinguish between native-form glycoproteins and misfolded ones is still unclear. In order to understand the recognition mechanism, artificial substrates were developed so far[17]. For example, Caramelo et al developed neoglycoproteins, which is an artificially glycosylated protein. By using these probes, they suggested that UGGT recognizes molten globule-like folding intermediates, which is partially folded glycoproteins, rather than fully unfolded ones[18,19].

Transportation of glycoproteins from the ER to Golgi apparatus

ERGIC-53 and VIPL regulate the transportation of correctly folded glycoproteins from the ER to Golgi apparatus[20-22]. These cargo receptors exist in the ER-Golgi intermediate compartment (ERGIC) and recognize the oligosaccharide of correctly folded glycoproteins. In terms of substrate specificity, VIPL can bind any deglucosylated high-mannose type oligosaccharide and not glucosylated one, but ERGIC-53 can bind both. These characters enable their transportation of substrate glycoproteins. On the other hand, vesicular integral protein of 36 kDa (VIP36) bind the substrate at lower pH, which is in contrast to ERGIC-53 and VIPL[23]. Considering this property, VIP36 may retrieve misfolded glycoprotein, which is accidently transported to Golgi apparatus and it work as post-ER quality control. In fact, Reiterer et al identified the glycoprotein α1-antitrypsin as a substrate of VIP36[24]. Whether this mechanism is general system or applied for α1-antitrypsin specifically should be revealed.

ER-associated degradation (ERAD)

In the ER, there are many irreparably misfolded glycoproteins unfortunately generated, but these are degraded by ER-associated degradation (ERAD) process[25]. ER degradation enhancing α-mannosidase-like protein 2 (EDEM2) removes a mannose residue from B-arm of high-mannose type oligosaccharide of misfolded glycoproteins and this is the start point of ERAD process[26]. EDEM3 or EDEM1 remove another mannose from C-arm of high mannose type oligosaccharide and this process result in the production of M7 oligosaccharide. Osteosarcoma amplified 9 (OS-9) and XTP3-transactivated gene B protein (XTP3-B) recognize exposed α-1,6 mannose residues (C-arm) and pass the misfolded glycoprotein to SEL1L and HRD1 membrane-anchored ubiquitin ligase complex[27]. This complex, including other molecules that are not shown here, is responsible for the retrotranslocation of misfolded glycoproteins to the cytosol and degradation via ubiquitin-proteasome system of misfolded glycoproteins.

Immunoglobulin heavy chain binding protein (BiP)

Not only CNX/CRT but also immunoglobulin heavy chain binding protein (BiP) is related to the folding of glycoprotein in ER. Molinari et al investigated which chaperones, BiP or CNX/CRT is a major pathway in the ER[28]. They found that BiP and CNX/CRT discriminate glycosylation position in order to bind nascent peptides and glycopeptides, respectively (Fig. 1-5). Nascent glycoproteins that have G1M9-oligosaccharide within 50 residues from N-terminal, interact with CNX/CRT. BiP cannot interact with such glycoproteins, because CNX/CRT can bind faster than BiP. In contract, the absence of oligosaccharides on protein leads a binding with BiP.

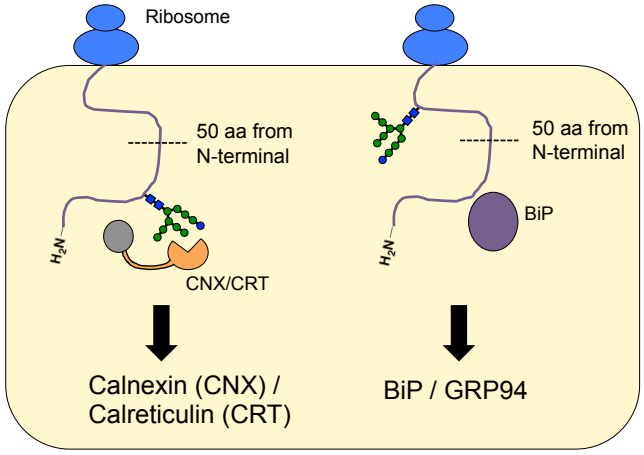

Figure 1-5. Chaperone selections are determined by the glycosylation position.

Chemical approach for elucidating the glycoprotein quality control system

So far the studies for glycoprotein quality control system have used several glycoprotein model of which oligosaccharide structure were heterogeneous, but recently, chemical approaches to yield homogeneous glycoproteins have been employed for the study of glycoprotein quality control system. Chemical synthesis can vary glycosylation position as well as oligosaccharide structure. Furthermore it can afford glycoprotein mimetic probes, which enable us to study at molecular level.

Studies of Ito group

Ito group synthesized high-mannose type oligosaccharide from M9 to G3M9 by their convergent method[29,30] (Fig. 1-6). They prepared short three glycan fragments and combined them to synthesize target compounds. Additionally, they prepared the library of high-mannose type oligosaccharide by using specific mannosidase digestion protocol toward M9 high-mannose type oligosaccharide. In this case, individual sugar at the non-reducing terminal of A, B and C-arm were masked with orthogonal three kinds of protecting groups against the enzymatic reactions[31,32]. Using these protecting groups, they can arrange which arm would be cleavage by enzyme.

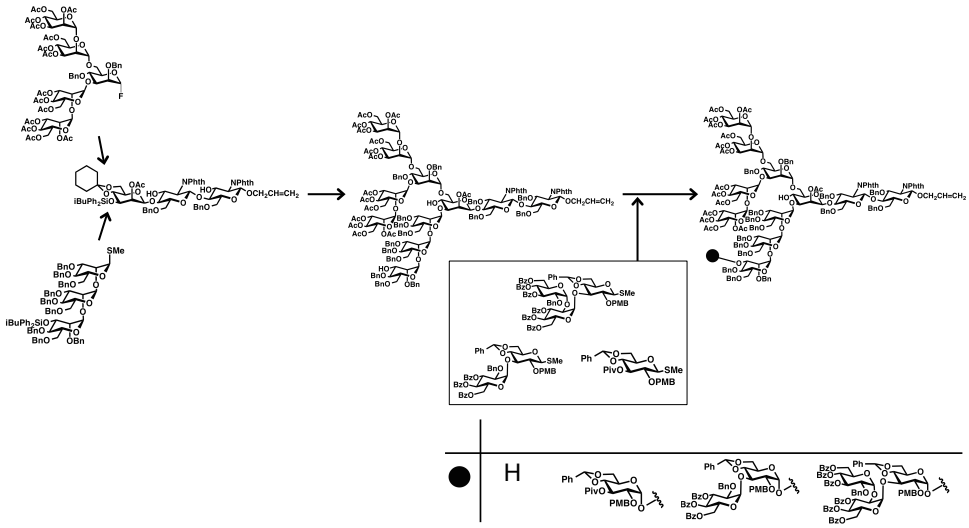

Figure 1-6. Systematic synthesis of high-mannose type oligosaccharide

They developed the preparation of glycoprotein mimic by combing dihydrofolate reductase (DHFR) and methotrexate (MTX)[33]. MTX is a strong inhibitor of DHFR, the K_D is less than 1 nM. They found that any oligosaccharide could be introduced to MTX. They also found that M9-MTX is excellent substrate of UGGT, but M9-MTX-DHFR is not[34]. This means M9-MTX-DHFR and M9-MTX can be used to mimic the structures of a native glycoprotein and misfolded glycoprotein respectively (Figure 1-7).

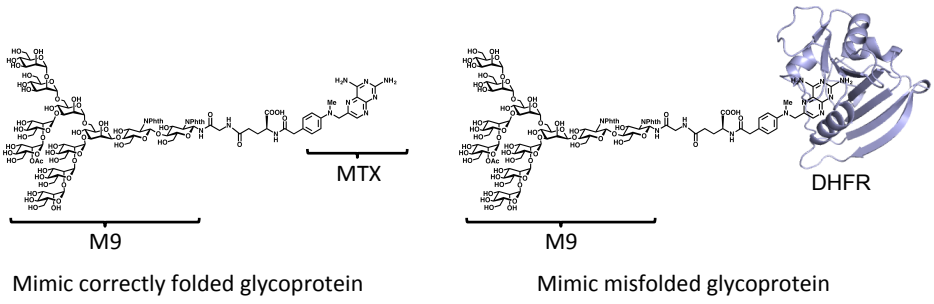

Figure 1-7. M9-MTX and M9-MTX-DHFR. MTX: methotrexate, DHFR: dihydrofolate reductase

They investigated substrate specificity of glucosidase II by using G2M9-MTX[35]. Glucosidase II cleaves two glucoses from G2M9 oligosaccharide. They revealed that the first cleavage is faster than second one. They also found that G1M9 oligosaccharide that was produced by fast cleavage was captured by CRT before the second cleavage. This result indicate that newly synthesized G1M9 glycoproteins enter the CNX/CRT cycle directly just after the cleavage of glucosidase II.

Studies of Kajihara group

The Izumi, Ito and Kajihara group synthesized native and misfolded interleukin-8 (IL-8) bearing M9 oligosaccharide by using isolated oligosaccharide from egg yolk and studied the substrate specificity of UGGT[36] (Fig. 1-8). IL-8 consists of 72 amino acid residues and is not naturally glycosylated. They introduced M9-oligosaccharide at the position, which don't disturb the tertiary structure of protein. These glycoproteins are synthesized by solid phase peptide synthesis and native chemical ligation. They prepared four kinds of glycosyl IL-8 derivatives, such as a native IL-8, two kinds of misfolded IL-8 which disulfide bonds are shuffled and the dimer of which disulfide bonds form between two homogeneous misfolded glycosyl-IL-8. UGGT could discriminate the difference of structures and transferred a glucose to misfolded IL-8 and the dimer, but not to native IL-8. The glucosylation-rate toward the dimer was greater than monomeric misfolded one. They showed that UGGT could recognize slight difference of protein conformations by using homogeneous glycoprotein probes.

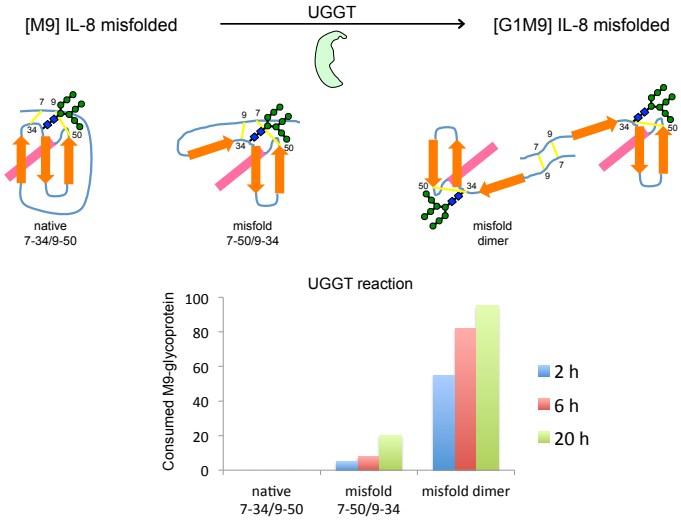

Figure 1-8. Chemical synthesis of four kinds of glycosyl IL-8 derivatives and UGGT assay using them

They also investigated whether UGGT can discriminate productive folding intermediates and unproductive ones by using chemically synthesized native and mutated glycosylated polypeptides of crambin[37]. Crambin is a small protein, consist of 46 amino acid residues, and not naturally glycosylated. M9-oligosaccharide was introduced at a suitable position. Mutated crambin could not fold correctly, because two of the six cysteines were mutated to serines. The folding in the ER could be reproduced by using the unique ability of crambin to fold without denature reagents like urea or guanidine (Gn). Native glycosylated polypeptide and mutated one were mixed and folded in the presence of UGGT. The folding process and glucosylation of UGGT were chased by LC-ESI MS. As a result, UGGT transferred a glucose to all the intermediate of both productive and unproductive. They concluded not only misfolded glycoprotein but also all folding intermediates are substrate of UGGT.

Studies will be described in this thesis

As I mentioned that previous glycoprotein substrates were not close to ideal substrates due to experimental limitations, because glycoprotein isolated were heterogeneous in high-mannose type oligosaccharide and synthetic glycoproteins were not natural type or small. Using more native and homogeneous glycoprotein will enable us to understand a glycoprotein quality control system. Based on these circumstances, I studied the synthesis of large and native glycoproteins for the understanding the glycoprotein quality control system.

Native glycoprotein models for my study

In order to elucidate the glycoprotein quality control system by using of homogeneous and native glycoprotein model, I selected erythropoietin (EPO), which is relatively large, consist of 166 amino acid residues, and has three *N*-linked oligosaccharides.

EPO is a hormone involved in maturation of red blood cells and used as a drug for anemia. Obtaining EPO bearing M9-oligosaccharide (Fig. 1-9) is difficult task by biological methods, because all the three oligosaccharide are converted to complex type when EPO is expressed in recombinant cells (Fig. 1-10). Chemical synthesis can overcome this problem for the study of glycoprotein quality control system.

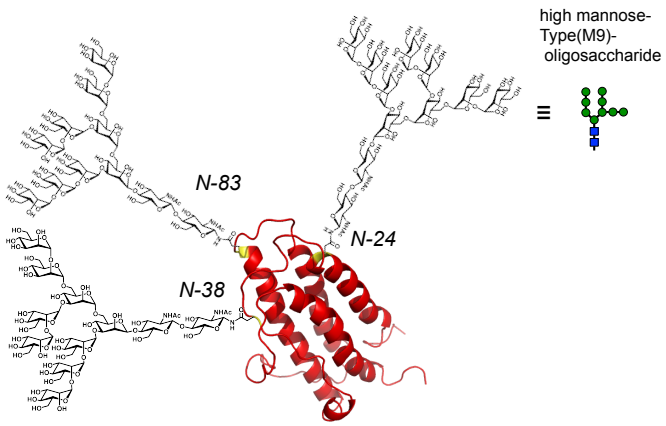

Figure 1-9. Erythropoietin bearing M9-high-mannose type oligosaccharides

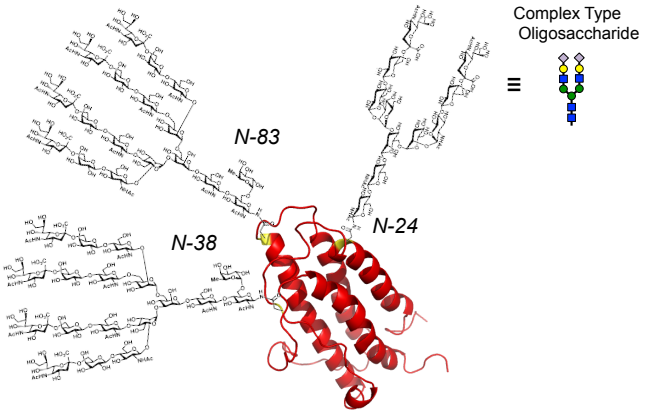

Figure 1-10. Erythropoietin bearing complex type oligosaccharides

EPO is a heavily glycosylated protein in spite of their protein-size and these oligosaccharides are essential for their biosynthesis. Yamaguchi et al investigated the effect of the number of oligosaccharides on the secretion of EPO[38]. They mutated the asparagine of glycosylation position to glutamine and estimated its effect on the secretion of EPO by ELISA assay (Table 1). As a result, the secretion of EPO is decreased according to the decrease of the oligosaccharides number. This result indicated that these oligosaccharides play an important role in the biosynthesis of EPO. But the reason of the decrease of secretion and the role of these oligosaccharides are still unclear.

Table 1 The relationship between the number of oligosaccharide on EPO and its secretion

Glycosylated site	Relative efficiency of EPO biosynthesis and secretion (%)
N-24, 38, 83	100
N-38, 83	70 ± 10
N-24, 83	60 ± 3
N-24, 38	78 ± 4
N-83	31 ± 4
N-38	58 ± 4
N-83	41 ± 7
No-oligosaccharide	10 ± 1

Purpose of my study

Based on these backgrounds, I synthesized EPO analogs bearing one or three high-mannose type oligosaccharides to investigate the role of these oligosaccharides in the biosynthetic pathway especially in the glycoprotein quality control system. Until now, only a few studies using glycosylation-defective glycoproteins were conducted for the studies of glycoprotein quality control system as well as biosynthetic pathway. Therefore, I designed to synthesize four kinds of EPO, which are EPO bearing one M9-high-mannose type oligosaccharide at the individual position of native three *N*-glycosylation positions and EPO bearing all three M9-high-mannose type oligosaccharides at the native three *N*-glycosylation positions (Fig. 1-11). The former types are also recognized as glycosylation-deficient glycoprotein models.

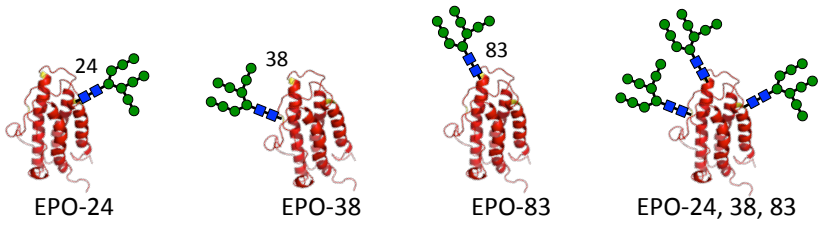

Figure 1-11. Correctly folded EPO bearing one or three oligosaccharides, synthetic targets of this study

Because chemical synthesis can be expected to yield misfolded glycoprotein due to disulfide bond scrambling along with correctly folded one, I designed my study to evaluate these misfolded forms to reveal the recognition mechanism of UGGT (Fig. 1-12). UGGT recognizes misfolded glycoproteins specifically. Therefore, misfolded glycoproteins are needed to study UGGT. I planned to use the disulfide bonds scramble method to prepare misfolded EPO analogs [30].

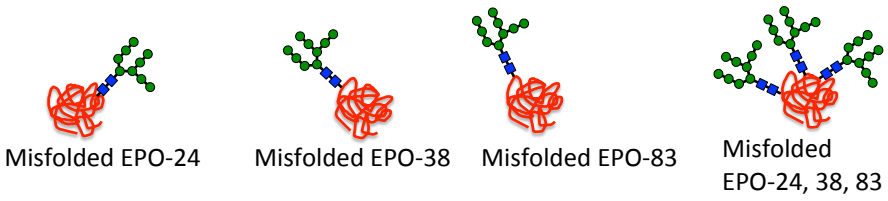

Figure 1-12. Misfolded EPO bearing one or three oligosaccharides, synthetic targets of this study

Chemical synthesis of such a large glycoprotein, however, is still challenging work. The establishment of efficient synthetic route is essential for the preparation of these EPO analogs. I intended to propose a unified route, which can be used for the synthesis of all kinds of EPO analogs.

I also studied the interaction of chaperones (CNX/CRT) and enzymes (glucosidase II and UGGT) to glycoprotein substrates in the glycoprotein quality control system in detail. Although the each functions of these CNX, CRT, UGGT and glucosidase II have been studied at some extent, the

sequence of interactions is still ambiguous, such as when and how to interact with glycoprotein substrates. One of the reasons is lack of glycoprotein probes to investigate it. I thought that my synthetic EPO analogs are possible to be suitable probe.

Contents of this thesis

This thesis consists of three chapters. I will discuss the chemical synthesis of EPO bearing high-mannose type oligosaccharide and the investigation of glycoprotein quality control system by using synthetic probes.

Chapter 1 describes the background and purpose of my study.

Chapter 2 will describe the general introduction of chemical synthesis of glycoprotein and my synthesis of EPO bearing one or three oligosaccharides. The characterizations of the synthetic EPO analogs were also mentioned here.

Chapter 3 will describe the result of UGGT and ER assay with synthetic EPO analogs. These experiments revealed the new insight of UGGT and the order of interaction of chaperones in glycoprotein quality control system.

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Chapter 2

Chemical Synthesis of EPO Analogs Bearing One or Three High-mannose Type Oligosaccharides and Their Characterizations

Introduction of chapter 2

This chapter will describe chemical synthesis of four kinds of EPO analogs using peptide and glycopeptide building blocks. The characterizations of these EPO analogs will be also described in this chapter. This chapter is consists of eight sections. The titles of each section are described below.

2-1. The methods used in the synthesis of EPO analogs
2-2. Synthetic strategy of erythropoietin bearing M9-high-mannose type oligosaccharides
2-3. The preparation of peptide and glycopeptide segments of EPO
2-4. Construction of EPO bearing three M9-high-mannose type oligosaccharides (EPO-24, 38, 83)
2-5. Construction of EPO bearing one M9-high-mannose type oligosaccharide (EPO-24)
2-6. Construction of EPO bearing oneM9-high-mannose type oligosaccharide (EPO-38)
2-7. Construction of EPO bearing one M9-high-mannose type oligosaccharide (EPO-83)
2-8. Characterization of synthetic EPO analogs (correctly folded and misfolded)

2-1. The methods used in the synthesis of EPO analogs

2-1-1. Solid phase peptide synthesis

Solid phase peptide synthesis (SPPS) method was developed by R. B. Merrifield in 1963[1]. This method uses the insoluble resin that is modified by functional groups as solid phase. Each amino acid is coupled to the resin from C-terminal sequentially. Excess reagents and by-products are removed by washing easily comparing with a solution phase synthesis methods.

This section show the simple procedure of the SPPS method. First, N-terminal protected amino acid is coupled to the resin. Second, next amino acid is coupled after removal of the protecting group and this cycle is repeated several times. Finally, desired peptide or peptide thioester are obtained after the removal of side chain protecting groups and the cleavage from the resin.

Fmoc (9-fluorenylmethoxycarbonyl) or Boc (*tert*-butoxycarbonyl) groups are used as protecting groups of N-terminal. Fmoc method is relatively safe and easy to handle (Fig. 2-1). Because

Fmoc group is removed by piperidine, which is weak base, and the cleavage doesn't need to use strong acids like Boc method. But this method cannot elongate peptide thioesters directly on a resin, because of the base labile property of thioesters. On the other hand, Boc method can construct peptide thioester directly on a resin, because Boc group is removed by acidic condition and thioester is stable under acidic conditions such as TfOH that can be used for the cleavage (Fig. 2-2). In this study, I used Fmoc method for the synthesis of C-terminal peptide segment and Boc method for the synthesis of other peptide and glycopeptide thioester segments[2].

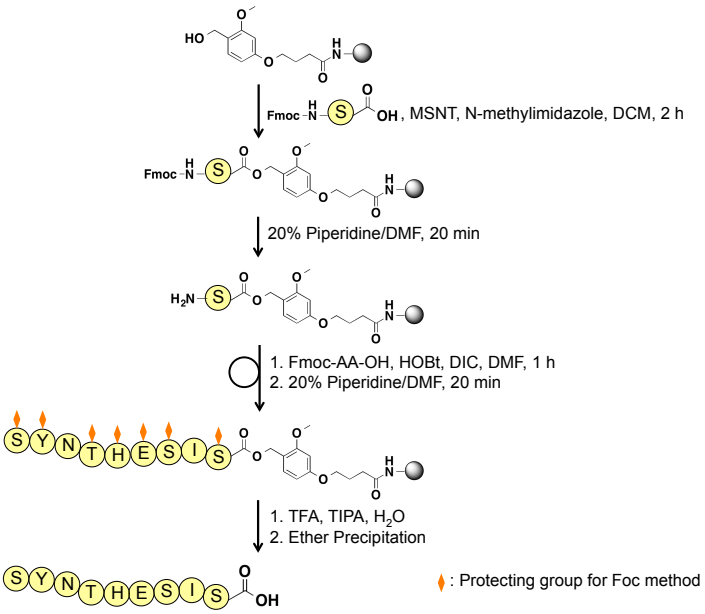

Figure 2-1. Solid phase peptide synthesis (Fmoc chemistry)

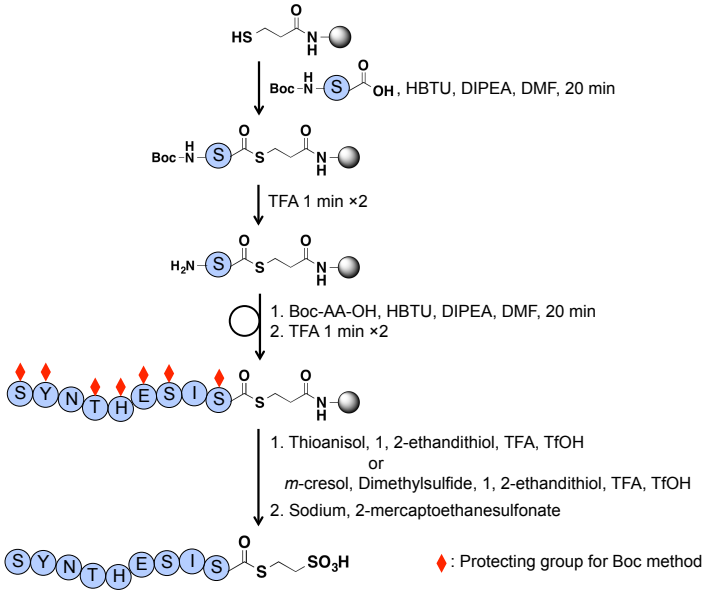

Figure 2-2. Solid phase peptide synthesis (Boc chemistry)

2-1-2. Native chemical ligation at cysteine and alanine site

Because it is difficult to construct long peptides (over 50 residues) on resin, peptide ligation methods are essential for the synthesis of proteins or glycoproteins. Native chemical ligation (NCL) is one of the potential methods to ligate peptides (Fig. 2-3)[3]. NCL is developed by S. B. H. Kent et al. and can couple unprotected peptides in solution phase. In this reaction, peptide thioesters and peptides bearing cysteine residue at the N-terminal are ligated and form peptide bond, through thioester exchange and subsequent intramolecular S-to-N acyl transfer. This method is utilized widely for protein or glycoprotein synthesis, because of the high specificity and yield. I also used this method for my synthesis.

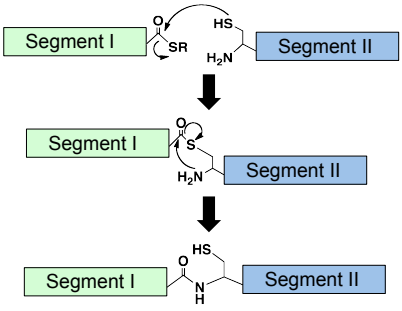

Figure 2-3. Mechanism of Native chemical ligation

I also used the selective reduction of cysteine residue to alanine at ligation site. Although NCL need cysteine residue at ligation, it is difficult to find optimal ligation site because cysteine residues exist only 1.7% in protein sequences[4]. To overcome the problem, many cysteine surrogates are reported[5]. Among them, I used the method developed by Danishefsky[6]. Cysteine residues at ligation site are reduced to alanine residues by phosphine radical reduction after NCL. By using this method, alanine residues can be used as ligation sites.

2-1-3. Preparation of asparagine derivative bearing M9-high-mannose type oligosaccharide

Our group established the method to isolate and purify homogeneous M9-high-mannose type oligosaccharide from egg yolks[7](Fig. 2-4). In this method, de-fat egg yolk is treated by proteases to generate Asparagine derivative bearing M9-high-mannose type oligosaccharide (M9-Asn) and purified by gel filtration as fast steps. Second, Fmoc group is introduced to M9-Asn for further purification and solid phase peptide synthesis. This Fmoc-M9-Asn is purified by ODS column, HPLC (NH_2-column), and gel filtration. The structure of obtained Fmoc-M9-Asn was determined by ESI-MS and NMR. We also reported the synthesis of glycopeptide thioesters by Boc method using this oligosaccharide. In our report, we used Fmoc-M9-Asn for Boc method and deprotected Fmoc group using Aimoto cocktail[8], but this deprotection have a possibility to cause racemization. Thus, I converted Fmoc-M9-Asn to Boc-M9-Asn in good yield and used it for the synthesis of glycopeptide thioester.

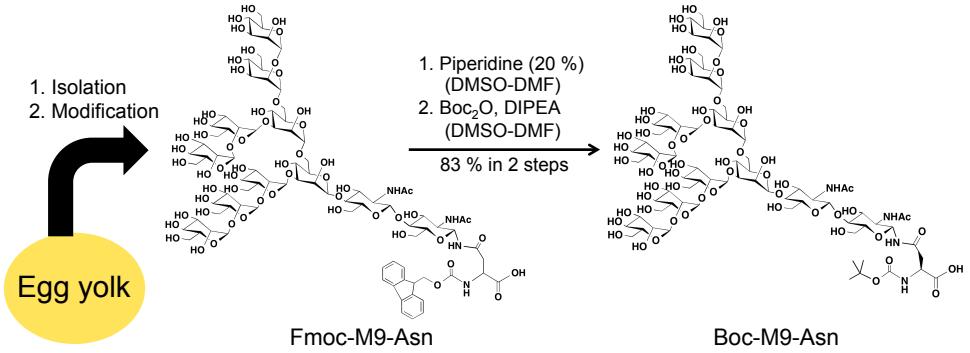

Figure 2-4. Isolation of homogeneous M9-high-mannose type oligosaccharide from egg yolk and modification by Boc group. DMSO; dimethyl sulfoxide, DMF; dimethylformamide, Boc_2O; di-tert-butyl dicarbonate, DIPEA; diisopropylethylamine

2-2. Synthetic strategy of erythropoietin bearing M9-high-mannose type oligosaccharides

2-2-1. Amino acid sequence of EPO and the strategy of peptide and glycopeptide ligations

Erythropoietin (EPO) consists of 166 amino acid residues that have two disulfide bonds (Fig. 2-5). Because synthesizing the whole glycosylated polypeptide as one chain is difficult, I divided it into six short peptide and glycopeptide segments as it is colored in Figure 2-5. After the preparation of all the segments, these were ligated sequentially, following the synthetic strategy as shown Figure 2-6. This synthetic strategy can synthesize both EPO analogs bearing one oligosaccharide and three oligosaccharides, just changing glycopeptide segments used. Finally, folding procedure lead them to correctly folded EPO and misfolded one. Solid phase peptide synthesis (Fmoc and Boc) and native chemical ligation were used for the preparation of segments and ligation of each segment, respectively.

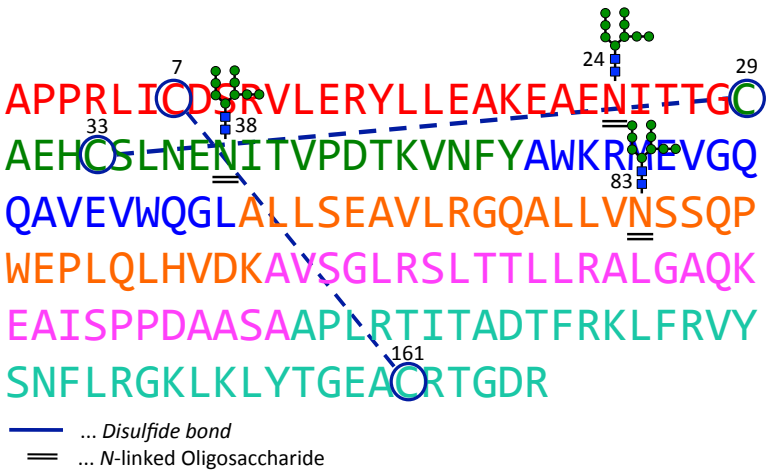

Figure 2-5. Sequence of erythropoietin

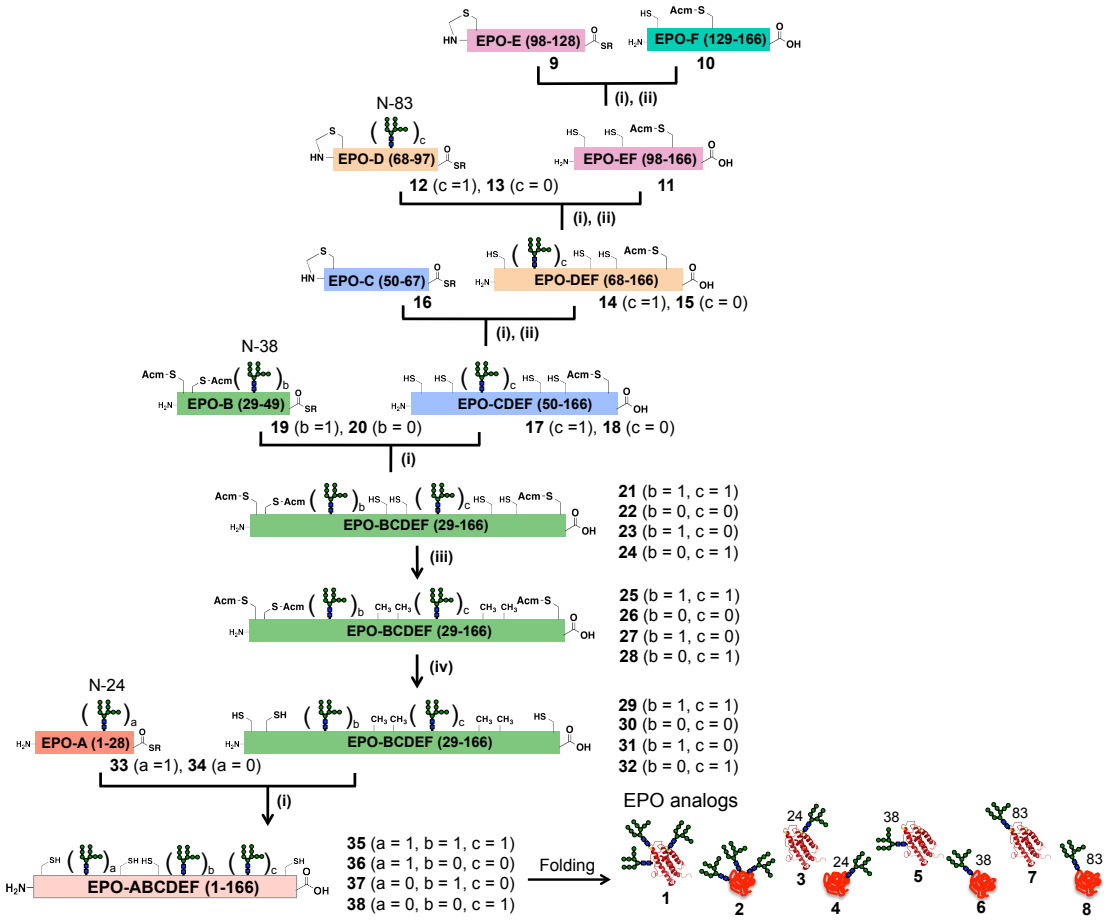

Figure 2-6. Synthetic strategy of erythropoietin analogs. Conditions: (i) native chemical ligation; (ii) removal of thiazolidine; (iii) desulfurization; (iv) removal of the Acm groups

2-2-2. Design for peptide segments for EPO synthesis

The whole glycosylated polypeptides of EPO were desingned to divide into 6 segments. Segment A to E were designed to prepare as a thioester form for NCL. Segment F was designed to prepare as a peptide form. In order to synthesize EPO bearing one or three oligosaccharides, I prepared both peptide and glycopeptide thioesters of Segment A (1-28), Segment B (29-49) and Segment D (68-97) (Fig. 2-7).

Figure 2-7. Divided segments to synthesize EPO

2-2-3. Mutation of potential glycosylation sites

Oligosaccharide defective EPO is known to be destabilized and prone to aggregate. Narhi et al. reported mutation, which prevent the aggregation[9]. They mutated asparagine residues, which are N-glycosylated sites, to lysine residues for the preparation of non-glycosylated EPO. This mutation changes isoelectric point of the non-glycosylated EPO and stabilizes it.

Actually, I synthesized both mutated EPO segment (EPO (50-166), K-83) and non-mutated one (EPO (50-166), N-83) as preliminary experiments and compared the isolated yields (Fig. 2-8). As a result, the isolation yield of final ligation of mutated segment with Lys83 was apparently improved rather than non-mutated Asn83 from 29.8% to 47.7%. Thus I synthesized EPO bearing one oligosaccharide with this mutation at the other potential glycosylation sites.

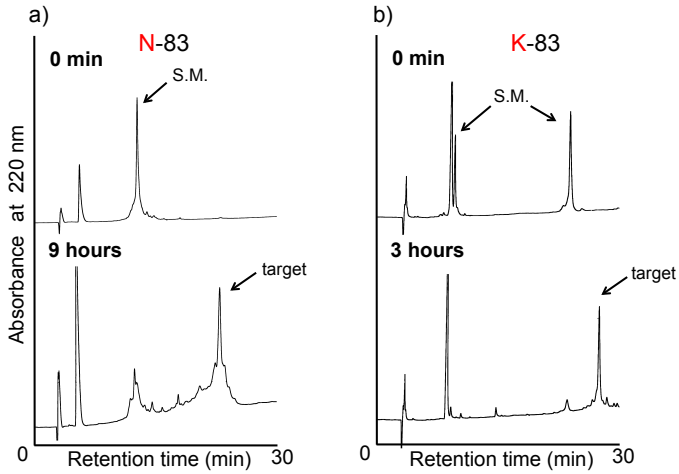

Figure 2-8. HPLC profiles of the synthesized EPO (50-166) by NCL. a) After 9 hours for the EPO (50-166) having Asn83. b) After 3 hours for the EPO (50-166) having Lys83.

2-3. The preparation of peptide and glycopeptide segments of EPO

2-3-1. The preparation of peptide thioester segments A, B, C, D and E by Boc method

I synthesized peptide thioester segments A, B, C, D and E by using Boc method. I used Nova PEG amino resin. Thiol-linker: S-trityl-3-mercaptopropionic acid was coupled by using HBTU as coupling reagent and *N, N*-diisopropylethylamine (DIPEA) in *N, N*-dimethlformamide (DMF). Trityl group was removed by the cocktail of 95% Trifluoroacetic acid (TFA), 2.5% H_2O and 2.5% triisopropylsilane. Then first amino acid was coupled with the same coupling condition as thiol-linker. Removal of Boc grope by TFA and subsequent coupling of next amino acid were conducted. By repeating this procedure, the whole sequences were constructed.

After the construction, the protecting groups of side chains were removed by the low acidic deprotection cocktail: TFA / Dimethylsulfide / *m*-cresol / Trifluoromethansulfonic acid (TfOH) (5:3:1:1) or high acidic deprotection cocktail: TFA / thioanisole / *m*-cresol / TfOH (20:2:1:2). The unprotected peptide was cleaved from the resin by thioester exchange reaction with 200 mM Sodium 2-mercaptoehanesulfonate (MESNa). These operations gave desired peptide thioester segment A, B, C, D and E. HPLC profiles and MASS spectra of obtained segments were shown from Figure 2-9 to Figure 2-13.

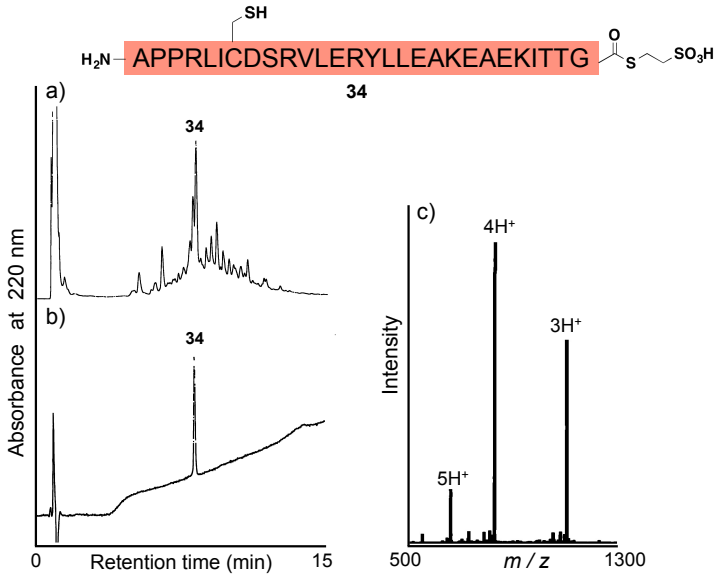

Figure 2-9. HPLC profiles and ESI-MS spectrum of purified peptide segment A. a) HPLC profile of crude compound **34**. b) HPLC profile of purified compound **34**. c) ESI-MS of compound **34**. ESI-MS: *m/z* calcd. for $C_{141}H_{240}N_{39}O_{45}S_3$: $[M+H]^+$ 3297.9, found for 3297.2 (deconvoluted).

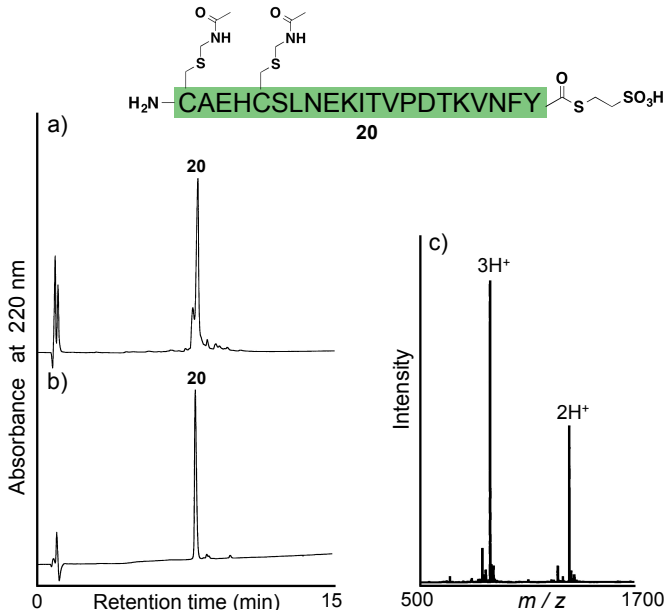

Figure 2-10. HPLC profiles and ESI-MS spectrum of purified peptide segment B. a) HPLC profile of crude compound **20**. b) HPLC profile of purified compound **20**. c) ESI-MS of compound **20**. ESI-MS: *m/z* calcd. for $C_{113}H_{178}N_{29}O_{38}S_4$: $[M+H]^+$ 2677.2, found for 2677.8 (deconvoluted).

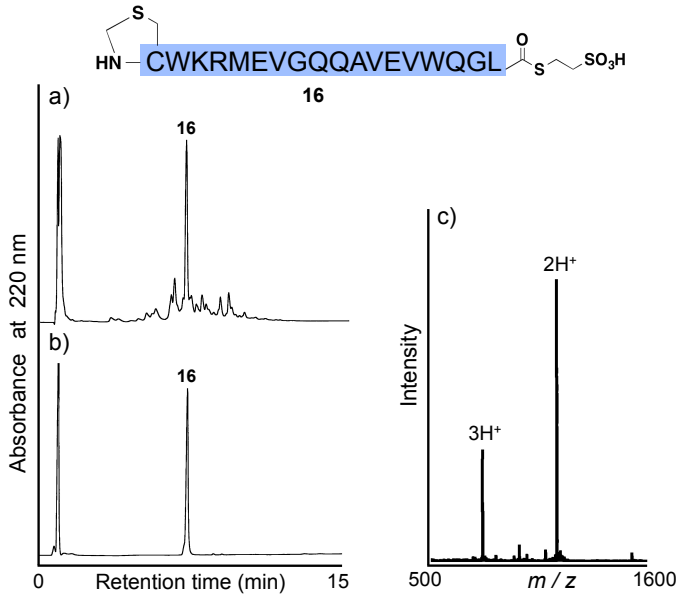

Figure 2-11. HPLC profiles and ESI-MS spectrum of purified peptide segment C. a) HPLC profile of crude compound **16**. b) HPLC profile of purified compound **16**. c) ESI-MS of compound **16**. ESI-MS: *m/z* calcd. for $C_{98}H_{152}N_{27}O_{28}S_4$: $[M+H]^+$ 2284.7 found for 2283.8 (deconvoluted).

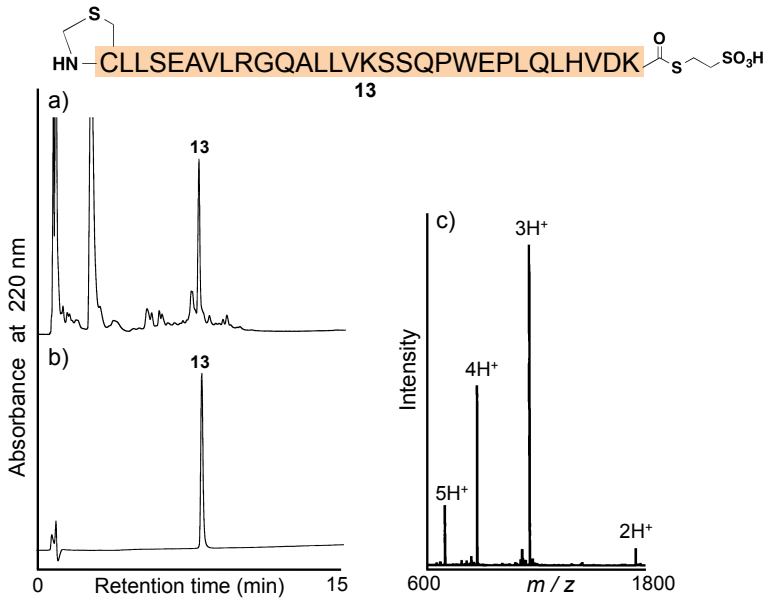

Figure 2-12. HPLC profiles and ESI-MS spectrum of purified peptide segment D. a) HPLC profile of crude compound **13**. b) HPLC profile of purified compound **13**. c) ESI-MS of compound **13**. ESI-MS: *m/z* calcd. for $C_{154}H_{254}N_{41}O_{45}S_3$: $[M+H]^+$ 3496.1 found for 3495.8 (deconvoluted).

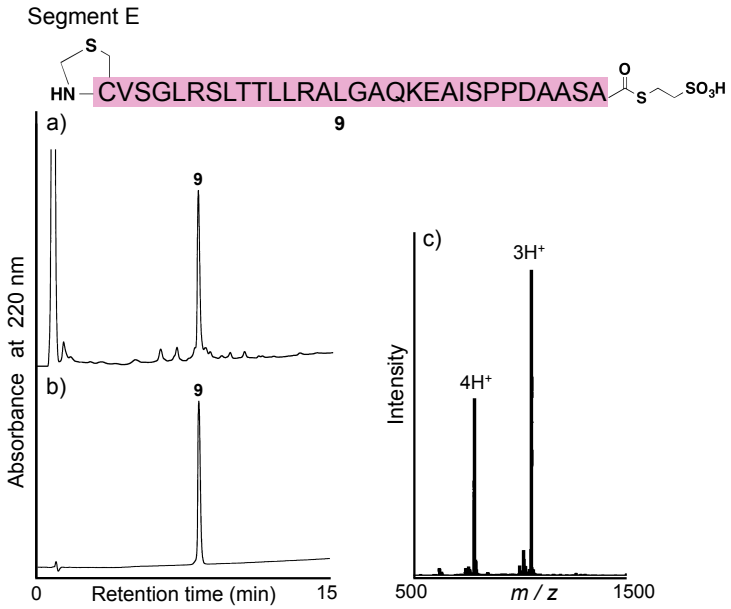

Figure 2-13. HPLC profiles and ESI-MS spectrum of purified peptide segment E. a) HPLC profile of crude compound **9**. b) HPLC profile of purified compound **9**. c) ESI-MS of compound **9**. ESI-MS: *m/z* calcd. for $C_{131}H_{227}N_{38}O_{44}S_3$: $[M+H]^+$ 3134.6 found for 3133.6 (deconvoluted).

2-3-2. The preparation of glycopeptide thioester segments B and D by Boc method

Glycopeptide thioester segments bearing M9-high-mannose type oligosaccharide were prepared by Boc method. Fmoc-M9-Asn was obtained by the isolation of M9-Asn from egg yolks and modification by Fmoc group as I mentioned in section 2-1-3. Fmoc-M9-Asn was converted to Boc-M9-Asn for using Boc method.

Glycopeptide thioester segments B and D were synthesized by using the Boc-M9-Asn. Before the coupling of the oligosaccharide was constructed on resin following the previously mentioned procedure of Boc method using amino PEGA resin. Boc-M9-Asn was coupled with PyBOP and DIPEA in the solution of DMF / dimethyl sulfoxide (DMSO) =1:1. Further amino acids were coupled with the 5 times diluted condition to prevent the esterification to the hydroxy group of oligosaccharide. After the construction, side chain protecting groups were removed by the low acidic deprotection cocktail. High acidic deprotection cocktail cannot be used for the glycopeptide synthesis, because this condition decomposes the oligosaccharide. The unprotected peptide was cleaved from the resin by thioester exchange reaction and the desired glycopeptide thioester segments B and D were obtained. HPLC profiles and MASS spectra of obtained segments were shown in Figure 2-14 and Figure 2-15.

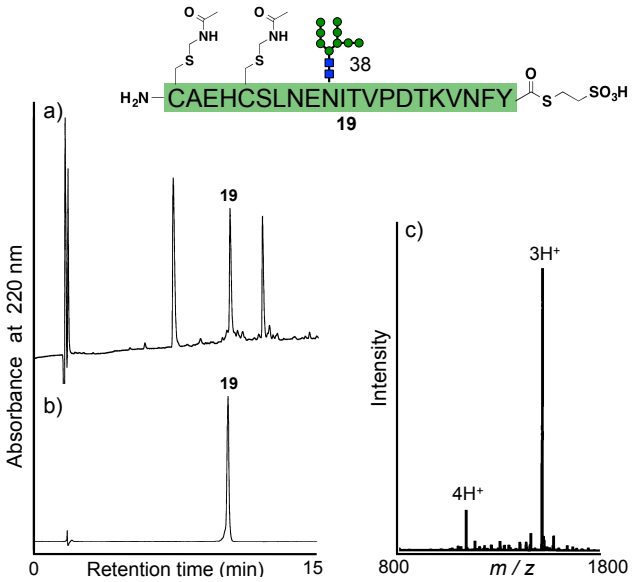

Figure 2-14. HPLC profiles and ESI-MS spectrum of purified glycopeptide segment B. a) HPLC profile of crude compound **19**. b) HPLC profile of purified compound **19**. c) ESI-MS of compound **19**. ESI-MS: *m/z* calcd. for $C_{181}H_{288}N_{31}O_{94}S_4$: $[M+H]^+$ 4530.6 found for 4529.4 (deconvoluted).

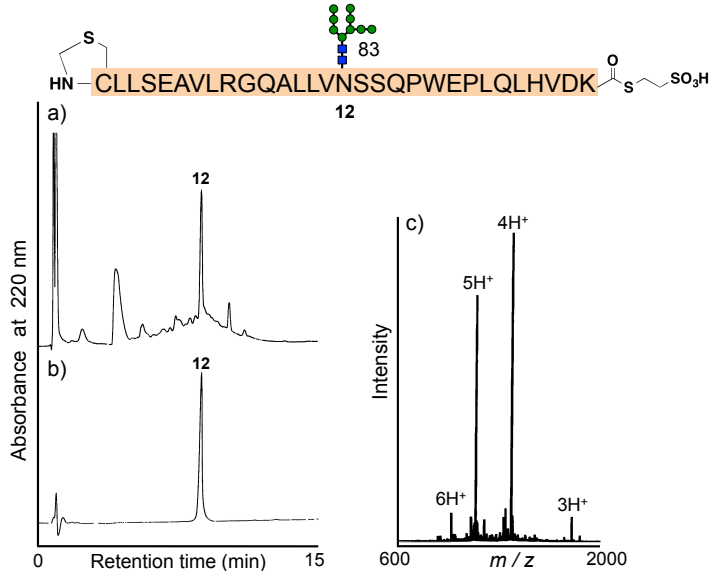

Figure 2-15. HPLC profiles and ESI-MS spectrum of purified glycopeptide segment D. a) HPLC profile of crude compound **12**. b) HPLC profile of purified compound **12**. c) ESI-MS of compound **12**. EIS-MS: *m/z* calcd. for $C_{222}H_{364}N_{43}O_{101}S_3$: $[M+H]^+$ 5346.7 found for 5347.0 (deconvoluted).

2-3-3. The preparation of glycopeptide thioester segment A by fragment-coupling method

Synthesis of N-terminal of EPO is known as difficult sequence to synthesize[10].Glycopeptide segment A is difficult to synthesize, because it has an oligosaccharide position at near N-terminal, thus nearly twenty amino acids should be coupled after the coupling of oligosaccharide in diluted coupling condition.

Therefore, I employed fragment-coupling approach. Carlo et al. have already employed the approach for the synthesis of glycopeptide bearing a complex type oligosaccharide by Fmoc method[11]. I applied it to the synthesis of glycopeptide thioester bearing M9-high-mannose type oligosaccharide by Boc method at the first time (Fig. 2-16).

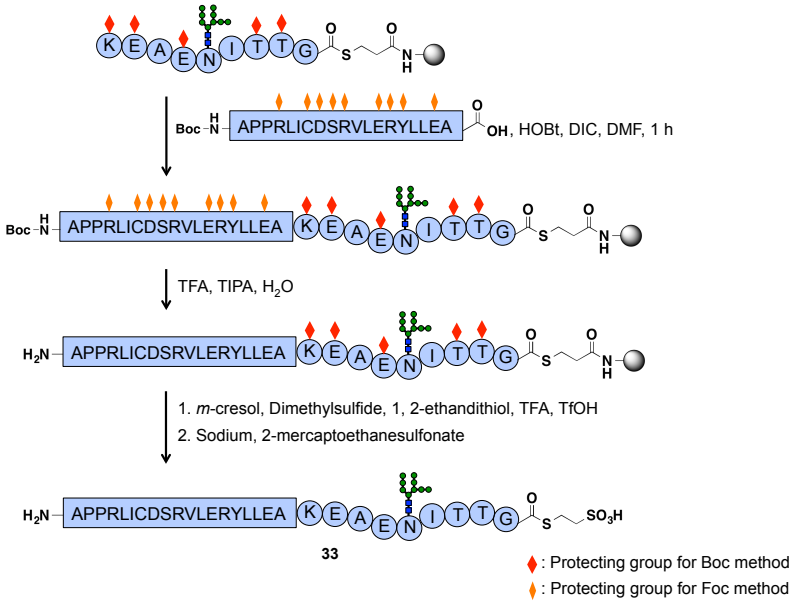

Figure 2-16. Fragment coupling approach for synthesis of glycopeptide segment A

The glycopeptide thioester on resin was constructed by Boc method. Protected peptide fragment was prepared by Fmoc method. This fragment was couple to the glycopeptide thioester on resin by using DIC and HOBt. The protecting groups used in Fmoc method were removed by TFA. The rest protecting groups used in Boc method were removed by the low acidic deprotection cocktail. Subsequence thiolysis reaction leads desired glycopeptide segment A. Successful HPLC profiles and MASS spectrum of obtained segment were shown in Figure 2-17.

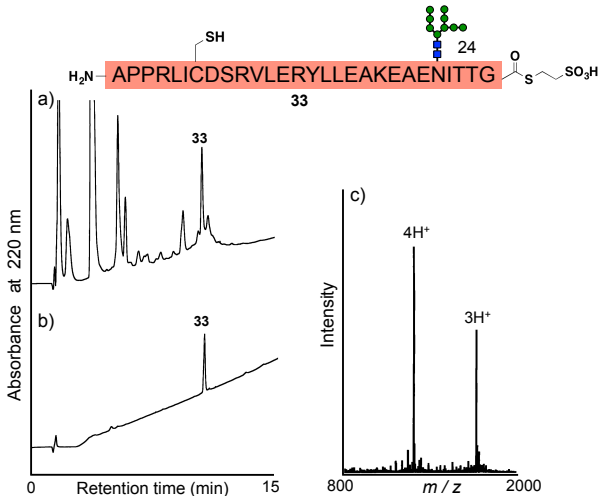

Figure 2-17. HPLC profiles and ESI-MS spectrum of purified glycopeptide segment A. a) HPLC profile of crude compound **33**. b) HPLC profile of purified compound **33**. c) ESI-MS of compound **33**. ESI-MS: *m/z* calcd. for $C_{209}H_{350}N_{41}O_{101}S_3$: $[M+H]^+$ 5149.4 found for 5148.3 (deconvoluted).

2-3-4. The preparation of peptide segment F by Fmoc method

I synthesized the peptide segment F by Fmoc method using HMPB-PEGA resin. First amino acid residue Fmoc-Arg (Pdf) was coupled with MSNT and *N*-metylimidazole in dichloromethan (DCM). After the coupling, Fmoc group was deprotected by 20% piperidine and next amino acid Fmoc-Asp (tBu) was couple with HOBt and *N, N'*-diisopropylcarbodiimide (DIC) in DMF. Repeating this procedure, the whole peptide segment was constructed on resin. The removal of side chain protecting groups and cleavage from the resin were conducted at the same time by 95% TFA, 2.5% TIPS and 2.5% H_2O. Thus the desired peptide segment F was obtained. HPLC profiles and MASS spectrum of obtained segment were shown in Figure 2-18.

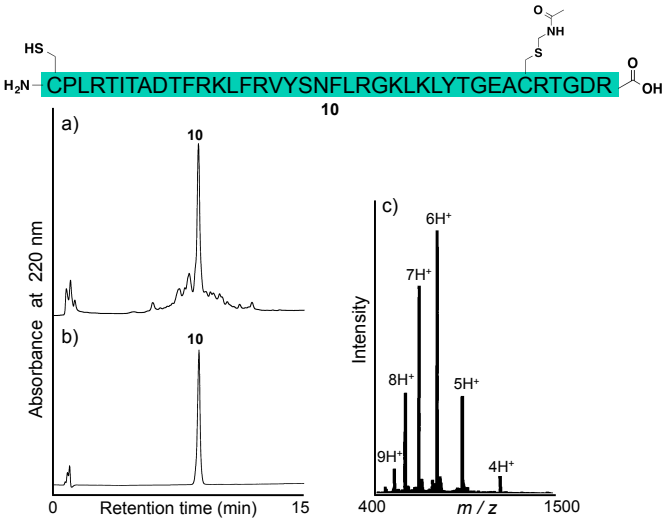

Figure 2-18. HPLC profiles and ESI-MS spectrum of purified peptide segment F. a) HPLC profile of crude compound **10**. b) HPLC profile of purified compound **10**. c) ESI-MS of compound **10**. EIS-MS: *m/z* calcd. for $C_{206}H_{335}N_{62}O_{56}S_2$: $[M+H]^+$ 4640.4 found for 4639.9 (deconvoluted).

In section 2-3, I mentioned the synthesis of peptide and glycopeptide thioester segments and peptide segment for the synthesis of EPO analogs bearing M9-high-mannose type oligosaccharides. The key glycopeptide thioesters were prepared efficiently by using Boc method. The other segments were also prepared successfully by Boc or Fmoc method. I will mention the ligation reactions using these segments in next section.

2-4. Construction of EPO bearing three M9-high-mannose type oligosaccharides (EPO-24, 38, 83)

Synthesis of EPO bearing three oligosaccharides is still challenging because of the highly glycosylation. So far, only a few glycoproteins that have several oligosaccharides were synthesized. In this section, I will mention the ligation reactions, desulfurization reaction, removal of Acm groups and the folding of glycosylated polypeptide for the synthesis of EPO bearing three oligosaccharides. As I mentioned above, each segments were reacted sequentially following the synthetic strategy (Fig. 2-6).

2-4-1. Ligation reaction between segment E and segment F

Segment E and F were ligated through native chemical ligation. Segment E and F were dissolved in 200 mM phosphate buffer containing 6 M guanidine (Gn), 40 mM 4-mercaptophenylacetic acid (MPAA) and 20 mM tris (2-carboxyethyl) phosphine hydrochloride

(TCEP-HCl). MPAA was used to accelerate the reaction. The reaction was started at pH 6.8 in room temperature and chased by HPLC and ESI-MS as shown in figure 2-19. The starting materials were consumed completely and the reaction was finished after 1 hours.

After the completion of the reaction, a thiazolidine, which is a protecting group of N-terminal cysteine was removed by 200 mM methoxyamine-HCl at pH4. The completion of the reaction was confirmed by HPLC and ESI-MS after 2 hours (Fig. 2-19). Desired segment EF was obtained after purification.

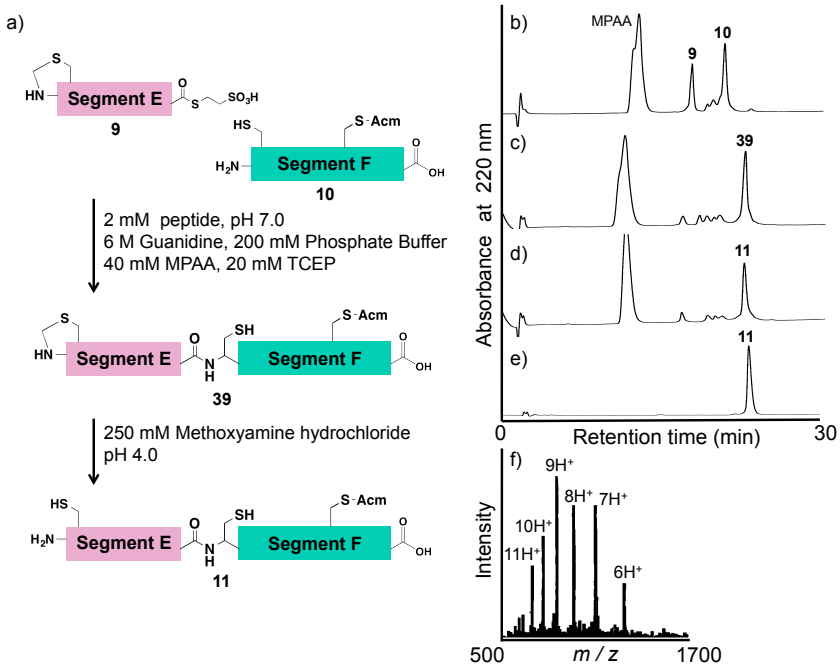

Figure 2-19. Analytical data of monitoring 1st-NCL. a) Scheme of peptide ligation reaction between compound **9** and **10**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 1 h. d) HPLC profile after treatment with methoxyamine-HCl. e) HPLC profile of purified compound **11**. f) ESI-MS of compound **11**. ESI-MS: *m/z* calcd. for $C_{334}H_{555}N_{100}O_{97}S_3$: $[M+H]^+$ 7619.8, found for 7620.2 (deconvoluted).

2-4-2. Ligation reaction between glycopeptide segment D and segment EF

Glycopeptide segment D and EF were ligated by the same procedure as section 2-4-1. Segment E and EF were dissolved in 200 mM phosphate buffer containing 6 M Gn, 40 mM MPAA and 20 mM TCEP-HCl. The reaction was started at pH 6.8 in room temperature and chased by HPLC and ESI-MS as shown in figure 2-20. The reaction was finished after 4 hours.

After the reaction, thiazolidine was removed by 200 mM methoxyamine-HCl at pH4. The completion of the reaction was confirmed by HPLC and ESI-MS after 2 hours (Fig. 2-20). Desired segment DEF was obtained after purification.

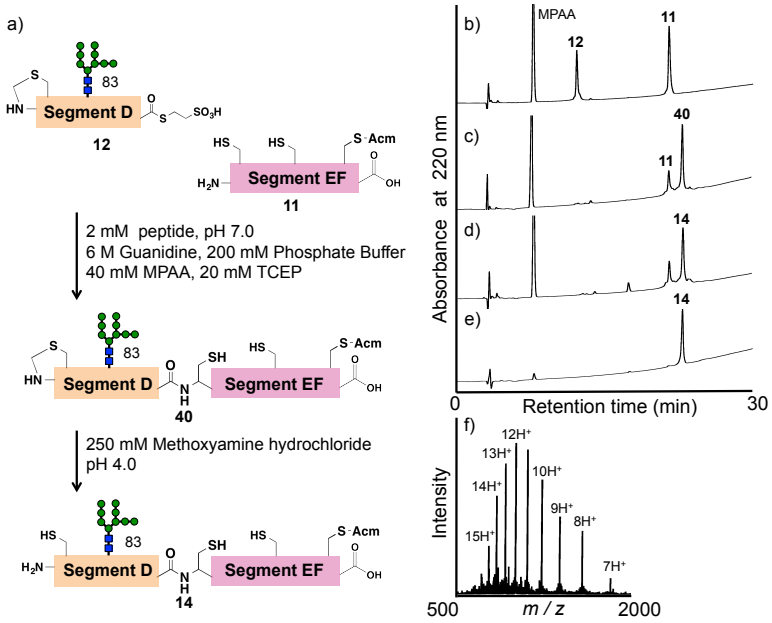

Figure 2-20. Analytical data of monitoring 2nd-NCL. a) Scheme of peptide ligation reaction between compound **11** and **12**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 4 h. d) HPLC profile after treatment with methoxyamine-HCl. e) HPLC profile of purified compound **14**. f) ESI-MS of compound **14**. ESI-MS: *m/z* calcd. for $C_{553}H_{912}N_{143}O_{195}S_4$: $[M+H]^+$ 12812.3, found for 12812.5 (deconvoluted).

2-4-3. Ligation reaction between segment C and segment DEF

Segment C and DEF were ligated by the same procedure as above. Segment C and DEF were dissolved in 200 mM phosphate buffer containing 6 M Gn, 40 mM MPAA and 20 mM TCEP-HCl. The reaction was started at pH 6.8 in room temperature and chased by HPLC and ESI-MS as shown in figure 2-21. The reaction was finished after 2.5 hours.

After the reaction, thiazolidine was removed by 200 mM methoxyamine-HCl at pH4. The completion of the reaction was confirmed by HPLC and ESI-MS after 2 hours (Fig. 2-21). Desired segment CDEF was obtained after purification.

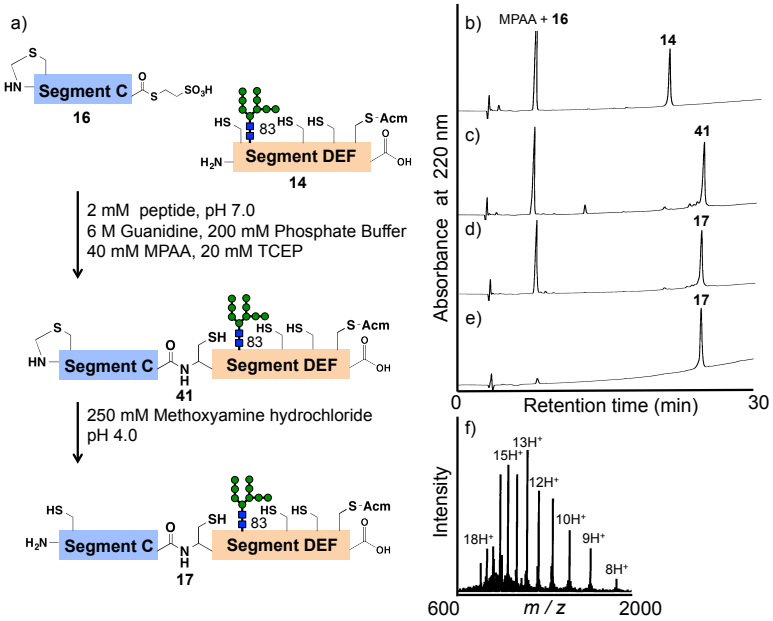

Figure 2-21. Analytical data of monitoring 3rd-NCL. a) Scheme of peptide ligation reaction between compound **14** and **16**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 2.5 h. d) HPLC profile after treatment with methoxyamine-HCl. e) HPLC profile of purified compound **17**. f) ESI-MS of compound **17**. ESI-MS: *m/z* calcd. for $C_{648}H_{1057}N_{170}O_{220}S_6$: $[M+H]^+$ 14941.8, found for 14942.8 (deconvoluted).

2-4-4. Ligation reaction between glycopeptide segment B and segment CDEF

Glycopeptide segment B and CDEF were ligated by the same procedure as above. Segment 2 and 3456 were dissolved in 200 mM phosphate buffer containing 6 M Gn, 40 mM MPAA and 20 mM TCEP-HCl. The reaction was started at pH 6.8 in room temperature and chased by HPLC and ESI-MS as shown in figure 2-22. The reaction was finished after 5 hours. It took a long time to complete the reaction. I assumed that this is because the segment became large and the reactivity was decrease. The desired segment BCDEF was obtained after purification.

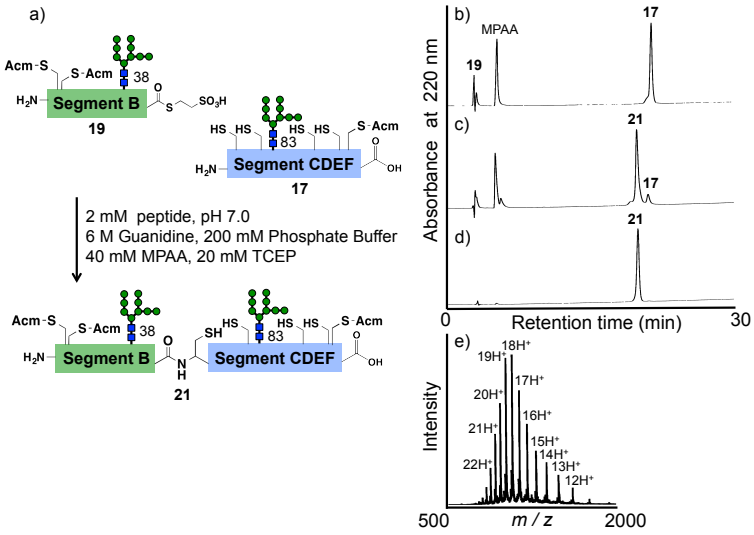

Figure 2-22. Analytical data of monitoring 4th-NCL. a) Scheme of peptide ligation reaction between compound **17** and **19**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 5 h. d) HPLC profile of purified compound **21**. e) ESI-MS spectrum of compound **21**. ESI-MS: *m/z* calcd. for $C_{827}H_{1338}N_{201}O_{311}S_8$: $[M+H]^+$ 19329.2, found for 19329.2 (deconvoluted).

2-4-5. Radical reductions of cysteine residues (position at 50, 68, 98 and 128) of compound **21** to alanine residues

Although a M9-glycopeptide segment BCDEF (compound **21**) has four cysteine residues, which is ligation positions, these are alanine residues in original sequence. Thus, these cysteines should be converted to alanines. As I mentioned in section 2-1-2, I used the radical reduction method, which is developed by Danishefsky group. M9-glycopeptide segment BCDEF was dissolved to phosphate buffer containing 6 M guanidine. TCEP (500 mM), 2-methyl-2-propanethiol as proton source and 2, 2'-azobis [2-(2-imidazoline-2-yl) propane] dihydrochloride (VA-044) (0.1 M) as a radical initiator were added to the solution and it was incubated at 37ºC for 4 h. This reaction was monitored by HPLC and ESI-MS as shown in Figure 2-23. The reduced segment BCDEF (compound **25**) was obtained after purification.

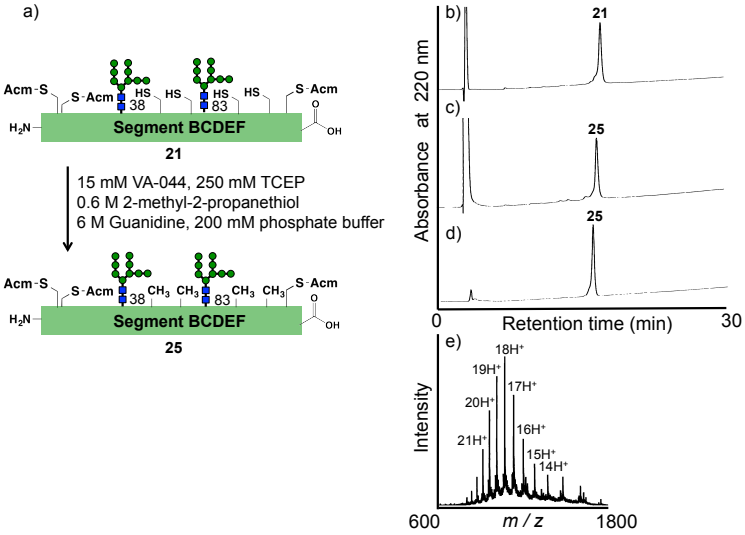

Figure 2-23. Analytical data of monitoring desulfurization of glycopeptide segment BCDEF. a) Scheme of desulfurization reaction of compound **21**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 4 h. d) HPLC profile of purified compound **25**. e) ESI-MS spectrum of compound **25**. ESI-MS: *m/z* calcd. for $C_{827}H_{1338}N_{201}O_{311}S_4$: $[M+H]^+$ 19200.9, found for 19200.9 (deconvoluted).

2-4-6. Deprotection of acetamidomethyl (Acm) groups (position at 29, 33 and 161) of compound **25**

The Acm groups, which protect cysteine residues needed for disulfide bonds formation, were removed by using silver acetate. Reduced M9-glycopeptide segment BCDEF (compound **25**) was dissolved to 90% acetic acid and silver acetate was added to the solution. After 4 h, excess amount of dithiothreitol (DTT) was added to quench the reaction. Then the complex of silver acetate and DTT was precipitated. After removal of the brawn precipitate by centrifugation, I confirmed the target by analyzing supernatant using HPLC and ESI-MS (Fig. 2-24). The Acm-deprotected segment BCDEF (compound **29**) was obtained after purification.

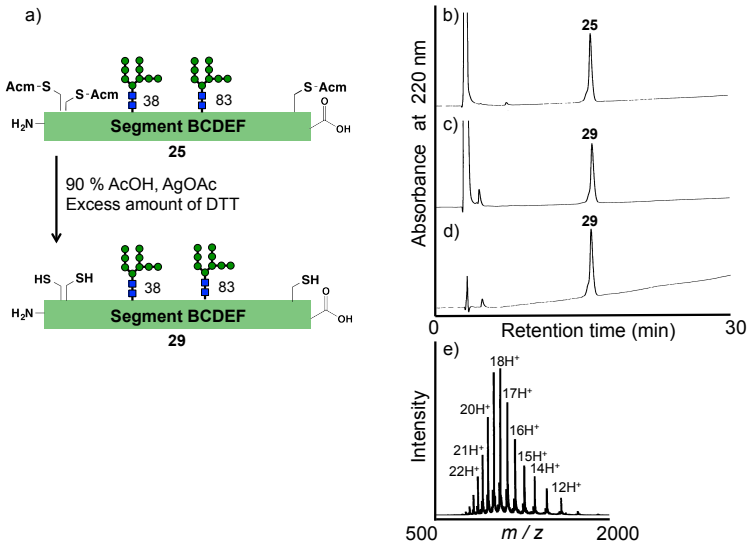

Figure 2-24. Analytical data of monitoring deprotection of Acm groups of glycopeptide segment BCDEF. a) Scheme of deprotection of Acm groups of compound **25**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 4 h. d) HPLC profile of purified compound **29**. e) ESI-MS spectrum after purification. ESI-MS: *m/z* calcd. for $C_{818}H_{1323}N_{198}O_{308}S_4$: $[M+H]^+$ 18987.7 found for 18987.4 (deconvoluted).

2-4-7. Ligation reaction between glycopeptide segment A and glycopeptide segment BCDEF

Segment A and BCDEF were ligated by the same procedure as above. Segment A and BCDEF were dissolved in 200 mM phosphate buffer containing 6 M Gn, 40 mM MPAA and 20 mM TCEP-HCl. The reaction was started at pH 7 in room temperature and chased by HPLC and ESI-MS as shown in figure 2-25. The reaction was finished after 10 hours. The whole glycosylated polypeptide of EPO (segment ABCDEF, compound **35**) was obtained after purification.

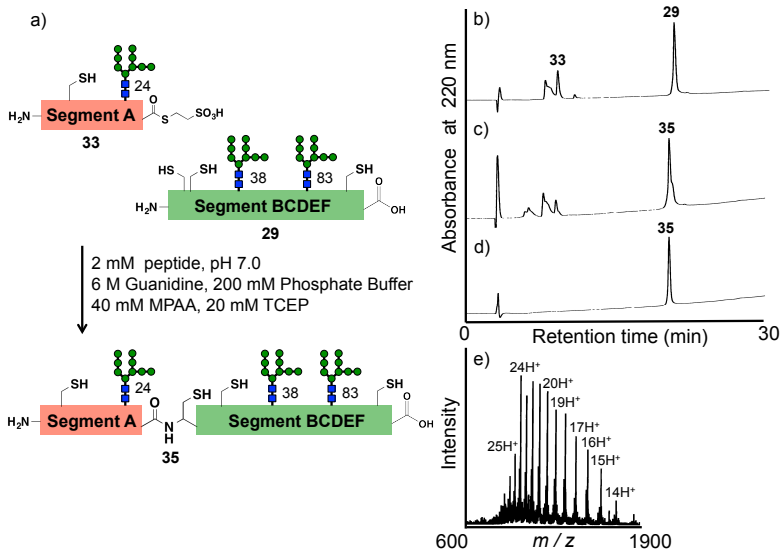

Figure 2-25. Analytical data of monitoring final NCL. a) Scheme of peptide ligation reaction between compound **29** and **33**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 10 h. d) HPLC profile of purified compound **35**. e) ESI-MS spectrum of compound **35**. ESI-MS: *m/z* calcd. for $C_{1025}H_{1666}N_{239}O_{406}S_5$: $[M+H]^+$ 23993.9, found for 23995.7 (deconvoluted).

2-4-8. Folding procedure of the whole glycosylated polypeptide (compound **35**) of EPO

Folding of the whole glycosylated polypeptide was conducted by using stepwise dialysis method, which is used as folding of EPO previously[12]. The glycosylated polypeptide (compound **35**) was dissolved to 100 mM tris buffer containing 6 M Gn and put it to dialysis membrane. The concentration of glycosylated polypeptide was adjusted to 0.1 mg/ml. The glycosylated polypeptide became completely linear by dissolving it to this solution. Decreasing the concentration of denature reagent by dialysis lead the glycosylated polypeptide to correct three-dimensional structure. HPLC and ESI-MS confirm the desired correctly folded EPO (compound **1**) (Figure 2-26). The change of peak pattern of ESI-MS was observed by the change of charge on protein surface after the folding. Misfolded EPO (compound **2**) was obtained simultaneously. Specific MS spectrum of it was not observed, which is typical MS pattern of aggregated protein. Correctly folded EPO and misfolded one were purified and isolated successfully.

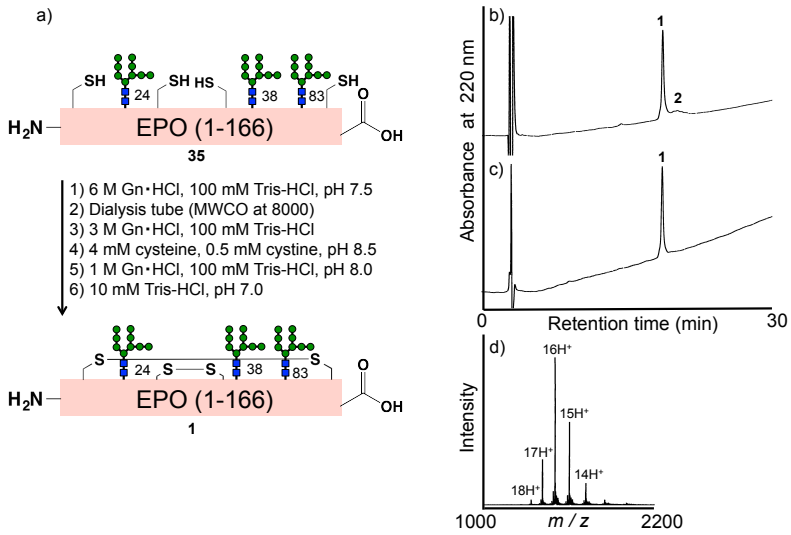

Figure 2-26. Analytical data of folding of segment ABCDEF. a) Scheme of the folding of compound **35**. b) HPLC profile after folding process. c) HPLC profile of purified correctly folded EPO (compound **1**). d) ESI-MS spectrum of correctly folded EPO (compound **1**). ESI-MS: *m/z* calcd. for $C_{1025}H_{1662}N_{239}O_{406}S_5$: $[M+H]^+$ 23989.5730, found for 23989.5022 (deconvoluted).

2-5. Construction of EPO bearing one M9-high-mannose type oligosaccharide (EPO-24)

EPO bearing one oligosaccharide was synthesized by the same procedure as the synthesis of EPO bearing three oligosaccharides.

2-5-1. Ligation reaction between segment E and segment F

This reaction was conducted by the same manner as section 2-4-1. This reaction showed a good reproducibility.

2-5-2. Ligation reaction between peptide segment D and segment EF

Peptide segment D and EF were ligated by the same procedure as above. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-27.

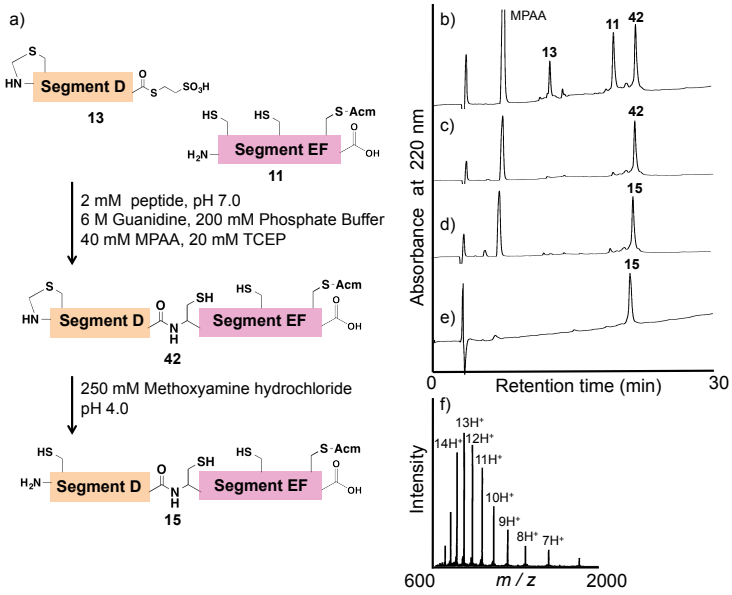

Figure 2-27. Analytical data of monitoring 2nd-NCL. a) Scheme of peptide ligation reaction between compound **11** and **13**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 2.5 h. d) HPLC profile after treatment with methoxyamine-HCl. e) HPLC profile of purified compound **15**. f) ESI-MS spectrum of compound **15**. ESI-MS: *m/z* calcd. for $C_{485}H_{802}N_{141}O_{139}S_4$: $[M+H]^+$ 10960.7, found for 10960.4 (deconvoluted).

2-5-3. Ligation reaction between peptide segment C and segment DEF

Peptide segment C and DEF were ligated by the same procedure as above. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-28.

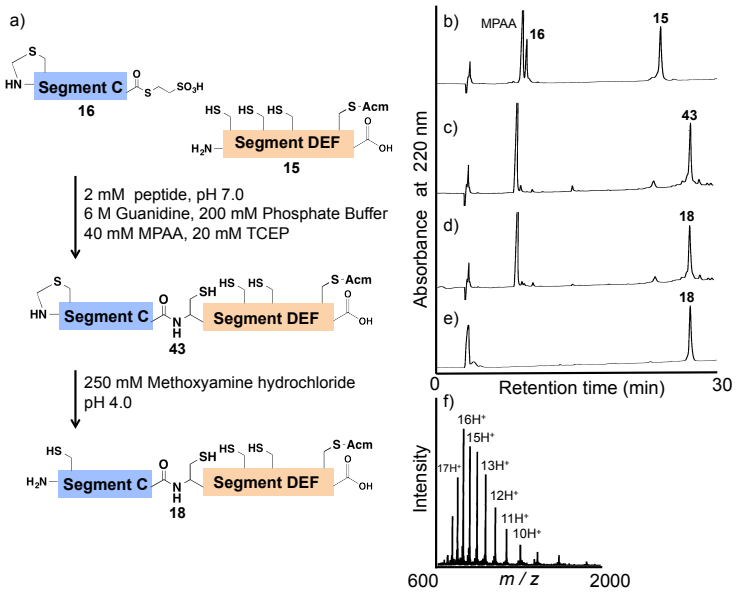

Figure 2-28. Analytical data of monitoring 3rd-NCL. a) Scheme of peptide ligation reaction between compound **15** and **16**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 3 h. d) HPLC profile after treatment with methoxyamine-HCl. e) HPLC profile of compound **18.** f) ESI-MS spectrum of compound **18**. ESI-MS: *m/z* calcd. for $C_{580}H_{947}N_{168}O_{164}S_6$: $[M+H]^+$ 13089.1, found for 13088.6 (deconvoluted).

2-5-4. Ligation reaction between peptide segment B and segment CDEF

Peptide segment B and CDEF were ligated by the same procedure as above. After the completion of the reaction, MESNa was added to remove the branched byproducts. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-29.

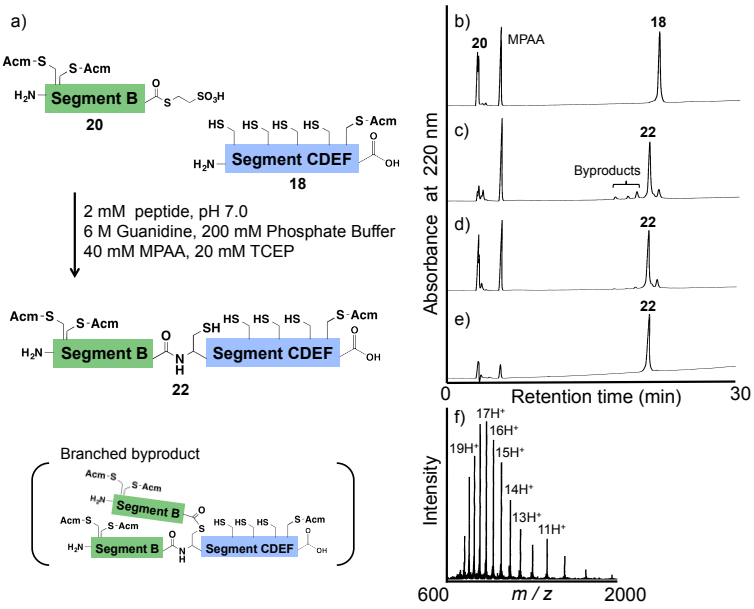

Figure 2-29. Analytical data of monitoring 4th-NCL. a) Scheme of peptide ligation reaction between compound **18** and **20**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 3.5 h. d) HPLC profile after treatment with MESNa. e) HPLC profile of compound **22**. f) ESI-MS spectrum of compound **22**. ESI-MS: *m/z* calcd. for $C_{691}H_{1118}N_{197}O_{199}S_8$: $[M+H]^+$ 15626.0, found for 15626.0 (deconvoluted).

2-5-5. Radical reductions of cysteine residues (position at 50, 68, 98 and 128) of compound **22** to alanine residues

Reductions of four cysteines of compound **22** to alanines were conducted by the same manner as the synthesis of EPO bearing three oligosaccharides. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-30.

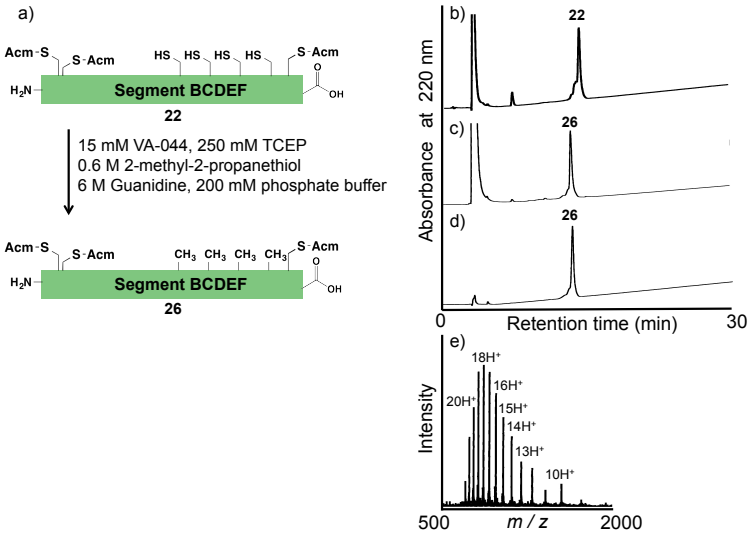

Figure 2-30. Analytical data of monitoring desulfurization of glycopeptide segment BCDEF. a) Scheme of desulfurization reaction of compound **22**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 5 h. d) HPLC profile of purified compound **26**. e) ESI-MS spectrum of compound **26**. ESI-MS: *m/z* calcd. for $C_{691}H_{1118}N_{197}O_{199}S_4$: $[M+H]^+$ 15497.8, found for 15498.6 (deconvoluted).

2-5-6. Deprotection of acetamidomethyl (Acm) groups (position at 29, 33 and 161) of compound **26**

Removals of four Acm groups of compound **26** were conducted by the same manner as the synthesis of EPO bearing three oligosaccharides. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-31.

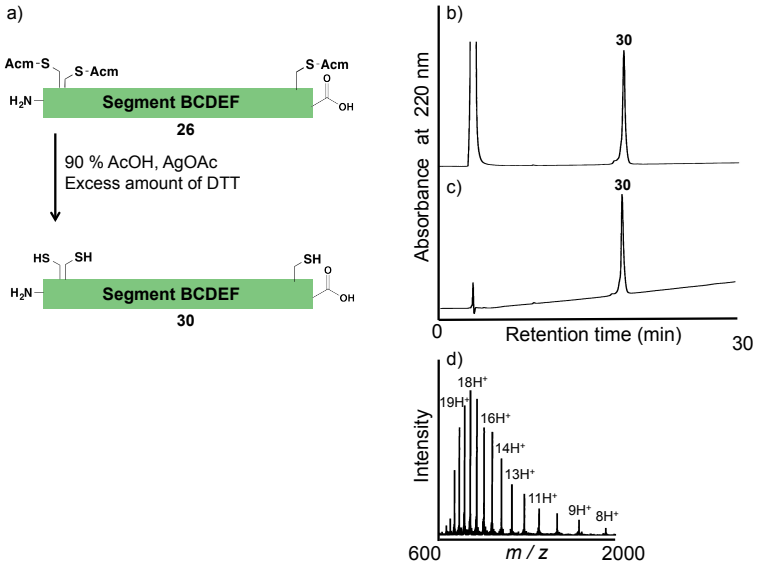

Figure 2-31. Analytical data of monitoring deprotection of Acm groups of glycopeptide segment BCDEF. a) Scheme of deprotection of Acm groups of compound **26**. b) HPLC profile after 4 h. c) HPLC profile of purified compound **30**. d) ESI-MS spectrum of compound **30**. ESI-MS: *m/z* calcd. for $C_{682}H_{1103}N_{194}O_{196}S_4$: $[M+H]^+$ 15284.5, found for 15284.6 (deconvoluted).

2-5-7. Ligation reaction between peptide segment A and glycopeptide segment BCDEF

Segment A and BCDEF were ligated by the same procedure as above. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-32.

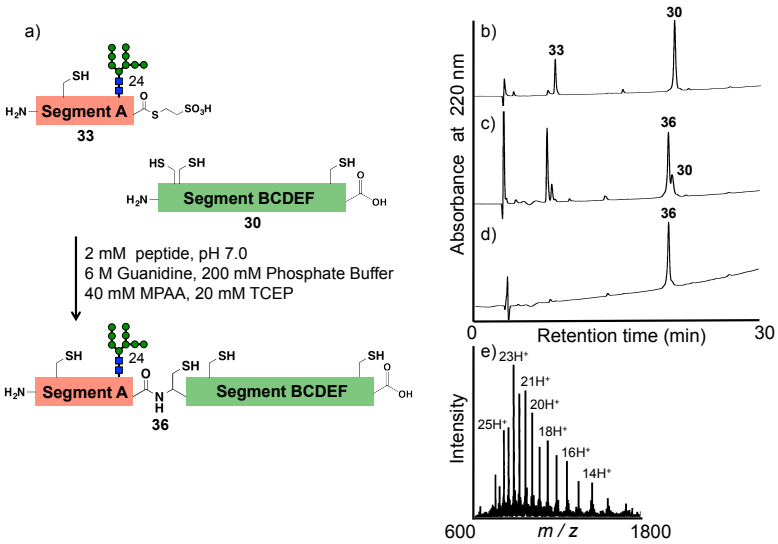

Figure 2-32. Analytical data of monitoring final NCL. a) Scheme of peptide ligation reaction between compound **30** and **33**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 10 h. d) HPLC profile of purified compound **36**. e) ESI-MS spectrum of compound **36**. ESI-MS: *m/z* calcd. for $C_{889}H_{1446}N_{235}O_{294}S_5$: $[M+H]^+$ 20290.8, found for 20290.8 (deconvoluted).

2-5-8. Folding procedure of the whole glycosylated polypeptide (compound **36**) of EPO

Folding of the whole glycosylated polypeptide (compound **36**) was conducted by the same procedure as section 2-4-8. Both correctly folded (compound **3**) and misfolded EPO (compound **4**) were obtained successfully. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-33.

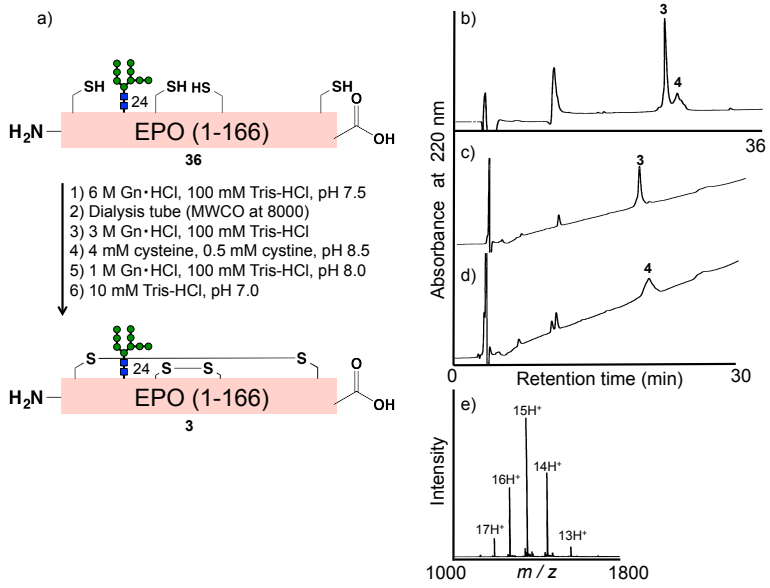

Figure 2-33. Analytical data of folding of segment ABCDEF. a) Scheme of folding of compound **36**. b) HPLC profile after folding process. c) HPLC profile of purified correctly folded EPO (compound **3**). d) HPLC profile of purified misfolded EPO (compound **4**). e) ESI-MS spectrum of compound **4**. FT-ICR-MS: *m/z* calcd. for $C_{889}H_{1442}N_{235}O_{294}S_5$: $[M+H]^+$ 20285.3948, found for 20285.4012 (deconvoluted)

2-6. Construction of EPO bearing one high-mannose type oligosaccharide (EPO-38)

EPO-38 was synthesized by the same procedure as the synthesis of EPO-24.

2-6-1. Ligation reaction between segment E and segment F

This reaction was conducted by the same manner as section 2-4-1 and 2-5-1. This reaction showed a good reproducibility.

2-6-2. Ligation reaction between segment D and segment EF

This reaction was conducted by the same manner as section 2-5-2. This reaction showed a good reproducibility.

2-6-3. Ligation reaction between segment C and segment DEF

This reaction was conducted by the same manner as section 2-5-3. This reaction showed a good reproducibility.

2-6-4. Ligation reaction between glycopeptide segment B and segment CDEF

Glycopeptide segment B and CDEF were ligated by the same procedure as above. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-34.

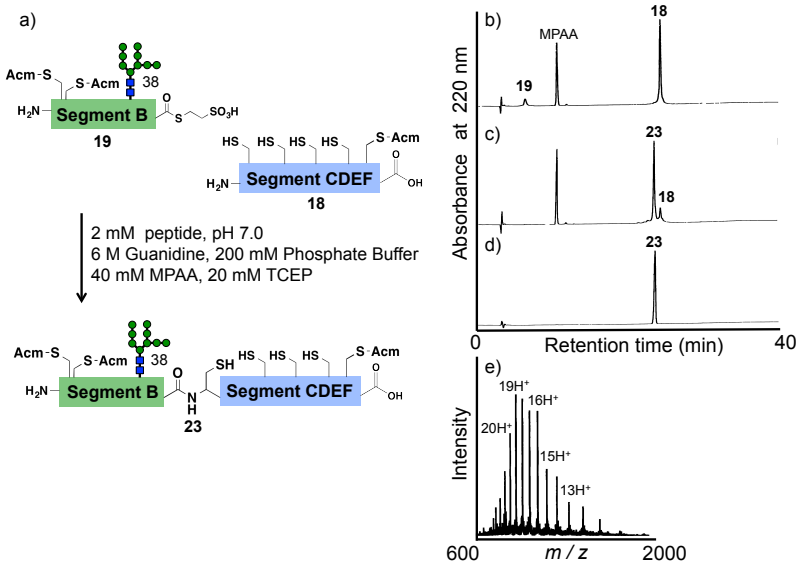

Figure 2-34. Analytical data of monitoring 4th-NCL. a) Scheme of peptide ligation reaction between compound **18** and **19**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 7 h. d) HPLC profile after treatment with methoxyamine-HCl. e) HPLC profile of purified compound **23**. f) ESI-MS spectrum of compound **23**. ESI-MS: *m/z* calcd. for $C_{759}H_{1228}N_{199}O_{255}S_8$: $[M+H]^+$ 17477.6, found for 17476.0 (deconvoluted).

2-6-5. Radical reductions of cysteine residues (position at 50, 68, 98 and 128) of compound **23** to alanine residues

Reductions of four cysteines of compound **23** to alanines were conducted by the same manner as the synthesis of EPO bearing three oligosaccharides. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-35.

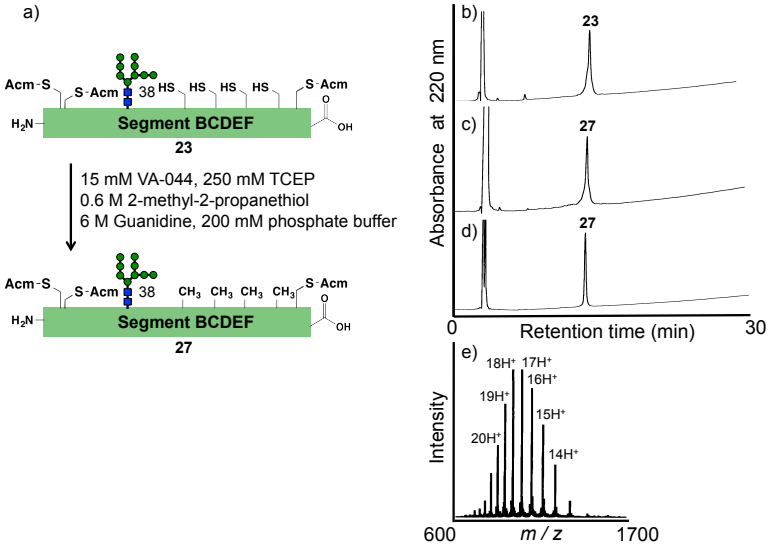

Figure 2-35. Analytical data of monitoring desulfurization of glycopeptide segment BCDEF. a) Scheme of desulfurization reaction of compound **23**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 3.5 h. d) HPLC profile of purifided compound **27**. e) ESI-MS spectrum of compound **27**. ESI-MS: *m/z* calcd. for $C_{759}H_{1228}N_{199}O_{255}S_4$: $[M+H]^+$ 17349.4, found for 17348.3 (deconvoluted).

2-6-6. Deprotection of acetamidomethyl (Acm) groups (position at 29, 33 and 161) of compound **27**

Removals of four Acm groups of compound **27** were conducted by the same manner as the synthesis of EPO bearing three oligosaccharides. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-36.

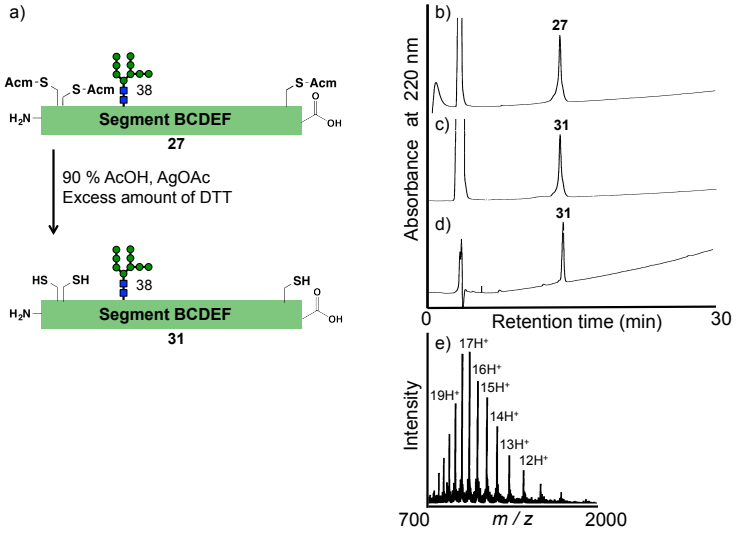

Figure 2-36. Analytical data of monitoring deprotection of Acm groups of glycopeptide segment BCDEF. a) Scheme of deprotection of Acm groups of compound **27**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 3.5 h. d) HPLC profile of purified compound **31**. e) ESI-MS spectrum of compound **31**. ESI-MS: *m/z* calcd. for $C_{750}H_{1213}N_{196}O_{252}S_4$: $[M+H]^+$ 17136.1, found for 17134.7 (deconvoluted).

2-6-7. Ligation reaction between peptide segment A and glycopeptide segment BCDEF

Segment A and BCDEF were ligated by the same procedure as above. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-37.

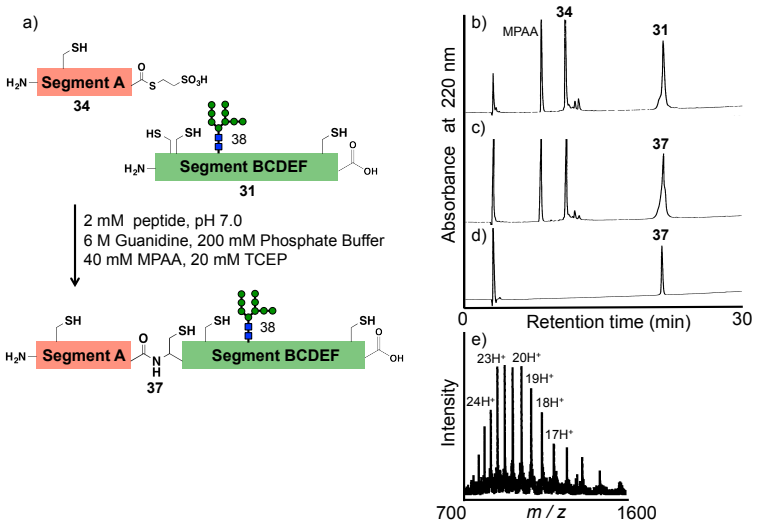

Figure 2-37. Analytical data of monitoring final NCL. a) Scheme of peptide ligation reaction between compound **31** and **34**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 5.5 h. d) HPLC profile of purified compound **37**. e) ESI-MS spectrum of compound **37**. ESI-MS: *m/z* calcd. for $C_{889}H_{1446}N_{235}O_{294}S_5$: $[M+H]^+$ 20290.8, found for 20289.2 (deconvoluted).

2-6-8. Folding procedure of the whole glycosylated polypeptide (compound **37**) of EPO

Folding of the whole glycosylated polypeptide (compound **37**) was conducted by the same procedure as above. Both correctly folded (compound **5**) and misfolded EPO (compound **6**) were obtained successfully. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-38.

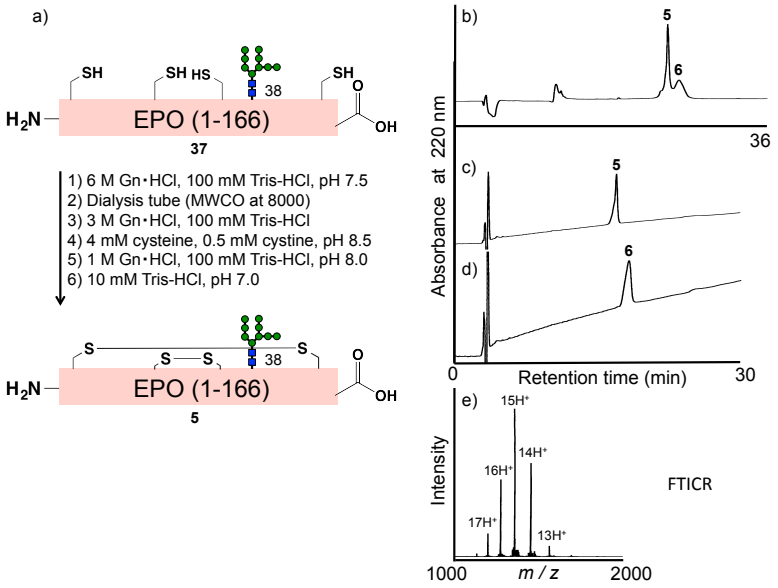

Figure 2-38. Analytical data of folding of segment ABCDEF. a) Scheme of folding of compound **37**. b) HPLC profile after folding process. c) HPLC profile of purified correctly folded EPO (compound **5**). d) HPLC profile of purified misfolded EPO (compound **6**). e) ESI-MS spectrum of compound **5**. FT-ICR-MS: *m/z* calcd. for $C_{889}H_{1442}N_{235}O_{294}S_5$: $[M+H]^+$ 20285.3948, found for 20285.3562 (deconvoluted)

2-7. Construction of EPO bearing one high-mannose type oligosaccharide (EPO-83)

EPO-83 was synthesized by the same procedure as the synthesis of EPO-24 and EPO-38.

2-7-1. Ligation reaction between segment E and segment F

This reaction was conducted by the same manner as section 2-4-1, 2-5-1 and 2-6-1. This reaction showed a good reproducibility.

2-7-2. Ligation reaction between segment D and segment EF

This reaction was conducted by the same manner as section 2-5-2 and 2-6-2. This reaction showed a good reproducibility.

2-7-3. Ligation reaction between segment C and segment DEF

This reaction was conducted by the same manner as section 2-5-3 and 2-6-3. This reaction showed a good reproducibility.

2-7-4. Ligation reaction between peptide segment B and segment CDEF

Peptide segment B and CDEF were ligated by the same procedure as above. After the completion of the reaction, MESNa was added to remove the branched byproducts. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-39.

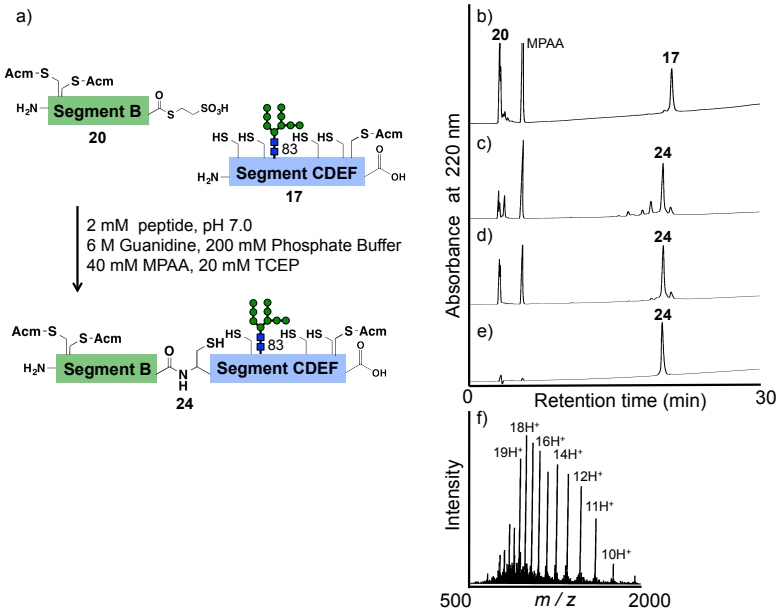

Figure 2-39. Analytical data of monitoring 4th-NCL. a) Scheme of peptide ligation reaction between compound **17** and **20**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 4.5 h. d) HPLC profile after treatment with MESNa. e) HPLC profile of purified compound **24**. f) ESI-MS spectrum of compound **24**. ESI-MS: *m/z* calcd. for $C_{759}H_{1228}N_{199}O_{255}S_8$: $[M+H]^+$ 17477.6, found for 17478.9 (deconvoluted).

2-7-5. Radical reductions of cysteine residues (position at 50, 68, 98 and 128) of compound **24** to alanine residues

Reductions of four cysteines of compound **24** to alanines were conducted by the same manner as the synthesis of EPO bearing three oligosaccharides. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-40.

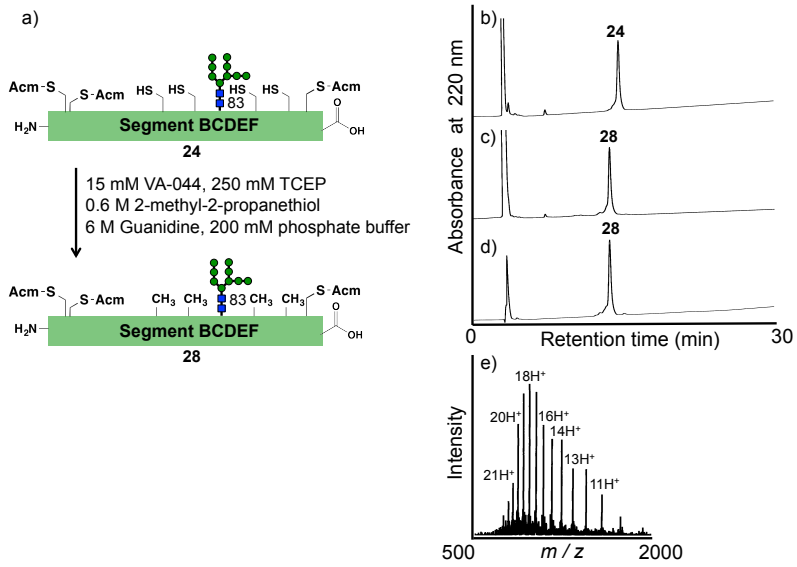

Figure 2-40. Analytical data of monitoring desulfurization of glycopeptide segment BCDEF. a) Scheme of desulfurization reaction of compound **24**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 3 h. d) HPLC profile of purified compound **28**. e) ESI-MS spectrum of compound **28**. ESI-MS: *m/z* calcd. for $C_{759}H_{1228}N_{199}O_{255}S_4$: $[M+H]^+$ 17349.4, found for 17349.7 (deconvoluted).

2-7-6. Deprotection of acetamidomethyl (Acm) groups (position at 29, 33 and 161) of compound **28**

Removals of four Acm groups of compound **28** were conducted by the same manner as the synthesis of EPO bearing three oligosaccharides. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-41.

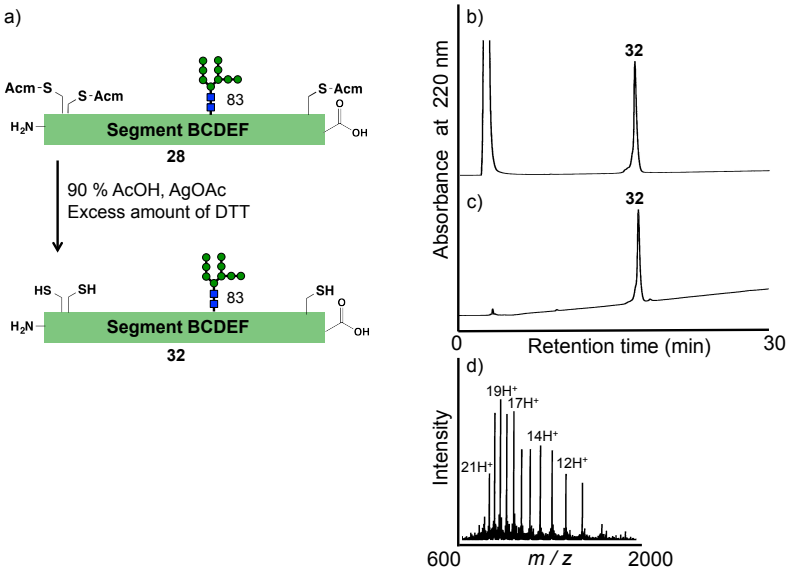

Figure 2-41. Analytical data of monitoring deprotection of Acm groups of glycopeptide segment BCDEF.

a) Scheme of deprotection of Acm group of compound **28**. b) HPLC profile after 4 h. c) HPLC profile of purified compound **32**. d) ESI-MS spectrum of compound **32**. ESI-MS: *m/z* calcd. for $C_{750}H_{1213}N_{196}O_{252}S_4$: $[M+H]^+$ 17136.1, found for 17136.5 (deconvoluted).

2-7-7. Ligation reaction between peptide segment A and glycopeptide segment BCDEF

Segment A and BCDEF were ligated by the same procedure as above. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-42.

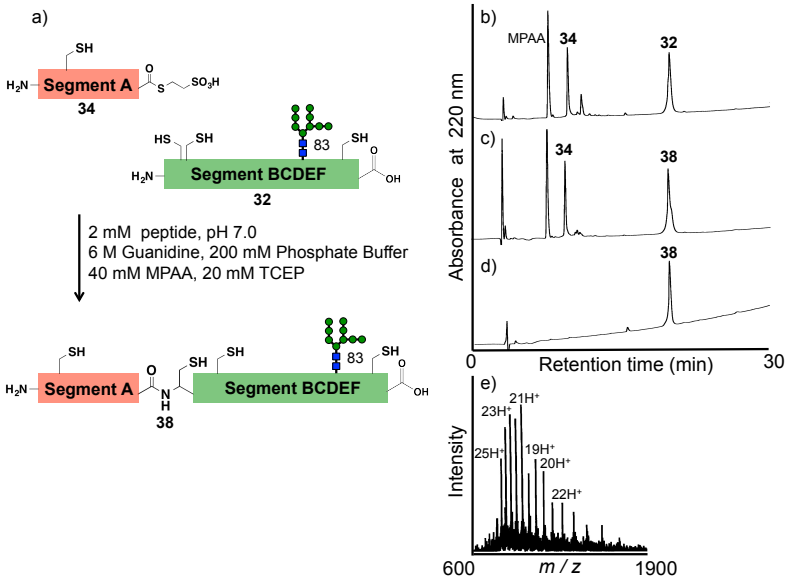

Figure 2-42. Analytical data of monitoring final NCL. a) Scheme of peptide ligation reaction between compound **32** and **34**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 10 h. d) HPLC profile of purified compound **38**. e) ESI-MS spectrum of compound **38**. ESI-MS: *m/z* calcd. for $C_{889}H_{1446}N_{235}O_{294}S_5$: $[M+H]^+$ 20290.8, found for 20290.9 (deconvoluted).

2-7-8. Folding procedure of the whole glycosylated polypeptide (compound **38**) of EPO

Folding of the whole glycosylated polypeptide (compound **38**) was conducted by the same procedure as above. Both correctly folded (compound **7**) and misfolded EPO (compound **8**) were obtained successfully. The HPLC profiles and MS spectrum of this reaction were shown in Figure 2-43.

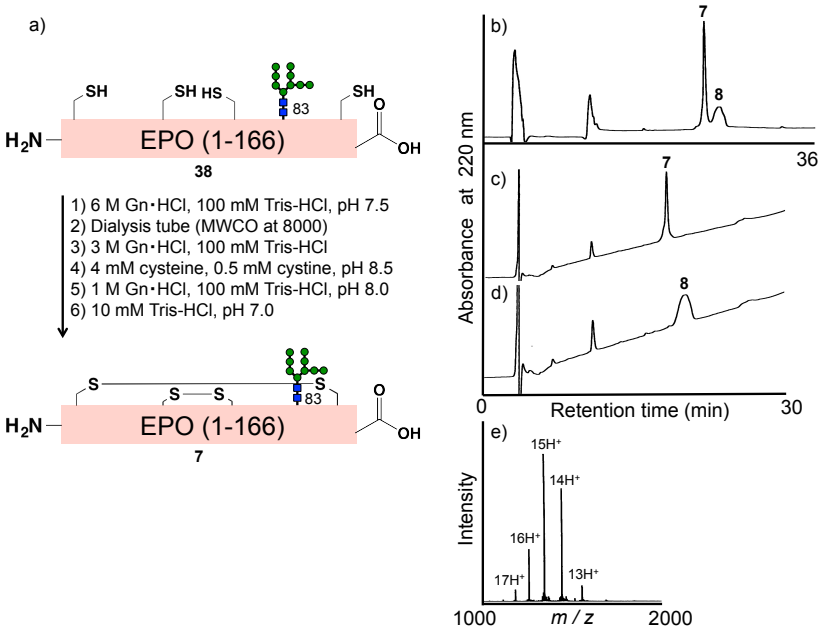

Figure 2-43. Analytical data of folding of segment ABCDEF. a) Scheme of folding of compound **38**. b) HPLC profile after folding process. c) HPLC profile of purified correctly folded EPO (compound **7**). d) HPLC profile of purified misfolded EPO (compound **8**). e) ESI-MS spectrum of compound **7**. FT-ICR-MS: *m/z* calcd. for $C_{889}H_{1442}N_{235}O_{294}S_5$: $[M+H]^+$ 20285.3948, found for 20285.3514 (deconvoluted)

2-8. Characterization of synthetic EPO analogs (correctly folded and misfolded)

2-8-1. Disulfide mapping

Disulfide bond positions of correctly folded and misfolded EPO-24, EPO-38, EPO-83 and EPO-24, 38, 83 were determined by trypsin digestion method. These EPO analogs were dissolved to 0.1 M phosphate buffer (pH 7.3) and suitable amount of trypsin were added to the solution. Trypsin is an enzyme that can hydrolyze amide bonds next to basic amino acids (lysine and arginine) specifically. This solution was incubated at 37ºC for 12 h and obtained peptide fragments were analyzed by LC-MS (from Fig. 2-44 to Fig. 2-51). The resultant fragments are different dependent on EPO analogs, because EPO-24, EPO-38 and EPO-83 have extra lysine residues at original glycosylation sites. Subsequently, this solution was treated by 20 mM TCEP, which is reducing reagent to confirm the disulfide bond positions (from Fig. 2-44 to Fig. 2-51).

As a result, it is confirmed that both correctly folded EPO analogs and misfolded ones have correct disulfide bonds. Glycopeptide fragments that don't have disulfide bond were also observed

after digestion of EPO-83 and EPO-24, 38, 83. The results of misfolded EPO analogs are unexpected, because I have planned to scramble the disulfide bonds of misfolded EPO. However, the properties of misfolded EPO analogs by HPLC and MS showed same disulfide bond network, but it might form aggregation. Therefore I used them to the next UGGT assay.

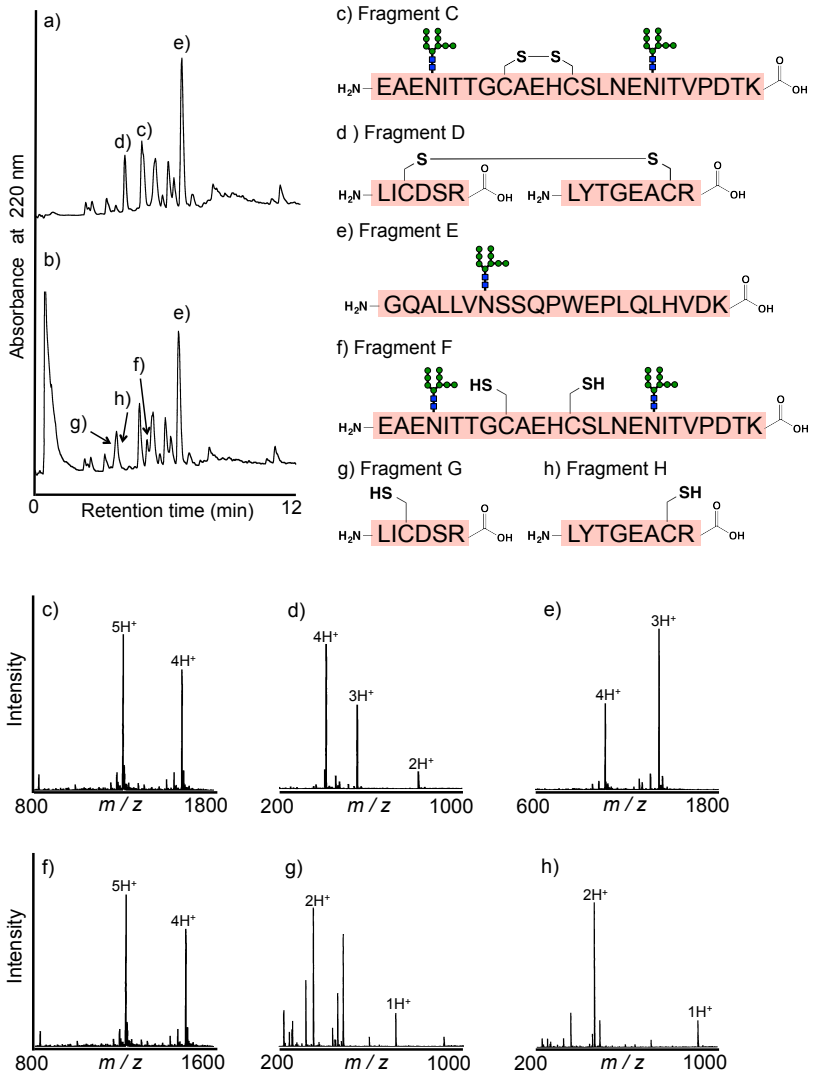

Figure 2-44. HPLC profiles and ESI-MS spectra of the resultant peptide fragments by trypsin digestion from correctly folded EPO-24, 38, 83 (compound **1**). a) HPLC profile after trypsin digestion. b) HPLC profile after TCEP treatment. c) ESI-MS spectrum of Fragment C: ESI-MS: *m/z* calcd. for $C_{249}H_{408}N_{35}O_{154}S_2$: $[M+H]^+$ 6420.2, found for 6419.8 (deconvoluted). d) ESI-MS spectrum of Fragment D: ESI-MS: *m/z* calcd. for $C_{66}H_{111}N_{20}O_{23}S_2$: $[M+H]^+$ 1616.8, found for 1616.3 (deconvoluted). e) ESI-MS spectrum of Fragment E: ESI-MS: *m/z* calcd. for $C_{176}H_{284}N_{31}O_{87}$: $[M+H]^+$ 4223.9, found for 4225.3 (deconvoluted). f) ESI-MS spectrum of Fragment F: ESI-MS: *m/z* calcd. for $C_{249}H_{410}N_{35}O_{154}S_2$: $[M+H]^+$ 6422.2, found for 6421.8 (deconvoluted). g) ESI-MS spectrum of Fragment G: ESI-MS: *m/z* calcd. for $C_{28}H_{52}N_9O_{10}S$: $[M+H]^+$ 706.8, found for 706.7 (deconvoluted). h) ESI-MS spectrum of Fragment H: ESI-MS: *m/z* calcd. for $C_{38}H_{62}N_{11}O_{13}S$: $[M+H]^+$ 912.4, found for 912.7 (deconvoluted).

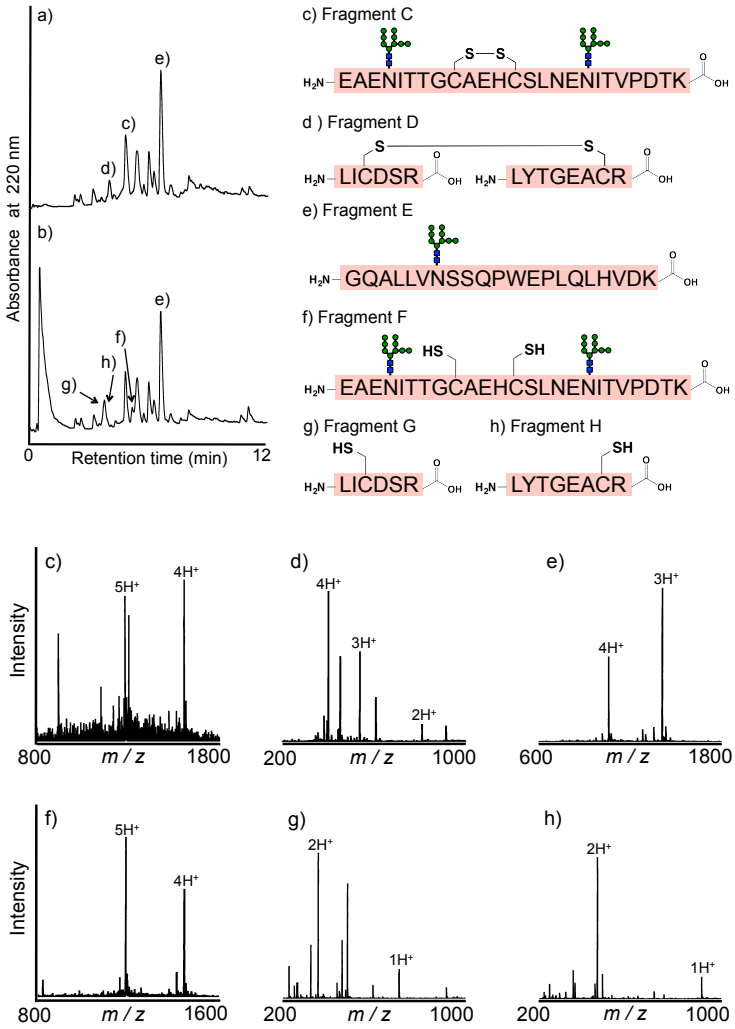

Figure 2-45. HPLC profiles and ESI-MS spectra of the resultant peptide fragments by trypsin digestion from misfolded EPO-24, 38, 83 (compound **2**). a) HPLC profile after trypsin digestion. b) HPLC profile after TCEP treatment. c) ESI-MS spectrum of Fragment C: ESI-MS: *m/z* calcd. for $C_{249}H_{408}N_{35}O_{154}S_2$: $[M+H]^+$ 6420.2, found for 6419.8 (deconvoluted). d) ESI-MS spectrum of Fragment D: ESI-MS: *m/z* calcd. for $C_{66}H_{111}N_{20}O_{23}S_2$: $[M+H]^+$ 1616.8, found for 1616.3 (deconvoluted). e) ESI-MS spectrum of Fragment E: ESI-MS: *m/z* calcd. for $C_{176}H_{284}N_{31}O_{87}$: $[M+H]^+$ 4223.9, found for 4225.7 (deconvoluted). f) ESI-MS spectrum of Fragment F: ESI-MS: *m/z* calcd. for $C_{249}H_{410}N_{35}O_{154}S_2$: $[M+H]^+$ 6422.2, found for 6421.7 (deconvoluted). g) ESI-MS spectrum of Fragment G: ESI-MS: *m/z* calcd. for $C_{28}H_{52}N_9O_{10}S$: $[M+H]^+$ 706.8, found for 706.6 (deconvoluted). h) ESI-MS spectrum of Fragment H: ESI-MS: *m/z* calcd. for $C_{38}H_{62}N_{11}O_{13}S$: $[M+H]^+$ 912.4, found for 912.7 (deconvoluted).

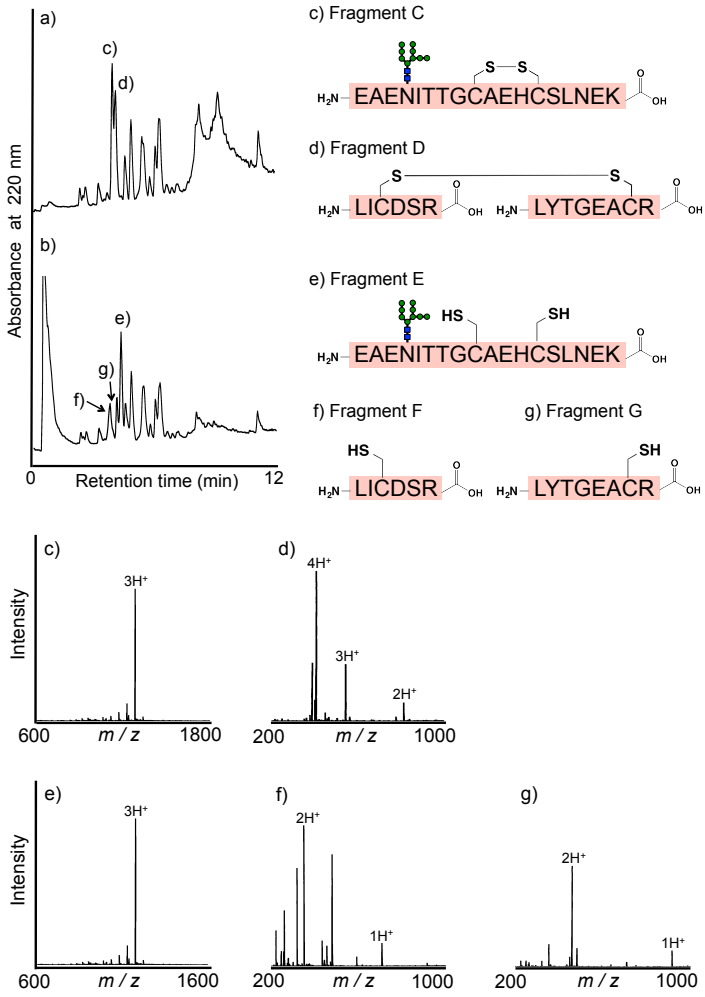

Figure 2-46. HPLC profiles and ESI-MS spectra of the resultant peptide fragments by trypsin digestion from correctly folded EPO-24 (compound **3**). a) HPLC profile after trypsin digestion. b) HPLC profile after TCEP treatment. c) ESI-MS spectrum of Fragment C: ESI-MS: *m/z* calcd. for $C_{147}H_{240}N_{25}O_{87}S_2$: $[M+H]^+$ 3813.7, found for 3815.6 (deconvoluted). d) ESI-MS spectrum of Fragment D: ESI-MS: *m/z* calcd. for $C_{66}H_{111}N_{20}O_{23}S_2$: $[M+H]^+$ 1616.8, found for 1616.3 (deconvoluted). e) ESI-MS spectrum of Fragment E: ESI-MS: *m/z* calcd. for $C_{147}H_{242}N_{25}O_{87}S_2$: $[M+H]^+$ 3815.7, found for 3817.6 (deconvoluted). f) ESI-MS spectrum of Fragment F: ESI-MS: *m/z* calcd. for $C_{28}H_{52}N_9O_{10}S$: $[M+H]^+$ 706.8, found for 706.7 (deconvoluted). g) ESI-MS spectrum of Fragment G: ESI-MS: *m/z* calcd. for $C_{38}H_{62}N_{11}O_{13}S$: $[M+H]^+$ 912.4, found for 912.7 (deconvoluted).

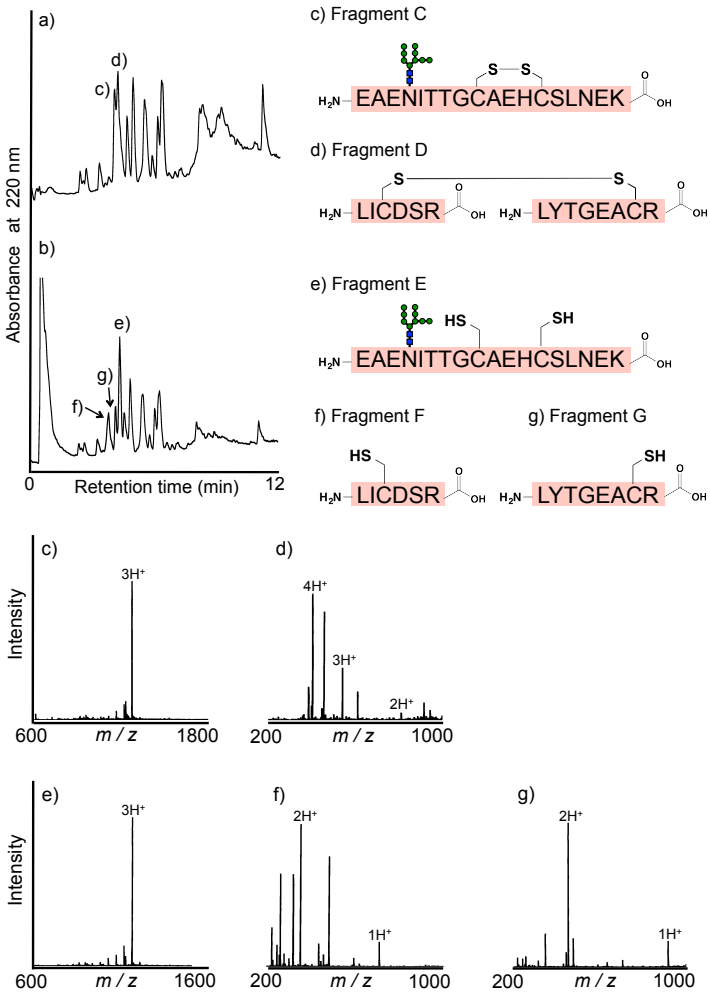

Figure 2-47. HPLC profiles and ESI-MS spectra of the resultant peptide fragments by trypsin digestion from misfolded EPO-24 (comound **4**). a) HPLC profile after trypsin digestion. b) HPLC profile after TCEP treatment. c) ESI-MS spectrum of Fragment C: ESI-MS: *m/z* calcd. for $C_{147}H_{240}N_{25}O_{87}S_2$: $[M+H]^+$ 3813.7, found for 3815.6 (deconvoluted). d) ESI-MS spectrum of Fragment D: ESI-MS: *m/z* calcd. for $C_{66}H_{111}N_{20}O_{23}S_2$: $[M+H]^+$ 1616.8, found for 1616.4 (deconvoluted). e) ESI-MS spectrum of Fragment E: ESI-MS: *m/z* calcd. for $C_{147}H_{242}N_{25}O_{87}S_2$: $[M+H]^+$ 3815.7, found for 3817.7 (deconvoluted). f) ESI-MS spectrum of Fragment F: ESI-MS: *m/z* calcd. for $C_{28}H_{52}N_9O_{10}S$: $[M+H]^+$ 706.8, found for 706.6 (deconvoluted). g) ESI-MS spectrum of Fragment G: ESI-MS: *m/z* calcd. for $C_{38}H_{62}N_{11}O_{13}S$: $[M+H]^+$ 912.4, found for 912.7 (deconvoluted).

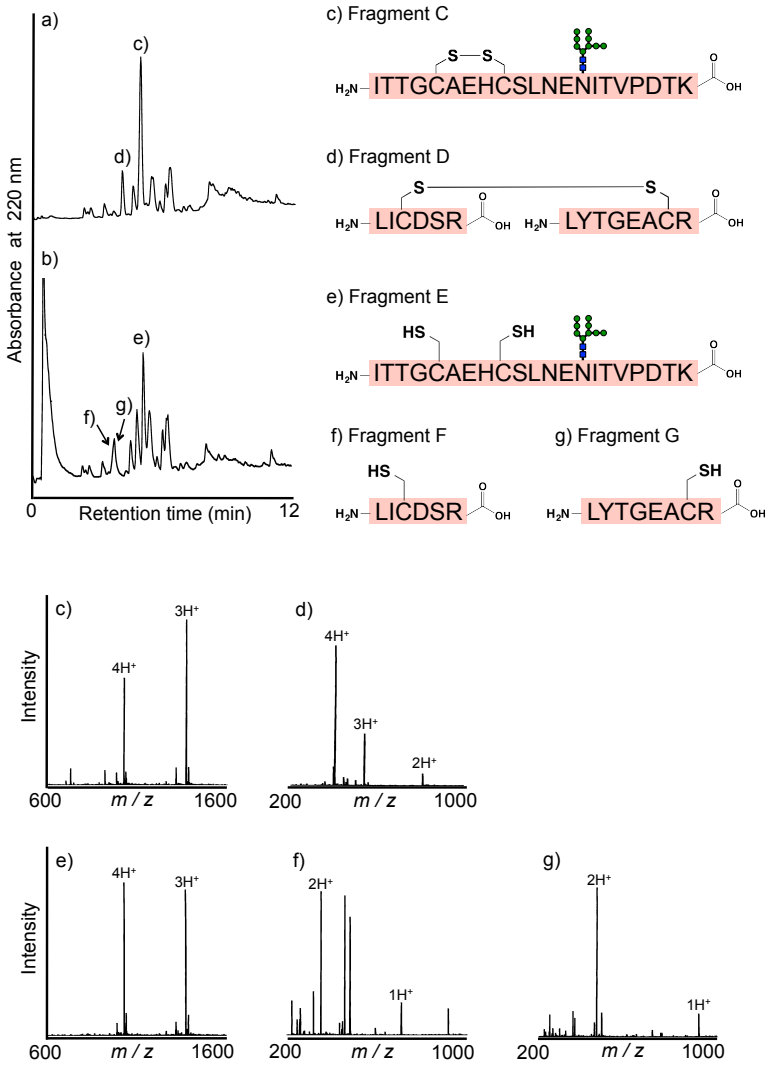

Figure 2-48. HPLC profiles and ESI-MS spectra of the resultant peptide fragments by trypsin digestion from correctly folded EPO-38 (compound **5**). a) HPLC profile after trypsin digestion. b) HPLC profile after TCEP treatment. c) ESI-MS spectrum of Fragment C: ESI-MS: *m/z* calcd. for $C_{162}H_{267}N_{28}O_{90}S_2$: $[M+H]^+$ 4108.7, found for 4809.6 (deconvoluted). d) ESI-MS spectrum of Fragment D: ESI-MS: *m/z* calcd. for $C_{66}H_{111}N_{20}O_{23}S_2$: $[M+H]^+$ 1616.8, found for 1616.3 (deconvoluted). e) ESI-MS spectrum of Fragment E: ESI-MS: *m/z* calcd. for $C_{162}H_{269}N_{28}O_{90}S_2$: $[M+H]^+$ 4110.7, found for 4111.6 (deconvoluted). f) ESI-MS spectrum of Fragment F: ESI-MS: *m/z* calcd. for $C_{28}H_{51}N_9O_{10}S$: $[M+H]^+$ 706.8, found for 706.7 (deconvoluted). g) ESI-MS spectrum of Fragment G: ESI-MS: *m/z* calcd. for $C_{38}H_{61}N_{11}O_{13}S$: $[M+H]^+$ 912.4, found for 912.7 (deconvoluted).

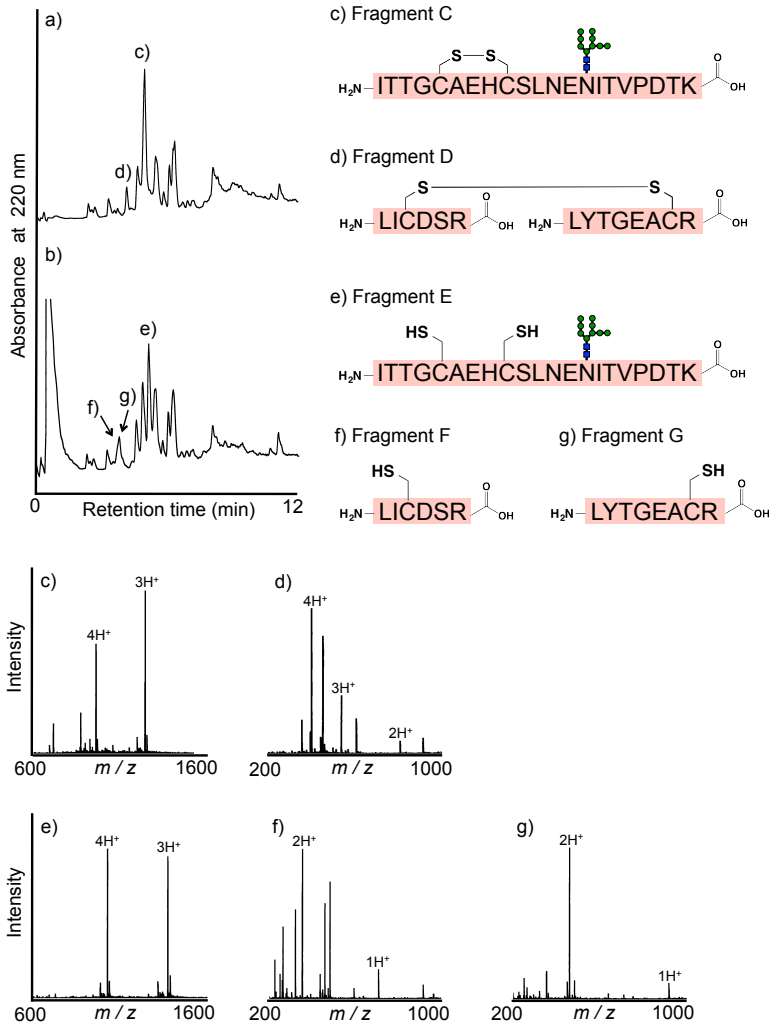

Figure 2-49. HPLC profiles and ESI-MS spectra of the resultant peptide fragments by trypsin digestion from misfolded EPO-38 (compound **6**). a) HPLC profile after trypsin digestion. b) HPLC profile after TCEP treatment. c) ESI-MS spectrum of Fragment C: ESI-MS: *m/z* calcd. for $C_{162}H_{267}N_{28}O_{90}S_2$: $[M+H]^+$ 4108.7, found for 4809.1 (deconvoluted). d) ESI-MS spectrum of Fragment D: ESI-MS: *m/z* calcd. for $C_{66}H_{111}N_{20}O_{23}S_2$: $[M+H]^+$ 1616.8, found for 1616.3 (deconvoluted). e) ESI-MS spectrum of Fragment E: ESI-MS: *m/z* calcd. for $C_{162}H_{269}N_{28}O_{90}S_2$: $[M+H]^+$ 4110.7, found for 4112.1 (deconvoluted). f) ESI-MS spectrum of Fragment F: ESI-MS: *m/z* calcd. for $C_{28}H_{52}N_9O_{10}S$: $[M+H]^+$ 706.8, found for 706.7 (deconvoluted). g) ESI-MS spectrum of Fragment G: ESI-MS: *m/z* calcd. for $C_{38}H_{62}N_{11}O_{13}S$: $[M+H]^+$ 912.4, found for 912.8 (deconvoluted).

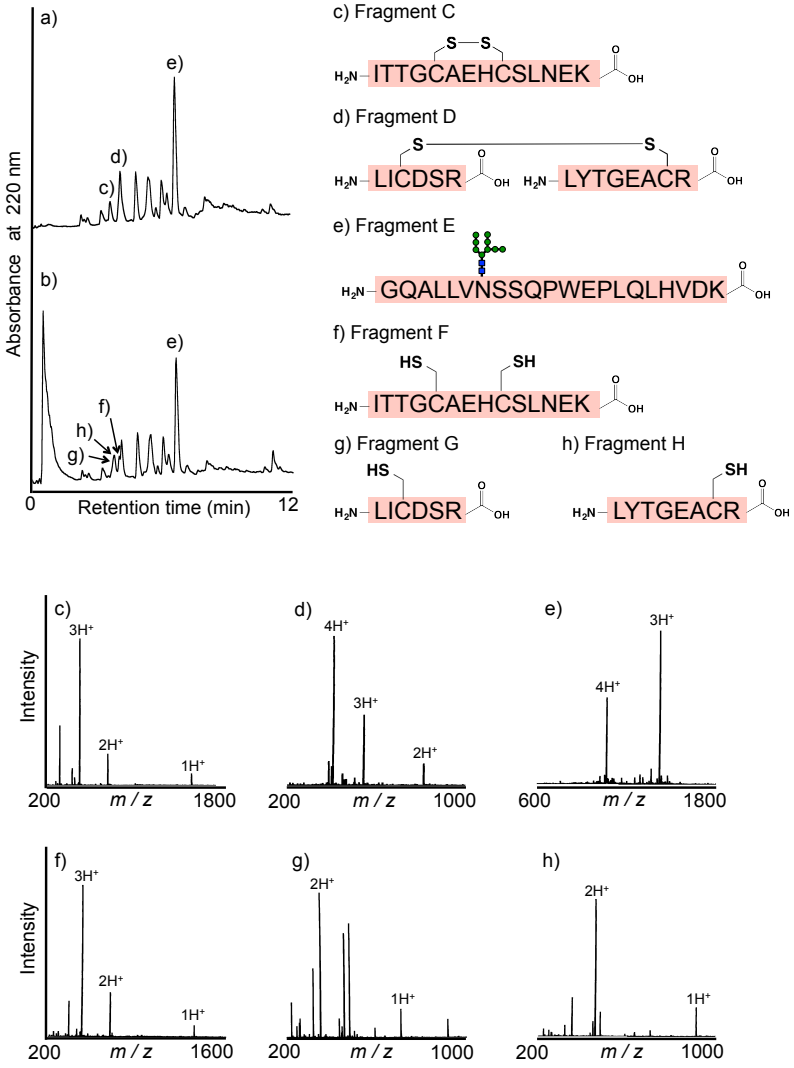

Figure 2-50. HPLC profiles and ESI-MS spectra of the resultant peptide fragments by trypsin digestion from correctly folded EPO-83 (compound **7**). a) HPLC profile after trypsin digestion. b) HPLC profile after TCEP treatment. c) ESI-MS spectrum of Fragment C: ESI-MS: *m/z* calcd. for $C_{60}H_{99}N_{18}O_{23}S_2$: $[M+H]^+$ 1504.7, found for 1504.1 (deconvoluted). d) ESI-MS spectrum of Fragment D: ESI-MS: *m/z* calcd. for $C_{66}H_{111}N_{20}O_{23}S_2$: $[M+H]^+$ 1616.8, found for 1616.3 (deconvoluted). e) ESI-MS spectrum of Fragment E: ESI-MS: *m/z* calcd. for $C_{176}H_{284}N_{31}O_{87}$: $[M+H]^+$ 4223.9, found for 4224.8 (deconvoluted). f) ESI-MS spectrum of Fragment F: ESI-MS: *m/z* calcd. for $C_{60}H_{101}N_{18}O_{23}S_2$: $[M+H]^+$ 1506.7, found for 1506.1 (deconvoluted). g) ESI-MS spectrum of Fragment G: ESI-MS: *m/z* calcd. for $C_{28}H_{52}N_9O_{10}S$: $[M+H]^+$ 706.8, found for 706.6 (deconvoluted). h) ESI-MS spectrum of Fragment H: ESI-MS: *m/z* calcd. for $C_{38}H_{62}N_{11}O_{13}S$: $[M+H]^+$ 912.4, found for 912.7 (deconvoluted).

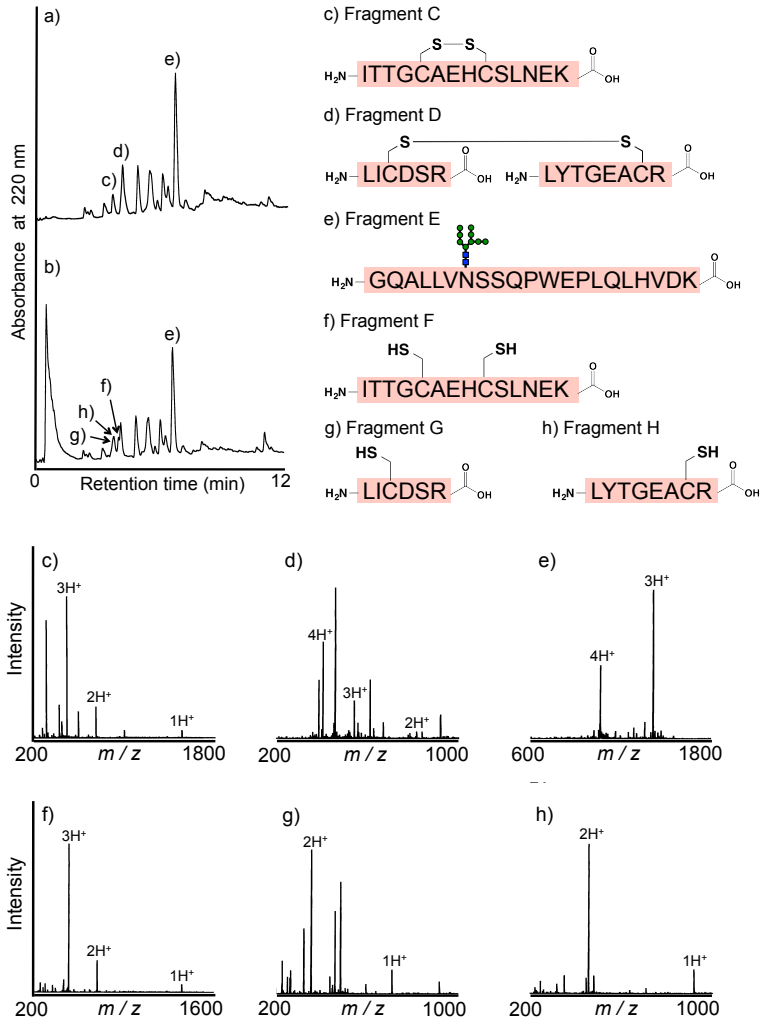

Figure 2-51. HPLC profiles and ESI-MS spectra of the resultant peptide fragments by trypsin digestion from misfolded EPO-83 (compound **8**). a) HPLC profile after trypsin digestion. b) HPLC profile after TCEP treatment. c) ESI-MS spectrum of Fragment C: ESI-MS: *m/z* calcd. for $C_{60}H_{99}N_{18}O_{23}S_2$: $[M+H]^+$ 1504.7, found for 1504.1 (deconvoluted). d) ESI-MS spectrum of Fragment D: ESI-MS: *m/z* calcd. for $C_{66}H_{111}N_{20}O_{23}S_2$: $[M+H]^+$ 1616.8, found for 1616.4 (deconvoluted). e) ESI-MS spectrum of Fragment E: ESI-MS: *m/z* calcd. for $C_{176}H_{284}N_{31}O_{87}$: $[M+H]^+$ 4223.9, found for 4224.7 (deconvoluted). f) ESI-MS spectrum of Fragment F: ESI-MS: *m/z* calcd. for $C_{60}H_{101}N_{18}O_{23}S_2$: $[M+H]^+$ 1506.7, found for 1506.1 (deconvoluted). g) ESI-MS spectrum of Fragment G: ESI-MS: *m/z* calcd. for $C_{28}H_{52}N_9O_{10}S$: $[M+H]^+$ 706.8, found for 706.7 (deconvoluted). h) ESI-MS spectrum of Fragment H: ESI-MS: *m/z* calcd. for $C_{38}H_{62}N_{11}O_{13}S$: $[M+H]^+$ 912.4, found for 912.7 (deconvoluted).

2-8-2. Measurement of circular dichroism (CD) spectra of EPO analogs

The CD spectra of correctly folded and misfolded EPO-24, EPO-38, EPO-83 and EPO-24, 38, 83 were measured to evaluate their secondary structures. These EPO analogs are dissolved to water and measured CD spectrums by CD spectroscopy. The all spectra of correctly folded EPO

analogs have a positive peak at 196 nm and negative peaks at 207 nm and 222 nm. This result indicated that correctly folded EPO analogs have α-helix structure (Fig. 2-52). These spectra are good agreement with previously reported spectra of EPO. The result of misfolded EPO analogs showed the similar spectrum patterns as correctly folded ones, although the intensities were relatively low (Fig. 2-53). This means that misfolded EPO analogs have not only correct disulfide bonds but also correct secondary structures.

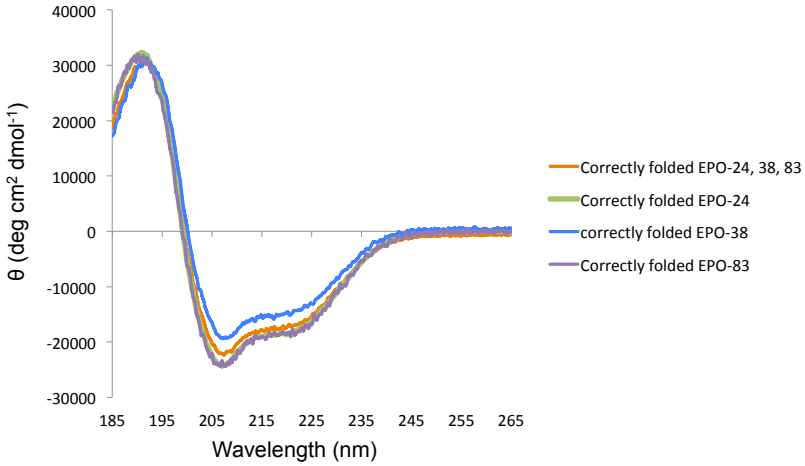

Figure 2-52. CD spectrum of correctly folded EPO bearing one or three oligosaccharides

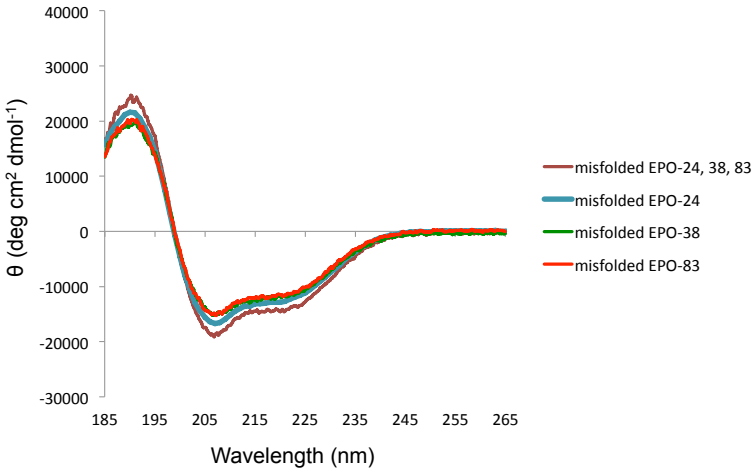

Figure 2-53. CD spectrum of misfolded EPO bearing one or three oligosaccharides

2-8-3. Measurement of biological activity of correctly folded EPO-38 and EPO-24, 38, 83

The *in vitro* biological activity of correctly folded EPO-38 and EPO-24, 38, 83 were measured by using TF-1 cell. TF-1 cell show growth dependency on EPO[13]. Commercial available EPO and only growth medium were used as positive and negative control, respectively. TF-1 cell and synthetic or commercial available EPO were incubated at 37ºC for 72 h under CO_2 atmosphere. The cell proliferation was evaluated by using WST-8. WST-8 is a tetrazolium salt, which emits fluorescent

formazan. As a result, synthetic EPO-24, 38, 83 and EPO-38 show suitable biological activity, comparable to commercial available ones (Fig. 2-54 and Fig. 2-55). This result proved that synthetic EPO analogs have correct three-dimensional structures.

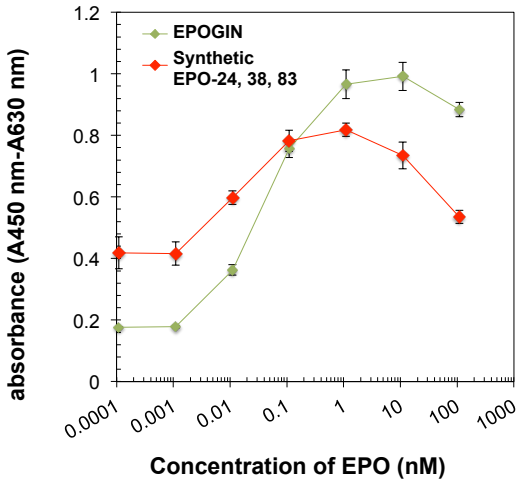

Figure 2-54. Activity of commercially available EPO and synthetic EPO-24, 38, 83

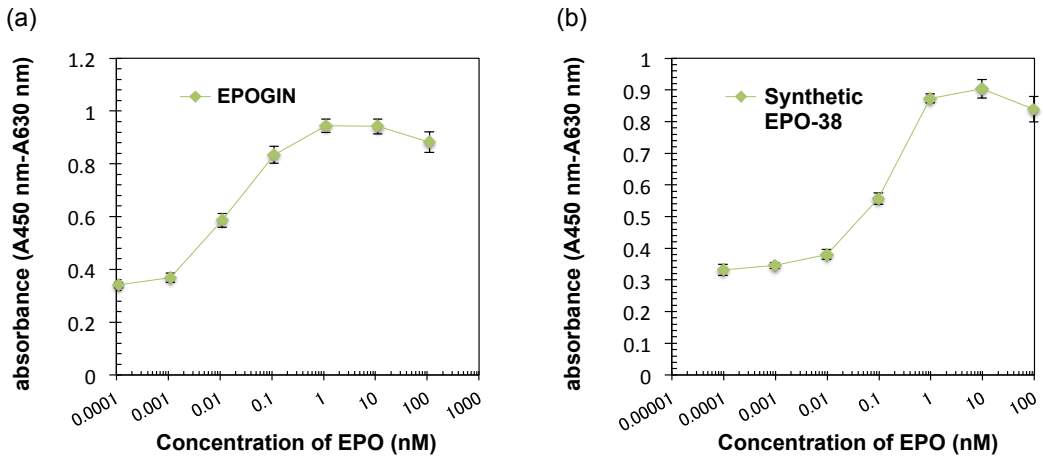

Figure 2-55. a) Activity of commercially available EPO. b) Activity of synthesized EPO-38

2-8-4. Native page of correctly folded and misfolded EPO-38

Correctly folded and misfolded EPO-38 were subjected to native page (Fig. 2-56). Native page is one of an electrophoresis method, which runs in non-denaturing conditions. Thus, the protein structure and complex are maintained in this analysis. The result of the analysis showed that misfolded EPO analog was aggregated and formed its dimer or trimer.

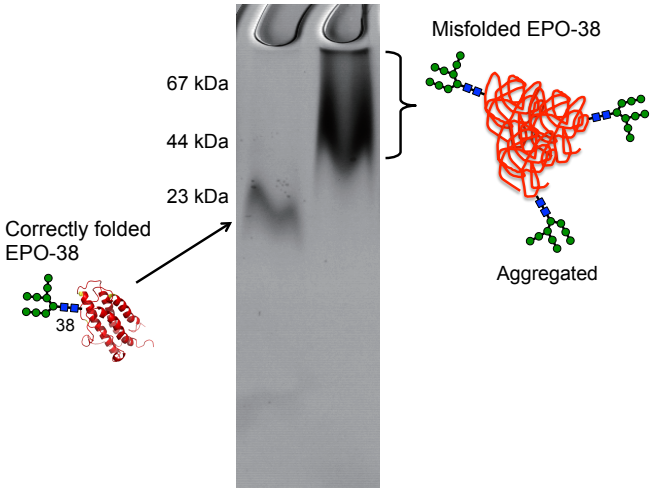

Figure 2-56. Native page of correctly folded and misfolded EPO-38.

2-8-5. Denaturing experiment of misfolded EPO-38

Because only a few are known about the structure of misfolded EPO, I evaluated the property of misfolded EPO by denaturing of it. Misfolded EPO-38 was dissolved the solution containing 20 mM TCEP and 6 M Gn and then this solution was incubated at room temperature. This solution was analyzed by LC-MS (Fig. 2-57). Although a part of MS peak of glycosylated polypeptide was observed, most of the peaks were as the disordered form. I examined some condition such as heating, TFA treatment and different kinds of reducing reagent, but misfolded EPO-38 have not returned to original glycosylated poly2-1-3peptide. According to these results, I concluded that misfolded EPO is aggregated irreversibly and it has apparently different property and structure from correctly folded EPO.

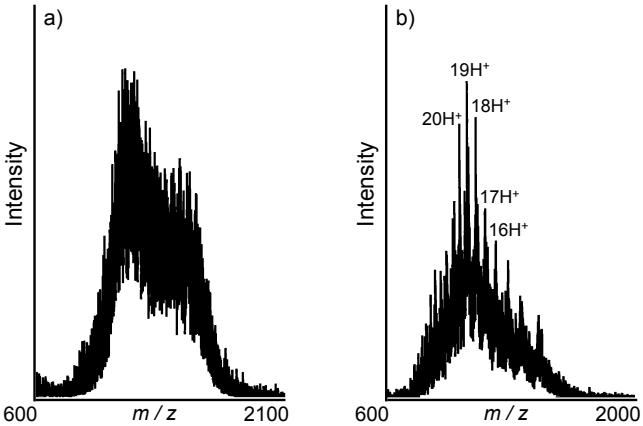

Figure 2-57. Analytical data of denaturing of misfolded EPO-38. a) ESI-MS spectrum of misfolded EPO-38. b) ESI-MS spectrum of misfolded EPO-38 after TCEP and guanidine treatment.

Summary of chapter 2

Eight kinds of EPO analog were synthesized by using solid phase peptide synthesis, native chemical ligation and *in vitro* folding. The characterization of these EPO analogs showed that both correctly folded EPO analogs and misfolded ones were obtained successfully. The analysis also showed that although misfolded EPO have α-helix structure and correct disulfide bonds, it is aggregated irreversibly.

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Chapter 3

UGGT Assay and ER Lysate Assay with Synthetic EPO Analogs

Introduction of chapter 3

This chapter will describe the result of recombinant UGGT assay with both correctly folded and misfolded synthetic EPO analogs. Chemical synthesis of another glycoprotein, interferon-β, as a reference, will also be described. UGGT assay with them to investigate the recognition mechanism of UGGT will be described. Additionally, the result of the assay with synthetic EPO analogs and ER lysate that contains all the players in a glycoprotein quality control system will be also discussed. This chapter consists of four sections. The titles of each section are described below.

3-1. Recombinant UGGT assay toward synthetic EPO analogs
3-2. Chemical synthesis of Interferon-β and UGGT assay toward Interferon-β
3-3. ER lysate assay toward synthetic EPO analogs
3-4. Discussion

3-1. Recombinant UGGT assay toward synthetic EPO analogs

3-1-1. UGGT assay toward misfolded EPO analogs

UGGT assay was performed with recombinant human UGGT in a solution containing M9-high-mannose type oligosaccharyl-protein, UDP-Glucose and appropriate amount of UGGT and this mixture was incubated at 37ºC. The glucosylation was estimated with reverse phase LC-MS. The ion peak area corresponding G1M9-oligosaccharyl-protein and M9-oligosaccharyl-protein were used for the estimation of glucosylated M9-glycoprotein and M9-glycoprotein.

First of all, misfolded EPO analogs bearing an oligosaccharide at any position were assayed with recombinant human UGGT expressed by yeast. As a result, UGGT transferred a glucose to misfolded M9-glycoproteins as I expected (Fig. 3-1). Although figure 3-1 showed the result of only misfolded EPO-38, misfolded EPO-24 and EPO-83 was the similar result. The case of misfolded EPO bearing all three oligosaccharides (EPO-24, 38, 83) showed the same glucosyl transfer ability (Fig. 3-1). According to these results and the previous study, we found that UGGT recognizes misfolded M9-glycoproteins and then transferred a glucose to them.

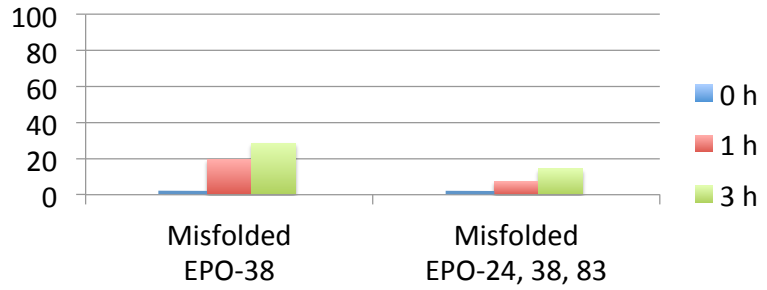

Figure 3-1. The rate of G1M9-glycosylated EPO analogs generated by recombinant UGGT (%)

3-1-2. UGGT assay with correctly folded EPO analogs

Next I examined UGGT assay with correctly folded EPO analogs bearing an oligosaccharide (EPO-24, EPO-38 and EPO-83). However, this assay showed an unexpected glucosyl transfer to these EPO analogs by recombinant UGGT (Fig. 3-2). The result of UGGT assays with correctly folded M9-EPO-24, EPO-38 and EPO-83 were almost same. I consider why UGGT unexpectedly transfer a glucose residue to correctly folded M9-EPO and I speculated that this is because hydrophobic surface was exposed by the lack of two oligosaccharides. In fact, the similar case was reported by Keith et al[1]. They reported that UGGT transferred a glucose to a correctly folded monomeric soybean agglutinin (SBA), while UGGT did not to natural form SBA that is a tetramer. Because this monomeric form is not a native form and the hydrophobic surface might exposed where is essential to form tetrameric form. The Keith group concluded that UGGT recognized the hydrophobic interfaces, which is hidden by other subunits in the structure of native tetramer.

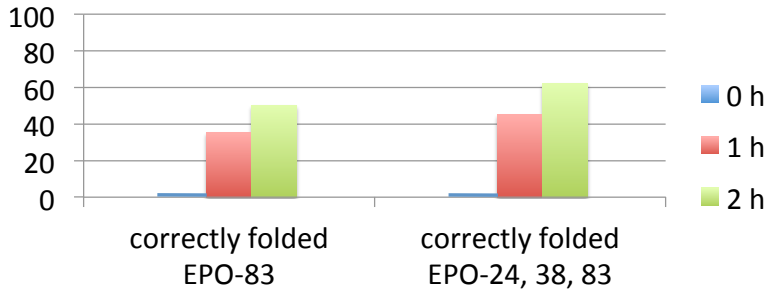

Figure 3-2. The rate of G1M9-glycosylated EPO analogs generated by recombinant UGGT (%)

As I speculated that glycosylation deficient EPO showed hydrophobic protein surface, then I examined UGGT assay with EPO analog bearing three oligosaccharides (EPO-24, 38, 83) in order to confirm whether three M9-oligosaccharides are key group for UGGT recognition.The result was that UGGT unexpectedly transferred glucoses to this EPO, which has correct protein structure and all three oligosaccharides (Fig. 3-2). The best of our knowledge, this is the first example that UGGT transfer a glucose to glycoproteins adopting native protein structure.

In order to investigate where M9-oligosaccharides were specifically glucosylated, the assay

of UGGT toward EPO-24, 38, 83 was performed. Identification for glucosylation position was performed by peptidase digestions with Glu-C and subsequent LC-MS analysis. Glu-C is an enzyme, which can cleave amide bonds at the C-terminal of either Aspartic acid or Glutamic acid specifically. This enzyme can digest EPO-24, 38, 83 into three glycopeptide and multiple peptide fragments. As a result, peptidase digestion and subsequent LC-MS analysis indicated that UGGT transferred glucose residues at any position with same amount (Fig. 3-3). This result indicates that UGGT doesn't have substrate specificity toward three oligosaccharides of EPO.

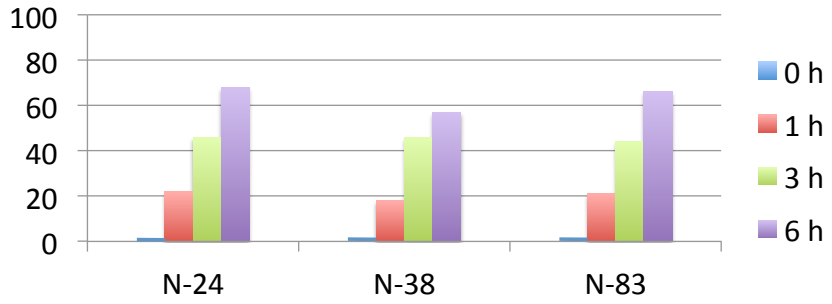

Figure 3-3. The rate of G1M9-oligosaccharide of EPO bearing three oligosaccharides at each glycosylation site of N-24, 38, 83 generated by recombinant UGGT (%)

3-2. Chemical synthesis of Interferon-β and UGGT assay with Interferon-β

3-2-1. Glycoprotein probes for the investigation of UGGT recognition mechanism in previous reports

In the previous section, I mentioned that UGGT transfer glucose residues to the correctly folded EPO and this section will discuss the substrate specificity of UGGT comparing with other UGGT substrates previously reported in the literature. I speculated that the reason of the unexpected glucosylation toward the correctly folded EPO is because of the different secondary structure of EPO comparing with other substrates used so far. As shown in Figure 3-4, almost all UGGT substrates in previous reports have β-sheet structures[2-7], but EPO has only α–helix. This fact led to the hypothesis that UGGT recognize β-sheet structures as a sigh of native glycoprotein form, because β-sheet structure have a wide area on the protein surface and interacts with bulk water. This characteristic nature of β-sheet may show a hydrophilic protein surface which may be recognized as correctly folded glycoprotein.

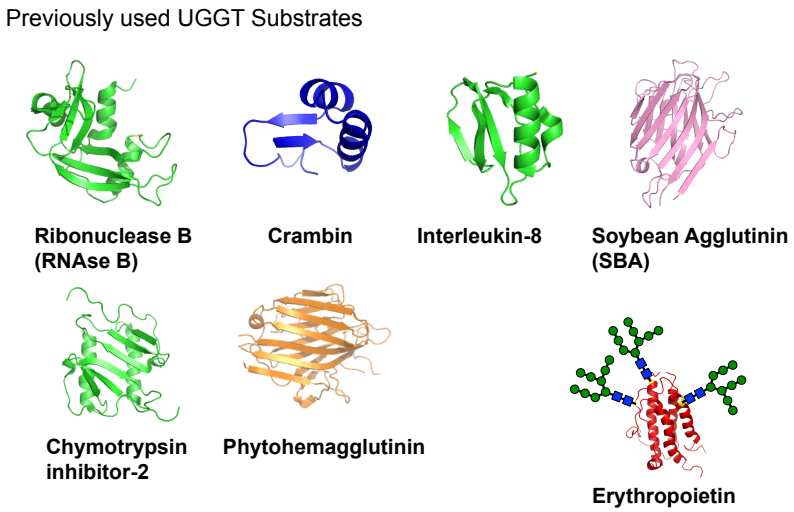

Figure 3-4. Previously used UGGT substrates and EPO

3-2-2. Characters of interferon-β (INF-β) and my synthetic strategy for the synthesis of INF-β bearing M9-high-mannose type oligosaccharide

To test the hypothesis that β-sheet is a key structure for UGGT recognition process, I examined to do assay with another α–helices bundle type glycoprotein. I selected and synthesized INF-β bearing M9-high-mannose type oligosaccharide, which has only α–helix structure. INF-β is a *N*-glycosylated cytokine possesses immunomodulating, antiviral and antitumor activities[8]. The synthesis of INF-β bearing M9-high-mannose type oligosaccharide is thought to be possible, because chemical synthesis of INF-β bearing complex type oligosaccharide have already reported from our group[9]. Changing of oligosaccharide structure often changes glycoprotein characteristic nature, so construction of whole glycopeptide and folding process should be carefully performed.

INF-β consists of 166 amino acid residues same as EPO (Fig. 3-5). INF-β has one disulfide bond and *N*-linked oligosaccharide. I synthesized it following the previous synthesis. I divide it into three segments as it is colored in figure 3-5. These segments were ligated from C-terminal sequentially (Fig. 3-6).

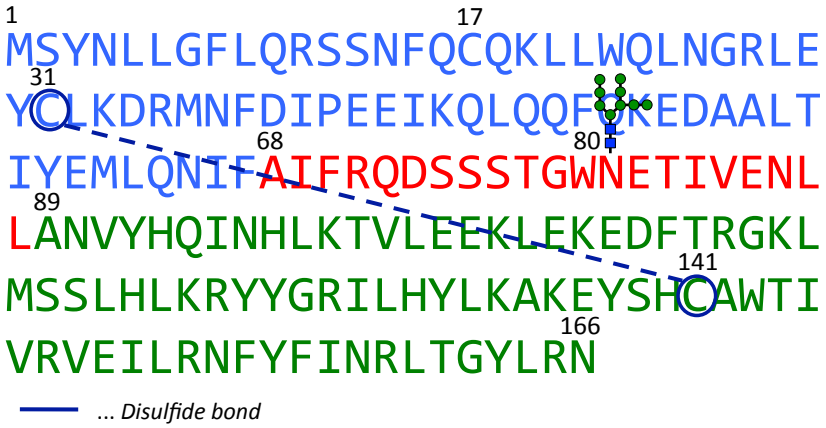

Figure 3-5. Sequence of INF-β

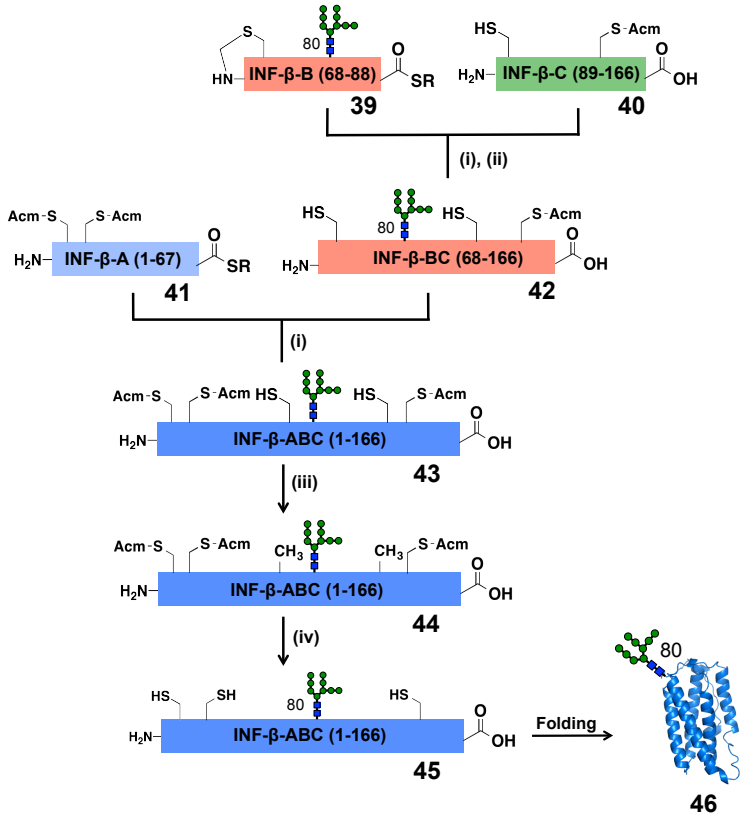

Figure 3-6. Synthetic strategy of INF-β bearing M9-high-mannose type oligosaccharide. Conditions: (i) native chemical ligation; (ii) removal of thiazolidine; (iii) desulfurization; (iv) removal of the Acm groups

3-2-3. The preparation of peptide and glycopeptide segments of INF-β

Peptide segment A and segment C were provided by Glytech, Inc. Glycopeptide segment B was synthesized by using the Boc-M9-Asn as I mentioned in section 2-3-2 (Fig. 3-7).

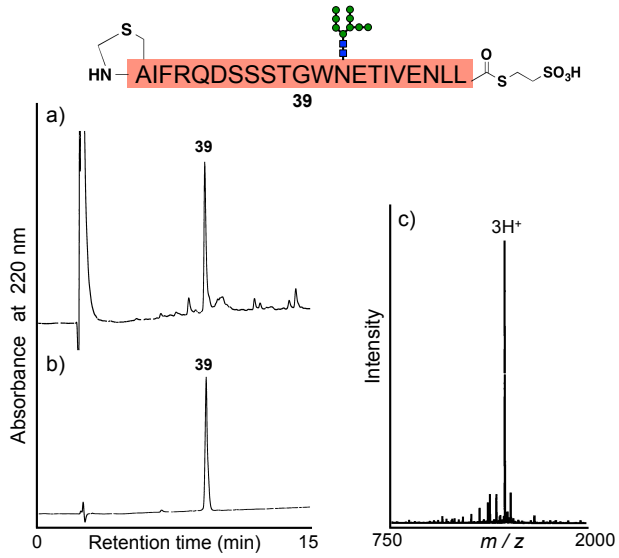

Figure 3-7. HPLC profile and ESI-MS spectrum of purified glycopeptide segment B. a) HPLC profile of crude compound **39**. b) HPLC profile of purified compound **39**. c) ESI-MS of compound **39**: *m/z* calcd. for $C_{177}H_{283}N_{30}O_{93}S_3$: $[M+H]^+$ 4415.5 found for 4414.0 (deconvoluted).

3-2-4. Ligation reaction between glycopeptide segment B and peptide segment C

Glycopeptide segment B and peptide segment C were ligated by the same procedure as the synthesis of EPO analogs. The HPLC profiles and MS spectrum of this reaction were shown in Figure 3-8.

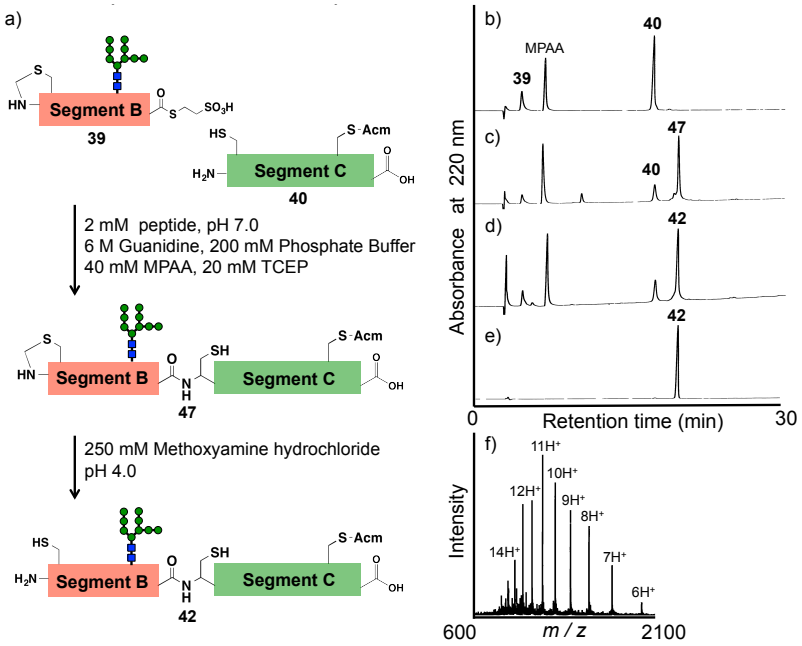

Figure 3-8. Analytical data of monitoring 1st-NCL. a) Scheme of peptide ligation between compound **39** and **40**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 26 h. d) HPLC profile after treatment with methoxyamine-HCl. e) HPLC profile of purified compound **42**. e) ESI-MS spectrum of compound **42**. ESI-MS: *m/z* calcd. for $C_{615}H_{965}N_{154}O_{204}S_4$: $[M+H]^+$ 13908.4, found for 13905.9 (deconvoluted).

3-2-5. Ligation reaction between segment A and segment BC

Peptide segment A and glycopeptide segment BC were ligated by the same procedure as above. The HPLC profiles and MS spectrum of this reaction were shown in Figure 3-9.

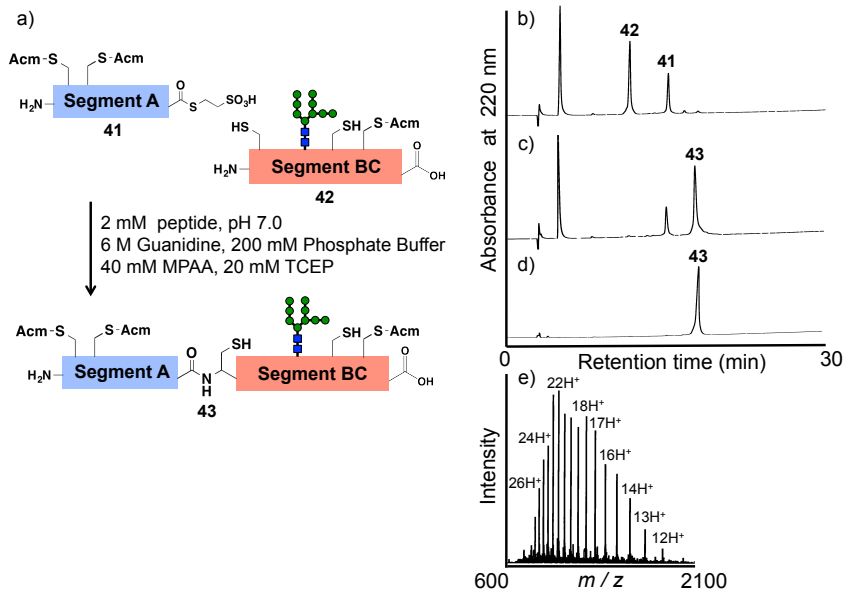

Figure 3-9. Analytical data of monitoring 2nd-NCL. a) Scheme of peptide ligation between compound **41** and **42**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 19 h. d) HPLC profile of purified compound **43**. e) ESI-MS spectrum of compound **43**. ESI-MS: *m/z* calcd. for $C_{987}H_{1540}N_{251}O_{310}S_9$: $[M+H]^+$ 22170.9, found for 22168.4 (deconvoluted).

3-2-6. Radical reductions of cysteine residues (position at 68 and 89) of compound **43** to alanine residues

Reductions of a cysteine to alanine of segment ABC were conducted by the same manner as the synthesis of EPO analogs. The HPLC profiles and MS spectrum of this reaction were shown in Figure 3-10.

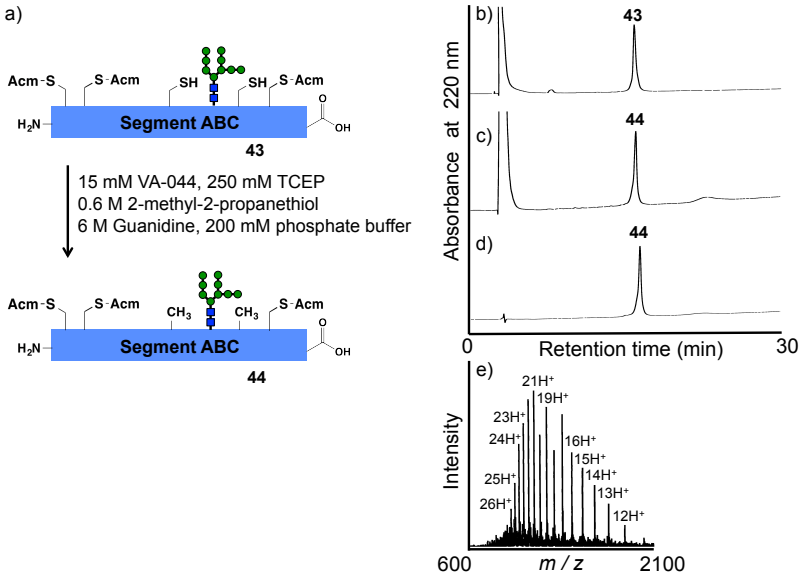

Figure 3-10. Analytical data of monitoring desulfurization of glycopeptide segment ABC. a) Scheme of desulfurization reaction of compound **43**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 3 h. d) HPLC profile of purified **44**. e) ESI-MS spectrum of compound **44**. ESI-MS: *m/z* calcd. for $C_{987}H_{1540}N_{251}O_{310}S_7$: $[M+H]^+$ 22106.8, found for 22104.6 (deconvoluted).

3-2-7. Deprotection of acetamidomethyl (Acm) groups (position at 17, 31 and 141) of compound **44**

Removals of three Acm groups of segment ABC were conducted by the same manner as the synthesis of EPO analogs. The HPLC profiles and MS spectrum of this reaction were shown in Figure 3-11.

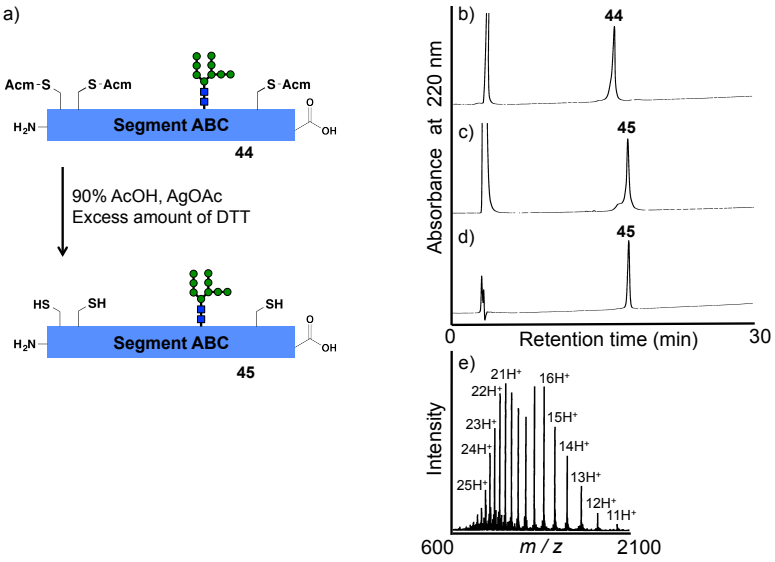

Figure 3-11. Analytical data of monitoring deprotection of Acm group of glycopeptide segment ABC. a) Scheme of deprotection of Acm groups of compound **44**. b) HPLC profile for stating point (t < 1 min). c) HPLC profile after 4 h. d) HPLC profile of purified compound **45**. e) ESI-MS spectrum of compound **45**. ESI-MS: *m/z* calcd. for $C_{978}H_{1525}N_{248}O_{307}S_7$: $[M+H]^+$ 21893.6, found for 21890.1 (deconvoluted).

3-2-8. Folding procedure of the whole glycosylated polypeptide (compound **45**) of INF-β

Folding of the whole glycopolypeptide (compound **45**) was conducted by the same procedure as described in the synthesis of EPO analogs. Because of the low solubility of INF-β glycopolypeptide, higher concentration of guanidine was used. Additionally, acetic acid (10 mM) was used as final folding buffer, instead of 10 mM tris-HCl. Only correctly folded INF-β was obtained. The HPLC profiles and MS spectrum of this reaction were shown in Figure 3-12.

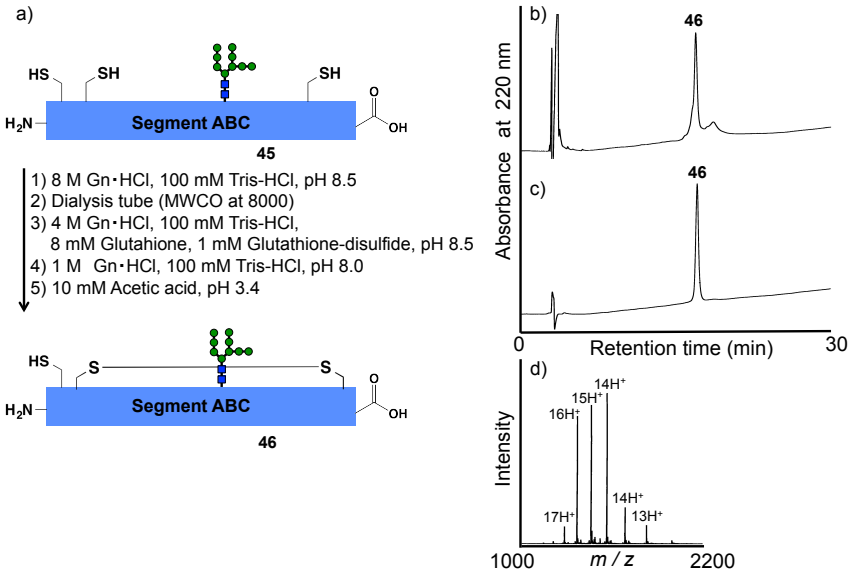

Figure 3-12. Analytical data of folding of segment ABC. a) Scheme of the folding of compound **45**. b) HPLC profile after folding process. c) HPLC profile of purified correctly folded INF-β (compound **46**). d) ESI-MS spectrum of correctly folded INF-β (compound **46**). FT-ICR-MS: *m/z* calcd. for $C_{978}H_{1523}N_{248}O_{307}S_7$: $[M+H]^+$ 21889.9489, found for 21889.9801 (deconvoluted)

3-2-9. Measurement of circular dichroism (CD) spectrum of synthetic INF-β

CD spectrum of synthetic INF-β was measured. The spectrum has a positive peak at 196 nm and negative peaks at 207 nm and 222 nm (Fig. 3-13). It means that synthetic INF-β has α-helix structure. The spectrum showed a good agreement with previous reports in terms of the shape and the intensity.

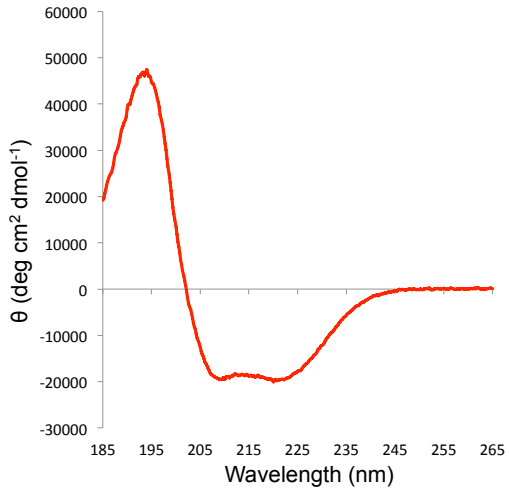

Figure 3-13. CD spectrum of synthetic INF-β

3-2-10. UGGT assay with synthetic INF-β

Synthetic INF-β thus obtained was then used for the assay with recombinant UGGT. The assays were performed with the same protocol as described in the assay of EPO analogs. As a result, UGGT didn't transfer a glucose to INF-β (Fig. 3-14). It means that UGGT recognize correctly folded INF-β as a native glycoprotein even it have no β–sheet structure. From this result, I concluded that my hypothesis was wrong.

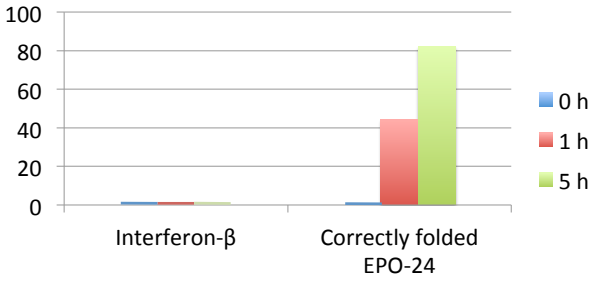

Figure 3-14. The rate of G1M9-glycosylated INF-β and EPO analog (positive control) generated by recombinant UGGT (%)

3-2-11. Evaluation of hydrophobicity of EPO and INF-β

According to the assay results of EPO analogs and INF-β, I considered the next hypothesis that hydrophobicity on the surface of these glycoproteins attributed to be a key recognition marker for UGGT. Figure 3-15 shows surface models of EPO and INF-β of which hydrophobic amino acid residues were colored as yellow. As shown in Figure 3-15, EPO model exhibit broad hydrophobic surface rather than INF-β model. I also evaluated the hydrophobicity of these glycoproteins by 1,8-anilinonaphthalene-sulfonate (ANS) assay[10]. ANS is a fluorescence probe that binds hydrophobic surface of proteins and this assay has been used as the gauge of hydrophobicity. As the results, ANS can bind with INF-β rather than EPO analogs (Fig. 3-16). These results indicated that INF-β exposes hydrophobic surface rather than EPO and this results was to be inconsistent with to the discussion using surface models (Fig. 3-16). Based on the result that UGGT transfer glucose residues to the EPO analogs specifically, I assumed that the spreading of hydrophobic protein surface through the glycoprotein molecule with hydrophobic amino acids seems to be a major reason for UGGT substrate specificity. Indeed, ANS can bind not only the hydrophobic patches on protein surface but also the patches in grooves or inside protein, but a wide range of hydrophobic surface may not be estimated by ANS. I assumed that glycoprotein model (Fig. 3-15) showing the broad hydrophobic surface of EPO seems to indicate a reason for UGGT substrate specificity.

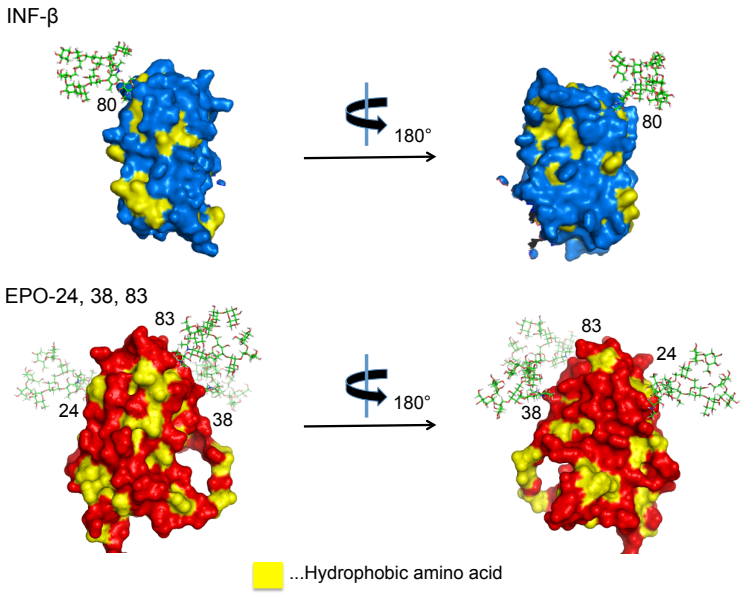

Figure 3-15. Surface models of EPO and INF-β

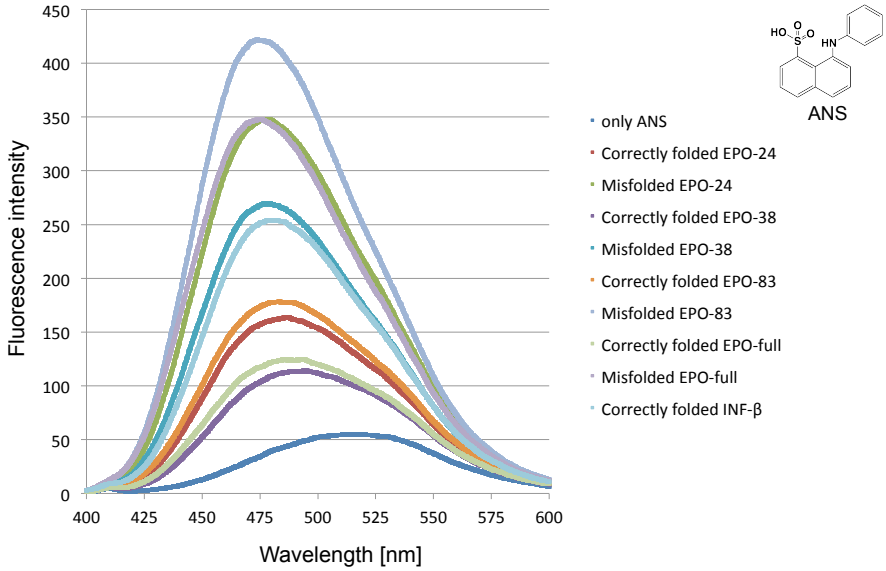

Figure 3-16. ANS assay of synthetic EPO analogs and synthetic INF-β

3-3. ER lysate assay with synthetic EPO analogs

3-3-1. ER lysate assay with correctly folded and misfolded EPO analogs

Next, I used synthetic EPO analogs toward assays with a glycoprotein quality control system isolated from rough ER lysate. Our group has already established this ER lysate assay toward misfolded glycoprotein and confirmed that this ER lysate accelerated the refolding of misfolded glycoproteins (unpublished results).

According to the procedure our group established, synthetic EPO analogs were added into the isolated rough ER lysate, which contains all the players in a glycoprotein quality control system. Rough ER lysate was isolated from rat liver by sucrose density gradient centrifugation. After the isolation, rough ER fraction was treated with detergent to slightly decompose ER membrane and this protocol reproduced the ER quality control system in test tube. Next, I examined ER lysate assays toward synthetic EPO analogs. All reactions were monitored by LC-MS analysis.

As a result of the reaction with ER lysate, a glucosylation to neither correctly folded nor misfolded EPO were not observed, although recombinant UGGT clearly transferred a glucose to these EPO analogs in a solution. (Fig. 3-17 and 3-18). Although biology textbooks and previous reports showed a structure of glucosylated misfolded glycoproteins by UGGT, my result is inconsistent with the previous discussion.

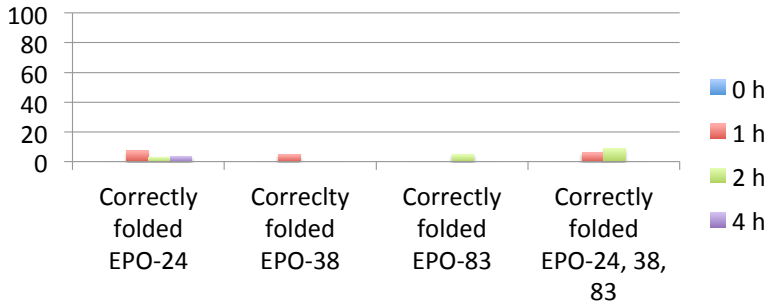

Figure 3-17. The rate of G1M9-glycosylated EPO analogs (correctly folded) generated by ER lysate (%)

The bars showed glucosyl transfer, but these value is close to noise.

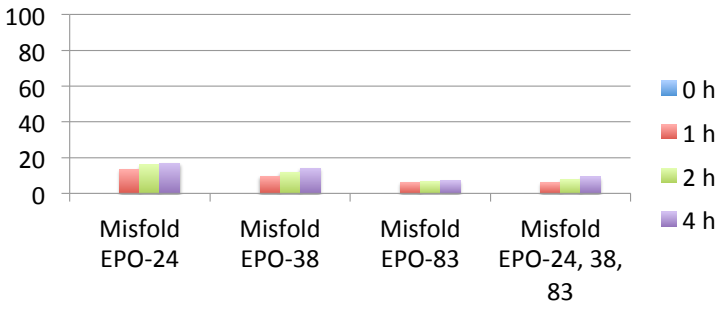

Figure 3-18. The rate of G1M9-glycosylated EPO analogs (misfolded) generated by ER lysate (%)

The bars showed glucosyl transfer (less than 20% yield), but these value is not high comparing with that of recombinant UGGT assay (over 60% yield, Fig. 3-2, 3-3)

3-3-2. ER lysate assay with correctly folded and misfolded EPO analogs using inhibitor of glucosidase II

Toward these unexpected results, I considered and then achieved a speculation that this is because glucosidase II hydrolyze external glucose, immediately after UGGT transfer a glucose. To test the hypothesis, I used an inhibitor of glucosidase II, 1-deoxynojirimycin (DNJ) toward the same ER lysate assays. When the inhibitor was added to the ER assay, glucosylated EPO analogs were clearly observed (Fig. 3-19 and 3-20). This evidence potentially indicated that there is a reversible glucosylation/deglucosylation reaction by the action of UGGT and glucosidase II, respectively. So far there was no report regarding this reversible reaction.

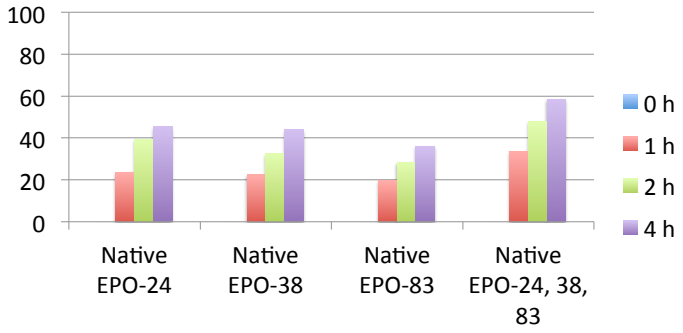

Figure 3-19. The rate of G1M9-glycosylated EPO analogs (correctly folded) generated by ER lysate with inhibitor of glucosidase II (%)

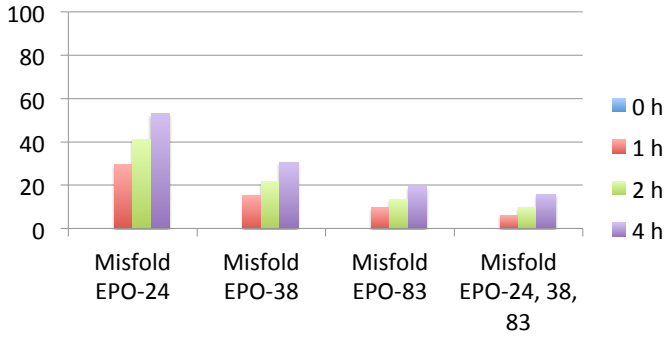

Figure 3-20. The rate of G1M9-glycosylated EPO analogs (misfolded) generated by ER lysate with inhibitor of glucosidase II (%)

3-3-3. Working hypothesis to elucidate how CNX/CRT interact with substrate G1M9-glycoproteins

However, this is also inconsistent with the explanation of glycoprotein quality control system described in the biology textbooks, because chaperone interaction needs G1M9-oligosaccharide for their recognition process. Therefore I also investigated the timing of interaction of CNX/CRT that specifically bind G1M9-glycoproteins. In order to study how CNX/CRT

bind with G1M9-glycoprotein during the fast reversible glucosylation/deglucosylation process, I made two working hypotheses.

The hypothesis 1 indicates that UGGT-glucosidase II-CNX/CRT interaction takes place randomly. While UGGT and glucosidase II are exchanging their substrate, once G1M9-glycosylated EPO is appeared, CNX/CRT recognize and interact to it by chance (Fig. 3-21(a)).

While the hypothesis 2 indicates that UGGT-Glucosidase II-CNX/CRT interaction takes place under the ordered sequence. G1M9-glycosylated EPO should be passed to CNX/CRT definitely, and CNX/CRT pass it to glucosidase II (Fig. 3-21(b)).

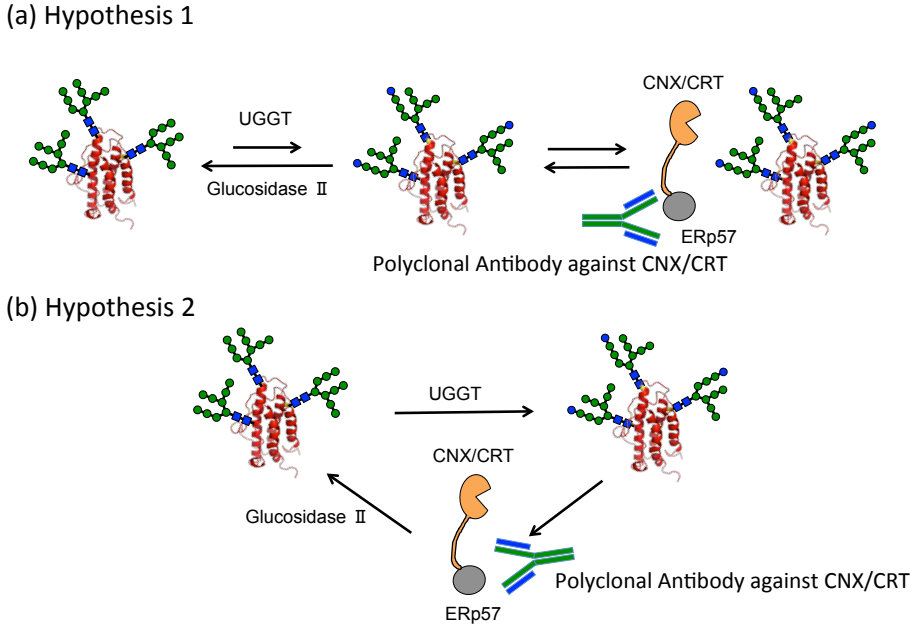

Figure 3-21. Working hypotheses about how CNX/CRT interact with G1M9-glycosylated EPO

3-3-4. ER lysate assay with correctly folded and misfolded EPO analogs using antibodies against CNX/CRT

To test which hypothesis is correct, I used polyclonal antibodies against CNX/CRT to neutralize and block the role of CNX/CRT. If hypothesis 1 is correct, EPO that don't have glucose residue will be observed. Because CNX/CRT do not interfere the reversible glucosylation/deglucosylation reaction by UGGT and Glucosidase II. If hypothesis 2 is correct, G1M9-glycosylated EPO will be observed in the absent of an inhibitor of glucosidase II. Because the antibodies inhibit the role of CNX/CRT and this inhibition results in the trouble to pass the G1M9-glycosylated EPO to glucosidase II. Therefore glucosidase II cannot hydrolyze a glucose residue of glycoproteins. So I examined ER lysate assays in the presence of both anti-CNX and CRT-polyclonal antibodies.

As a result, G1M9-glycosylated EPO was observed and this result indicates that UGGT,

CNX/CRT and glucosidase II are working in ordered-sequence like hypothesis 2 (Fig. 3-22). I also examined the same antibody experiments toward misfolded EPO and the experiments also gave the same result that the increase of G1M9-glycosyl misfolded EPO was observed (Fig. 3-23).

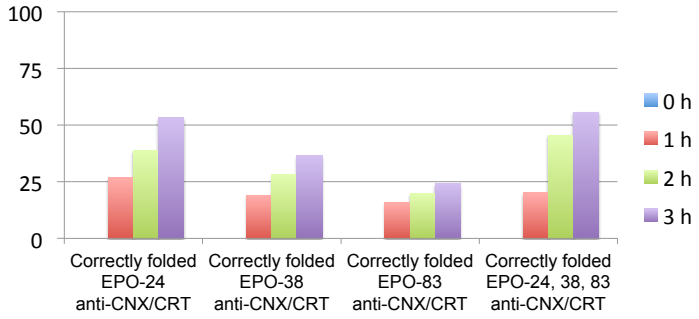

Figure 3-22. The rate of G1M9-glycosylated EPO analogs (correctly folded) generated by ER lysate with anti-CNX/CRT (%)

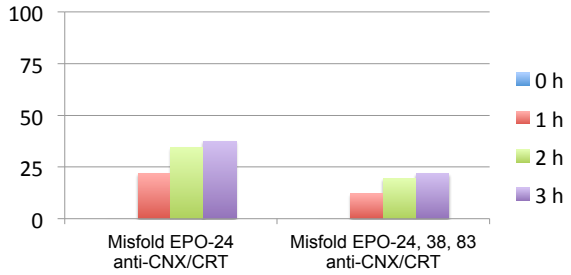

Figure 3-23. The rate of G1M9-glycosylated EPO analogs (misfolded) generated by ER lysate with anti-CNX/CRT (%)

3-3-5. ER lysate assay with correctly folded and misfolded EPO analogs using either antibody against CNX or CRT

In order to investigate which chaperones CNX or CRT is concerning with the UGGT-Glucosidase II-CNX/CRT cycle, I used either anti-CXN-polyclonal antibody or anti-CRT-polyclonal antibody to block the role of CNX or CRT. When one of both antibodies was used, the antibody could not stop the UGGT-glucosidase II-CNX/CRT cycle (Fig. 3-24). However, the result of the ER assay in the presence of both antibodies indicates that UGGT-glucosidase II-CNX/CRT cycle was inhibited. Considering these results, both antibodies against CNX and CRT are simultaneously needed to block the cycle completely and this result showed that CNX and CRT rescue each other in order to accelerate glycoprotein-refolding process. Therefore even if the antibody blocks the role of CNX specifically, CRT can contribute to accelerate UGGT-glucosidase II-CNX/CRT cycle as a part of the cycle instead and vice versa.

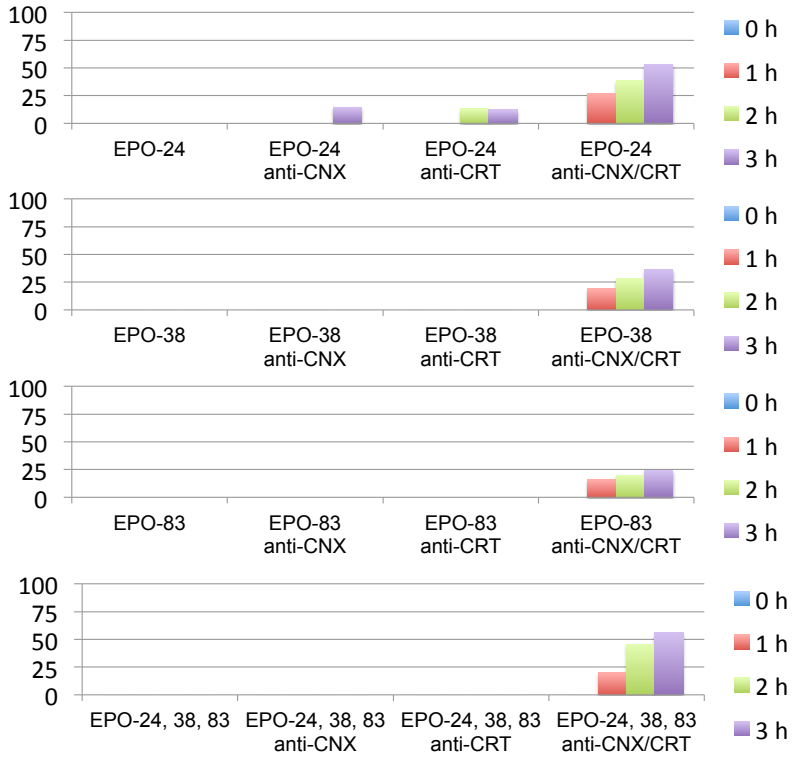

Figure 3-24. The rate of G1M9-glycosylated EPO analogs (correctly folded) generated by ER lysate with anti-CNX or anti-CRT or anti-CNX/CRT (%)

3-3-6. The velocity of UGGT-glucosidase II-CNX/CRT cycle

As far as I observed the UGGT-glucosidase II-CNX/CRT cycle, it appeared to be a catalytic process and therefore I estimated their catalytic activity. We could estimate the protein quantity in a ER lysate used for a assay and the quantity of product, G1M9-glycoprotein based of MS intensity. Using these values, I estimated that the velocity of the UGGT-glucosidase II-CNX/CRT cycle was 41.6 ng/s. The concentration of cytokine in the blood is around 100 pg/ml[11]. Considering the total amounts of rat blood (16 ml), it is thought to be a reasonable velocity.

3-3-7. ER lysate assay with correctly folded and misfolded EPO analogs using antibodies against several kinds of chaperone.

I also investigated whether other chaperones or enzymes contribute to the glycoprotein quality control cycle or not. I used several antibodies against many components act as players in a glycoprotein quality control system such as protein disulfide isomerase (PDI), protein disulfide isomerase-related protein (PDIR), ERp5, ERp29, ERp57, ERp72 and BiP. However, no antibody can block the glycoprotein quality control cycle (Fig. 3-25). Hence only UGGT, CNX/CRT and

Glucosidase II contribute to the glycoprotein quality control cycle exclusively as far as I studied.

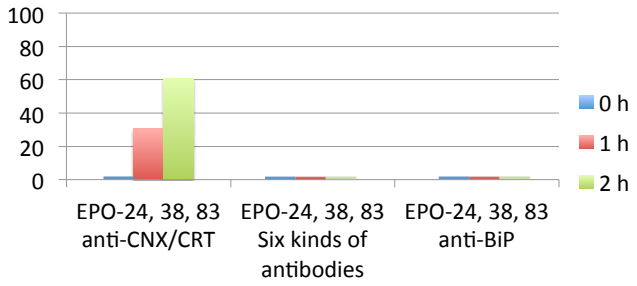

Figure 3-25. The rate of G1M9-glycosylated EPO analogs (correctly folded) generated by ER lysate with antibodies against several kinds of chaperone (%)

3-4. Discussion

So far UGGT has been acknowledged to transfer a glucose residue to misfolded glycoproteins specifically, but my experiments showed that UGGT could transfer a glucose to a correctly folded EPO bearing an oligosaccharide that lacks two oligosaccharides. In terms of this unexpected result, I supposed that UGGT recognize the exposed hydrophobic surface because of the lack of oligosaccharides at the native glycosylation site. In fact, ANS assay showed that EPO bearing an oligosaccharide have higher hydrophobicity than EPO bearing three oligosaccharides (Fig. 3-16). This result can explain the previous report that the secretions of EPO decrease dependent on the decreasing of oligosaccharides as mentioned in chapter 1 (Table 1). Based on my results that UGGT make a tag, G1M9-high-mannose type oligosaccharide on the correctly folded EPO, UGGT may trap the oligosaccharide deficient EPO and keep it in the glycoprotein quality control system. This keeping may prevent the secretion. Actually, congenital disorder of glycosylation (CDG) causes oligosaccharide deficiency[12]. Several reports indicated that the secretions of some glycoproteins decrease due to oligosaccharide deficient[13]. Similar situation may occur in some disease. The lack of oligosaccharides of glycoproteins exhibit hydrophobic protein surfaces and this leads aggregation in some case as shown Figure 2-8, thus glycoprotein quality control system may monitor hydrophobicity to avoid production of such hydrophobic proteins.

The glucosylation by UGGT for correctly folded EPO bearing three oligosaccharides was quite an unexpected result. This EPO is an absolutely native glycoprotein form in ER. However, EPO is a relatively hydrophobic glycoprotein. Indeed, ANS assay of EPO-24, 38, 83 showed the hydrophobicity of it compared to the negative control (ANS only). It is estimated that UGGT recognize the hydrophobic surface of EPO that cannot be covered by three oligosaccharides.

Because UGGT recognized correctly folded EPO bearing three high-mannose type

oligosaccharides, a correctly folded EPO should be classified as unsuitable glycoproteins based on the function of glycoprotein quality control system. The correctly folded EPO bearing three oligosaccharide is an essential cytokine in our body and therefore the question of how correctly folded EPO bearing three high-mannose type oligosaccharides at the 24, 38, 83 secrets to outside of ER in our body remains unanswered.

Considering the previous reports, I assumed that EPO-24, 38, 83 is bound by CRT tightly in ER because of the cluster effect of three oligosaccharides and this binding between EPO and CRT makes EPO a hydrophilic glycoprotein particle that should be recognized as suitably folded glycoproteins. The EPO is transported to Golgi apparatus covering the hydrophobic surface by hydrophilic-CRT. Then G1M9 oligosaccharide of EPO is trimmed to M8 by mannosidase (Fig. 3-26). I assumed that Golgi endo-mannosidase is a key to solve this question. Golgi endo-mannosidase cleaves internally the glucose-substituted mannose residue of oligosaccharide of glycoproteins in Golgi apparatus[14]. After the processing, these glycoproteins are normally secreted to outside[15]. Molecular chaperon CRT, which binds G1M9 oligosaccharides, exists in the same place as Golgi endo-mannosidase. It is presumed that some CRT-bound G1M9-glycoprotein is transported to Golgi apparatus and cleaved both glucose and mannose residues by Golgi endo-mannosidase to release from CRT[16,17]. I would like to continue the experiments to confirm this interesting and exciting hypothesis.

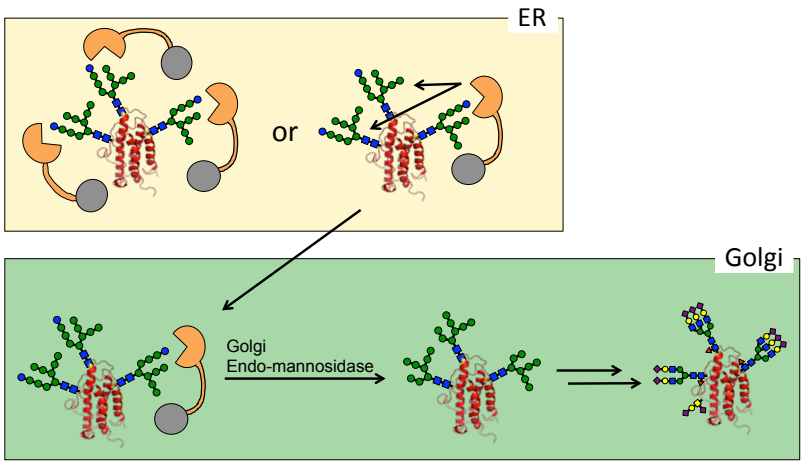

Figure 3-26. Hypothesis of biosynthetic pathway of EPO-24, 38, 83

Using anti-CNX/CRT polyclonal antibodies, this antibody blocking experiments clearly showed that glucosylation was elevated toward synthetic EPO analogs. This result gave an insight into the interaction cycle of UGGT, CRT/CNX and glucosidase II. According to my all experimental results, UGGT, CNX/CRT and glucosidase II work in order in glycoprotein quality control system. The key to find this cycle was the character of these correctly folded EPO analogs that is recognized by UGGT as misfolded-type glycoprotein and therefore kept in this glycoprotein quality control

system. In my group, our colleagues examined same experiments with another misfolded glycoprotein probes, but these misfolded glycoprotein probes fold into correct structure in this ER lysate and escape from the cycle immediately. EPO is supposed to be in borderline between mature glycoprotein and immature one based on hydrophobic nature of protein surface.

However, the mechanism how antibodies against CNX/CRT block the glucosidase II activity is still unclear. In fact, CNX/CRT inhibit the activity of glucosidase II *in vitro* (unpublished data). I assumed that CNX/CRT and glucosidase II make a complex in ER and antibodies against CNX/CRT blocked this complex. This research will be continued.

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Conclusions

EPO bearing M9-high-mannose type oligosaccharides were chemically synthesized at the first time. The unified synthetic strategy leads three kinds of EPOs bearing an M9-high-mannose type oligosaccharide at the individual glycosylation site (EPO-24, EPO-38 and EPO-83) and EPO bearing three oligosaccharides at three glycosylation sites (EPO-24, 38, 83) as described in chapter 1. Fragment coupling method was used for the preparation of key glycopeptide thioester. Both correctly folded EPO and misfolded EPO were also synthesized. Because EPO bearing high-mannose type oligosaccharides transiently emerges in the ER only, the preparation of these by biotechnological methods is difficult. My synthesis proved that chemical synthesis is a potential method to prepare the variety of homogeneous glycoproteins.

I found the first example that UGGT transfer a glucose to the correctly folded glycoprotein. All the synthetic EPO analogs having native protein structure were glucosylated by UGGT. Considering the result of UGGT assay with synthetic INF-β that have a similar secondary structure to EPO, secondary structure is not affect to the recognition of UGGT. From the observation of protein surface model and the result of ANS assay, it is estimated that UGGT recognize exposed hydrophobic surface that cannot be covered by three oligosaccharides.

ER lysate assay with synthetic EPO analogs revealed that UGGT and glucosidase II exchange their substrates constantly. Glucosylated EPO was not observed unless an inhibitor of glucosidase II was used, because of the fast substrate exchange.

I also found that UGGT, CNX/CRT and glucosidase II interact with the substrate glycoprotein under the ordered sequence. So far, the order of interaction was unclear whether it is in random order or in order. Our experimental results could answer the long-standing question. The unique character of EPO enabled me to find the ordered cycle. Moreover, the cycle velocity was estimated and it was suitable for the production of cytokine in the biosynthetic pathway.

These EPOs bearing high-mannose type oligosaccharides I synthesized are highly potential probes to investigate glycoprotein quality control system.

General and abbreviation.

2-(*1H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-benzotriazole (HOBt) and Boc-amino acids were purchased from Peptide Institute Inc. Boc-Cys(Trt) was purchased from Watanabe Chemical Ind. S-Trityl-mercaptopropionic acid was purchased from Oakwood Products Inc. Trifluoroacetic acid (TFA), triisopropylsilane (TIPS), 1,2-ethanedithiol (EDT), sodium 2-mercaptoethanesulfonate (MESNa), N,N'-diisopropylcarbodiimide (DIC), N,N-diisopropylethylamine (DIPEA), dibutyldicarbonate (Boc_2O) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Tokyo Chemical Ind. N,N-dimethylformamide (DMF), dichloromethane (DCM), dimethyl sulfoxide (DMSO), piperidine, m-cresol, thioanisole, trifluoromethanesulfonic acid (TfOH), diethyl ether, dimethylsulfide (DMS) , 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044), Cystine, Glutathione (reduced), Glutathione-dusulfide and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) were purchased from Wako Pure Chemical. Fmoc-amino acids, Boc-Arg(di-Z), Boc-His(DNP), Boc-Asn(Xan), 1-(mesitylene-2-sulfonyl)-3-nitro-*1H*-1,2,4-trizole (MSNT), amino-PEGA resin, Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and 4-(4-Hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) were purchased from Novabiochem. Guanidine hydrochloride (Gn·HCl), HPLC grade acetonitrile and L-Cysteine hydrochloride hydrate were purchased from Kanto Chemical Co. Inc. 2-Bromoaceto-phenone, 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(*3H*)-one (DEPBT) and 4-mercaptophenylacetic acid (MPAA) were purchased from Sigma-Aldrich. 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 tetrazolium monosodium salt (WST-8) were purchased from DOJIN. RP-HPLC analyses were performed using Vydac C18, C8 (GRACE Co.), Cadenza CD-C18 (Imtakt) or Proteonavi or Capcell pak (Shiseido) were used for analytical or/and semipreparative HPLC. Proteonavi (Shiseido) were used in LC-MS analysis. LC-MS and ESI-MS spectra were recorded on a Bruker Daltonics amaZon-mass spectrometer system and Bruker Daltonics Esquire 3000 mass spectrometer, respectively. High-resolution MS spectra were recorded on a FTICR-MS (Bruker solariX XR)

General procedure for Boc solid phase peptide synthesis (SPPS)

Boc-amino acids used were Gly, Ala, Val, Leu, Ile, Pro, Ser(Bzl), Thr(Bzl), Met, Asp(Bzl), Glu(Bzl), Asn(Xan), Gln, His(DNP), Lys(Cl-Z), Arg(di-Z), Arg(Mtr), Phe, Tyr(Br-Z), Trp(CHO) , Cys(Acm), Cys(MBzl) and Thz.

Peptide-α-thioesters were prepared using improved *in situ* neutralization Boc SPPS protocol on Nova PEG amino resin[1]. Nova PEG amino resin was pre-washed by MeOH (1mim*2), DMF (1min*2), DCM (1min*3), 1%TFA/DCM (1min*3), 5% DIPEA/DCM (1min*3) and DCM (1min*3). HBTU (72.1 mg, 0.19 mmol), DIPEA (70.0 μL, 0.4 mmol) and *S*-trityl-3-mercaptopropionic acid (80.0 mg, 0.2 mmol) were dissolved in DMF (1.0 mL) and pre-activated for 30 seconds. This solution was added to Nova PEG amino resin (50 μmol) to incorporate 3-mercaptopropionic acid to Nova PEG amino resin. *S*-Trityl group was removed by treatment of the resin with TFA for 1.5 min twice. The first Boc amino acid (Boc-AA, 0.2 mmol) was pre-activated with HBTU (72.1 mg, 0.19 mmol) and DIPEA (70.0 μL, 0.4 mmol) in DMF (1.0 mL) for 30 seconds and then the solution was added to the resin and the suspension was gently shaken for 20 min at ambient temperature. After the coupling of first amino acid, the following Boc-AA couplings were performed by same manner with the first Boc-AA coupling. For the Boc deprotection, the peptidyl resin was treated with TFA for 1.5 min twice followed by careful washing with DCM (ca. 70-100 mL) and DMF (ca. 70-100 mL).

After the coupling of the last amino acid residue, the resin was washed with DMF and DCM. To the resin (ca. 10 μmol) was added a solution of TFA (1.2 mL), *m*-cresol (0.12 mL) and DMS (0.36 mL) at 0ºC followed by slow addition of TfOH (0.12 mL). Then the suspension was shaken for 1 hour at 0ºC. The suspension was filtered and then the resulting resin was treated again with the same manner to complete the side-chain deprotection. In the case of incompletion of the deprotection by this process, strong acidic conditions employing TFA : TfOH : EDT : thioanisole = 20 : 2 : 1 : 2 for 1 hour at 0ºC were able to use. However this strong condition was only for peptide-α-thioester and not for glycopeptide-α-thioester. An aliquot of the resin was treated with sodium phosphate buffer (0.2 M, pH 7.0, 2.0 mL) containing 6 M Gn-HCl and 0.2 M MESNa for 12 h to perform thiolysis at the ambient temperature. Purification of the resultant solution containing peptide-α-thioester by preparative HPLC (Vydac C4 or C8 or C18 Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN at moderate ratio, over 30 min at the flow rate of 2.5 mL/min) afforded peptide-α-thioester.

Synthesis of EPO peptide segments A, B, C, D, and E (compound 34, 20, 16, 13 and 9).

Synthesis of peptide-α-thioester A, B, C, D and E (compound **34**, **20**, **16**, **13** and **9**) were performed by the same manner described in the general procedure for Boc SPPS. The analytical results are shown from Figure 2-9 to Figure 2-13.

Preparation of Boc-M9-high-mannose type oligosaccharide (Boc-M9)

Fmoc M9-high-mannose type oligosaccharide (Fmoc-M9) was prepared following the method that we have already reported[2]. Prepared Fmoc-M9 (55 mg, 24.8 μmol) was dissolved to DMSO-DMF (4 / 1, v / v, 3.0 ml). Piperidine (750 μl, 7.59 mmol) was added to the solution for deprotection of Fmoc group. After 1 h, the solution was poured into chilled Et_2O, and the resultant white precipitate was collected. This precipitate was dissolved to water and lyophilized. The resultant white foam was dissolved to DMSO-DMF (4 / 1, v / v, 3.0 ml). DIPEA (41 μl, 230 μmol) and Boc_2O (52 μl, 226 μmol) were added to the solution. After 1 h, the solution was poured into chilled Et_2O, and the resultant white precipitate was collected.

This precipitate was dissolved to water and lyophilized. The resultant white foam was dissolved to water and applied to ion-exchange column (Dowex W50×8 i.d. 10 ×80 mm, eluted with H_2O). The resultant fraction was lyophilized and Boc-M9 was yielded 43 mg as white foam. The yield was ca. 82.7% as the isolated yield.

Preparation of glycopeptide-α-thioester bearing M9-high-mannose type oligosaccharide

Peptide-α-thioesters on the resin before coupling of high-mannose-M9-oligosaccharide were prepared using general procedure for Boc SPPS as described above (ca. 4 μmol scale). Coupling of M9-high-mannose type oligosaccharide (8.4 mg, 4 μmol) was performed by using DEPBT (2.4 mg, 8.0 μmol) or PyBOP (4 mg, 8 μmol) and DIPEA (8.7 μL, 49 μmol) in DMF-DMSO (1/1, v/v, 133 μL) for 14 h at ambient temperature. After this coupling step, following Boc deprotection were performed following general procedure. The coupling of next Boc-AA was performed by using diluted condition such as Boc-AA (40 μmol, 40 mM), HBTU (38 μmol) and DIPEA (80 μmol) in DMF (1 mL) for 4 μmol scale synthesis. This coupling was performed for 20 min.

After the coupling of the last amino acid residue, the protecting groups of side chain were deprotected. The glycopeptide-α-thioester was cleaved from the resin and analyzed by the same method mentioned

above. Then the desired compound was afforded.

Synthesis of EPO glycopeptide segment B and D (compound 19 and 12)

Synthesis of glycopeptide segment B and D (compound **19** and **12**) were performed by the same manner described above. The analytical results are shown in Figure 2-14 and Figure 2-15.

Synthesis of EPO glycopeptide segment A (compound 33)

Glycopeptide-α-thioester on the resin before coupling of a protected peptide fragment was prepared using the same method as described above (ca. 4 μmol scale). The protected-peptide fragment was constructed by conventional Fmoc method using HMPB linker, which is cleaved by weak acid. The protected-peptide was cleaved from the resin by using the solution TFE : AcOH = 1:1. The reaction solution was evaporated and dried in vacuo. Coupling of this protected-peptide fragment (21 mg, 6 μmol) was performed by using DIC (0.92 μl, 6 μmol) and HOBt (0.81 mg, 6 μmol) in DMF (150 μL) for 1 h at ambient temperature. This coupling was conducted three times. The removal of protecting groups used in Fmoc method were performed by the cocktail containing 95% TFA, 2.5% triisopropylsilane and 2.5% water. The rest protecting groups used in Boc method were deprotected by the low acidic condition employing TFA : TfOH : *m*-cresol : dimethylsulfide = 5 : 1 : 1 : 3 for 1 hour at 0ºC. The resultant resin was treated with sodium phosphate buffer (0.2 M, pH 7.0, 0.4 mL) containing 6 M Gn-HCl and 0.2 M MESNa for 12 h to perform thiolysis at the ambient temperature. Purification of the resultant solution containing glycopeptide-α-thioester by preparative HPLC (Capcell Pak C18 Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 75 : 25 to 20 : 80, 35 min at the flow rate of 2.5 mL/min) afforded desired glycopeptide segment A (compound **33**). The analytical results are shown in Figure 2-17.

Synthesis of peptide segment F (compound 10)

Peptide segment F (compound **10**) was synthesized by using of conventional Fmoc-based SPPS. The analytical result is shown in Figure 2-18.

Synthesis EPO-24, 38, 83

Native chemical ligation (NCL) between segment E (EPO (98-127)-peptide-α-thioester, compound 9) and segment F (EPO (128-166)-peptide, compound 10).

Peptide segment F (compound **10**, 20.0 mg, 4.3 μmol) was dissolved in a ligation buffer (1070 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (7.1 mg, 42 μmol) and 20 mM TCEP (6.1 mg, 21 μmol) at pH 6.8. The peptide-α-thioester segment E (compound **9**, 14.0 mg, 4.4 μmol) was added to the solution and this solution was left for 3 h at ambient temperature. The reaction was monitored by RP-HPLC (Cadenza CD-C18 Φ4.6 × 10 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 70 : 30 to 30 : 70 for 15 min by 1 mL/min flow rate). After completion of the reaction, *N*-terminal thiazolidine (Thz) moiety of the product was converted into Cys residue as a one-pot reaction by adjusting pH to 4.0 with methoxyamine-HCl. After 2 h, the reaction mixture was subjected to RP-HPLC purification (Vydac C18 Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 62 : 38 to 30 : 70 for 35 min at the flow rate of 3 mL/min). The product (glycopeptide segment EF, compound **11**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 14.0 mg as white foam. The yield of the first NCL between segment E (compound **9**) and F (compound **10**) was ca. 41.8% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{334}H_{555}N_{100}O_{97}S_3$: $[M+H]^+$ 7619.8, found for 7620.2 (deconvoluted).

Native chemical ligation (NCL) between segment D (EPO (68-97)- M9-glycopeptide-α-thioester, compound 12) and segment EF (EPO (98-166)-peptide, compound 11).

The M9-glycopeptide-α-thioester segment D (compound **12**, 5.6 mg, 1.05 μmol) was dissolved in a ligation buffer (524 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (3.5 mg, 21 μmol) and 20 mM TCEP (3.0 mg, 10 μmol) at pH 6.9. The peptide segment EF (compound **11**, 7.9 mg, 1.04 μmol) was added to the solution and this solution was left for 4 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 70 : 30 to 35 : 65 for 30 min by 1 mL/min flow rate). After completion of the reaction, *N*-terminal thiazolidine (Thz) moiety of the product was converted into Cys residue followed with the same manner as mentioned above. After 2 h, the reaction mixture was subjected to RP-HPLC purification (Vydac C4 Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 57: 43 to

40 : 60 for 35 min by 2.5 mL/min flow rate). The product (glycopeptide segment DEF, compound **14**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 5.0 mg as white foam. The yield of the second NCL between segment D (compound **12**) and EF (compound **11**) was ca. 37.6% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{553}H_{912}N_{143}O_{195}S_4$: $[M+H]^+$ 12812.3, found for 12812.5 (deconvoluted).

Native chemical ligation (NCL) between segment C (EPO (50-67)-peptide-α-thioester, compound 16) and segment DEF (EPO (68-166)-M9-glycopeptide, compound 14).

The peptide-α-thioester segment C (compound **16**, 1.5 mg, 0.66 μmol) was dissolved in a ligation buffer (195 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (1.3 mg, 7.8 μmol) and 20 mM TCEP (1.1 mg, 7.8 μmol) at pH 6.9. The glycopeptide segment DEF (compound **14**, 5 mg, 0.39 μmol) was added to the solution and this solution was left for 2.5 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 70 : 30 to 30 : 70 for 30 min by 1 mL/min flow rate). After completion of the reaction, *N*-terminal thiazolidine (Thz) moiety of the product was converted into Cys residue followed with the same manner as mentioned above. After 2 h, the reaction mixture was subjected to RP-HPLC purification (Vydac C4 Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 52 : 48 to 35 : 65 for 35 min by 2.5 mL/min flow rate). The product (glycopeptide segment CDEF, compound **17**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 3.6 mg as white foam. The yield of the third NCL between segment C (compound **16**) and DEF (compound **14**) was ca. 61.7% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{648}H_{1057}N_{170}O_{220}S_6$: $[M+H]^+$ 14941.8, found for 14942.8 (deconvoluted).

Native chemical ligation (NCL) between segment B (EPO (29-49)- M9-glycopeptide-α-thioester, compound 19) and segment CDEF (EPO (50-166)-M9-glycopeptide, compound 17).

The M9-glycopeptide-α-thioester segment B (compound **19**, 1.2 mg, 0.26 μmol) was dissolved in a ligation buffer (120 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (0.81 mg, 4.8 μmol) and 40 mM TCEP (1.37 mg, 4.8 μmol) at pH 7.0. The M9-glycopeptide segment CDEF (compound **17**, 3.6 mg, 0.24 μmol) was added to the solution and this solution was left for 5 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1%

TFA : 0.1% TFA in 90% MeCN = 60 : 40 to 30 : 70 for 30 min by 1 mL/min flow rate). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 35 : 65 for 35 min by 2.5 mL/min flow rate). The product (glycopeptide segment BCDEF, compound **21**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 2.0 mg as white foam. The yield of the forth NCL between segment B (compound **19**) and CDEF (compound **17**) was ca. 47.6% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{827}H_{1338}N_{201}O_{311}S_8$: $[M+H]^+$ 19329.2, found for 19329.2 (deconvoluted).

Desulfurization of segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 21)

M9-glycopeptide BCDEF (compound **21**, 1.0 mg, 0.052 μmol) was dissolved in a 200 mM sodium phosphate buffer (pH 7.0, 220 μl) containing 7.5 M Gn-HCl and 0.25 M TCEP. To the solution, 2-methyl-2-propanethiol (19.8 μl), 0.1 M VA-044 (39.8 μl) was added. The reaction mixture was stirred for 3 h at 37ºC. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 55 : 45 to 32 : 68 for 30 min at the flow rate of 1 mL/min). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 38 : 62 for 35 min by 1 mL/min flow rate). The product (Reduced M9-glycopeptide segment BCDEF, compound **25**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 0.5 mg as white foam. The yield of the reduction of segment BCDEF (compound **21**) was ca. 50.4% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{827}H_{1338}N_{201}O_{311}S_4$: $[M+H]^+$ 19200.9, found for 19200.9 (deconvoluted).

Deprotection of Acetamidomethyl (Acm) group of segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 25)

Above desulfurized M9-glycopeptide BCDEF (compound **25**, 0.65 mg, 0.034 μmol) was dissolved in 90% acetic acid solution (141 μL) containing AgOAc (0.82 mg) and the mixture was stirred for 4 h at ambient temperature. After centrifugation of the reaction mixture, the supernatant was collected in a new tube and was added DTT (0.95 mg). After stirring for 5 min, the resultant precipitate was removed by centrifugation and the supernatant was subjected to RP-HPLC purification (Proteonavi

Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 38 : 62 for 35 min at the flow rate of 2.5 mL/min). The purified M9-glycopeptide BCDEF (compound **29**) was characterized by ESI-MS. This product was lyophilized and afforded 0.4 mg as white foam. The yield of the segment BCDEF (compound **29**), which was deprotected Acm groups was ca. 62.5% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{818}H_{1323}N_{198}O_{308}S_4$: $[M+H]^+$ 18987.7, found for 18987.4 (deconvoluted).

Native chemical ligation (NCL) between segment A (EPO (1-28)- M9-glycopeptide-α-thioester, compound 33) and segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 29).

The M9-glycopeptide-α-thioester segment A (compound **33**, ca. 0.4 mg, 0.78 μmol) was dissolved in a ligation buffer (20 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (ca. 0.13 mg, 0.8 μmol) and 40 mM TCEP (ca. 0.23 mg, 0.8 μmol) at pH 7.0. The glycopeptide segment BCDEF (compound **29**, 0.8 mg, 0.042 μmol) was added to the solution and this solution was left for 10 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 70 : 30 to 20 : 80 for 30 min by 1 mL/min flow rate). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 38 : 62 for 35 min by 2.5 mL/min flow rate). The product (EPO glycopeptide segment ABCDEF, compound **35**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded as white foam. ESI-MS: *m/z* calcd. for $C_{1025}H_{1666}N_{239}O_{406}S_5$: $[M+H]^+$ 23993.9, found for 23995.7 (deconvoluted).

Folding of EPO M9-glycosylated polypeptide (compound 35)

Folding of EPO M9-glycosylated polypeptide ABCDEF (compound **35**) was performed by use of stepwise dialysis method. EPO M9-glycosylated polypeptide ABCDEF(compound **35**) was dissolved in a Tris-HCl buffer (100 mM, pH 7.5) containing 6 M Gn-HCl. The concentration of the EPO M9-glycosylated polypeptide ABCDEF (compound **35**) was adjusted to suitable concentration (ca. 0.1 mg/mL). This solution was poured into the dialysis tubing (MWCO at 8,000 Spectra/Por®) and then dialyzed against the first folding buffer (3 M Gn-HCl, 100 mM Tris-HCl, pH 8.5) containing 4 mM cysteine and 0.5 mM cystine for redox system and left for 12 h at 4ºC. Then, the external buffer solution was replaced to the second folding buffer solution (1 M Gn-HCl, 100 mM Tris-HCl, pH 8.0) and dialysis was performed for 20 h. Finally, the external buffer was discarded and changed again to

the third folding buffer solution (10 mM Tris-HCl, pH 7.0) The dialysis was performed for 24 h. The correctly folded and misfolded EPO analogs were purified by RP-HPLC (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 30 : 70 for 35 min at the flow rate of 2.5 mL/min). Fractions containing correctly folded EPO **1** and misfolded EPO **2** were collected and then lyophilized. The purity of correctly folded EPO **1** and misfolded EPO **2** was confirmed by HPLC and ESI-MS spectroscopy. The isolated amounts of correctly folded EPO **1** and misfolded EPO **2** were ca. 124.7 μg and ca. 25.8 μg respectively, estimated by the Bradford protein assay. FT-ICR-MS: *m/z* calcd. for $C_{1025}H_{1662}N_{239}O_{406}S_5$: $[M+H]^+$ 23989.5730, found for 23989.5022 (deconvoluted).

Synthesis EPO-24

Native chemical ligation (NCL) between segment D (EPO (68-97)-peptide-α-thioester, compound 13) and segment EF (EPO (98-166)-peptide, compound 11).

The peptide-α-thioester segment D (compound **13**, 11.3 mg, 3.20 μmol) was dissolved in a ligation buffer (810 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (5.4 mg, 32 μmol) and 20 mM TCEP (4.6 mg, 16 μmol) at pH 6.9. The peptide segment EF (compound **11**, 24.7 mg, 3.24 μmol) was added to the solution and this solution was left for 3 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 65 : 35 to 30 : 70 for 30 min by 1 mL/min flow rate). After completion of the reaction, *N*-terminal thiazolidine (Thz) moiety of the product was converted into Cys residue followed with the same manner as mentioned above. After 2 h, the reaction mixture was subjected to RP-HPLC purification (Vydac C4 Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 30 : 70 for 35 min by 3 mL/min flow rate). The product (peptide segment DEF, compound **15**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 12.5 mg as white foam. The yield of the second NCL between segment D (compound **13**) and EF (compound **11**) was ca. 35.2% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{485}H_{802}N_{141}O_{139}S_4$: $[M+H]^+$ 10960.7, found for 10960.4 (deconvoluted).

Native chemical ligation (NCL) between segment C (EPO (50-67)-peptide-α-thioester, compound 16) and segment DEF (EPO (68-166)-peptide, compound 15).

The peptide-α-thioester segment C (compound **16**, 2.58 mg, 1.13 μmol) was dissolved in a ligation buffer (565 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (3.8 mg, 22.6 μmol) and 20 mM TCEP (3.2 mg, 11.3 μmol) at pH 6.9. The peptide segment DEF (compound **15**, 12.4 mg, 1.13 μmol) was added to the solution and this solution was left for 3 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 70 : 30 to 30 : 70 for 30 min by 1 mL/min flow rate). After completion of the reaction, *N*-terminal thiazolidine (Thz) moiety of the product was converted into Cys residue followed with the same manner as mentioned above. After 2 h, the reaction mixture was subjected to RP-HPLC purification (Vydac C4 Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 54 : 46 to 30 : 70 for 35min by 2.5 mL/min flow rate). Then the product (peptide segment CDEF, compound **18**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 7.1 mg as white foam. The yield of the third NCL between segment C (compound **16**) and DEF (compound **15**) was ca. 47.7% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{580}H_{947}N_{168}O_{164}S_6$: $[M+H]^+$ 13089.1, found for 13088.6 (deconvoluted).

Native chemical ligation (NCL) between segment B (EPO (29-49)- peptide-α-thioester. Compound 20) and segment CDEF (EPO (50-166)-peptide, compound 18).

The peptide-α-thioester segment B (compound **20**, 1.60 mg, 0.60 μmol) was dissolved in a ligation buffer (120 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (0.81 mg, 4.8 μmol) and 40 mM TCEP (1.38 mg, 4.8 μmol) at pH 7.0. The peptide segment CDEF (compound **18**, 4.2 mg, 0.32 μmol) was added to the solution and this solution was left for 3.5 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 60 : 40 to 30 : 70 for 30 min by 1 mL/min flow rate). 0.2 M MESNa (3.9 mg, 23.7 μmol) was added to the reaction mixture to remove the oxidized and branched by-products. After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 54 : 46 to 35 : 65 for 35 min by 2.5 mL/min flow rate). The product (glycopeptide segment BCDEF, compound **22**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 2.4 mg as white foam. The yield

of the forth NCL between segment B (compound **20**) and CDEF (compound **18**) was ca. 48% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{691}H_{1118}N_{197}O_{199}S_8$: $[M+H]^+$ 15626.0, found for 15626.0 (deconvoluted).

Desulfurization of segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 22)

Peptide segment BCDEF (compound **22**, 1.2 mg, 0.077 μmol) was dissolved in a 200 mM sodium phosphate buffer (pH 7.0, 220 μl) containing 7.5 M Gn-HCl and 0.25 M TCEP. To the solution, 2-methyl-2-propanethiol (19.8 μl) and 0.1 M VA-044 (39.8 μl) was added. The reaction mixture was stirred for 3 h at 37ºC. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 53 : 47 to 30 : 70 for 30 min at the flow rate of 1 mL/min). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 38 : 62 for 35 min by 1 mL/min flow rate). The product (Reduced glycopeptide segment BCDEF, compound **26**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 0.8 mg as white foam. The yield of the reduction of glycopeptide segment BCDEF (compound **22**) was ca. 67.8% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{691}H_{1118}N_{197}O_{199}S_4$: $[M+H]^+$ 15497.8, found for 15498.6 (deconvoluted).

Deprotection of Acetamidomethyl (Acm) group of segment BCDEF (EPO (29-166)- peptide, compound 26)

Above desulfurized peptide BCDEF (compound **26**, 0.8 mg, 0.052 μmol) was dissolved in 90% acetic acid solution (141 μL) containing AgOAc (0.82 mg) and the mixture was stirred for 4 h at ambient temperature. After centrifugation of the reaction mixture, the supernatant was collected in a new tube and was added DTT (0.95 mg). After stirring for 5 min, the resultant precipitate was removed by centrifugation and the supernatant was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 38 : 62 for 35 min at the flow rate of 1 mL/min). The purified glycopeptide BCDEF (compound **30**) was characterized by ESI-MS. This product was lyophilized and afforded 0.5 mg as white foam. The yield of the segment BCDEF (compound **30**), which was deprotected Acm group was ca. 63.5% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{682}H_{1103}N_{194}O_{196}S_4$: $[M+H]^+$ 15284.5, found for 15284.6 (deconvoluted).

Native chemical ligation (NCL) between segment A (EPO (1-28)- M9-glycopeptide-α-thioester, compound 33) and segment BCDEF (EPO (29-166)- peptide, compound 30).

The M9-glycopeptide-α-thioester segment A (compound **33**, ca. 0.4 mg, 0.078 μmol) was dissolved in a ligation buffer (33 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (ca. 0.22 mg, 1.32 μmol) and 40 mM TCEP (ca. 0.38 mg, 1.32 μmol) at pH 7.0. The peptide segment BCDEF (compound **30**, 1 mg, 0.065 μmol) was added to the solution and this solution was left for 10 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 70 : 30 to 20 : 80 for 30 min by 1 mL/min flow rate). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 38 : 62 for 35 min by 1 mL/min flow rate). The product (EPO glycopeptide segment ABCDEF, compound **36**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 0.6 mg as white foam. The yield of the final NCL between segment A (compound **33**) and BCDEF (compound **30**) was ca. 45% as the isolated yield. ESI-MS: *m/z* calcd for $C_{889}H_{1446}N_{235}O_{294}S_5$: $[M+H]^+$ 20290.8, found for 20290.8 (deconvoluted).

Folding of EPO M9-glycosylated polypeptide (compound 36)

Folding of EPO M9-glycosylated polypeptide ABCDEF (compound **36**) was performed by using of stepwise dialysis method. EPO M9-glycosylated polypeptide ABCDEF (compound **36**) was dissolved in a Tris-HCl buffer (100 mM, pH 7.5) containing 6 M Gn-HCl. The concentration of the EPO M9-glycosylated polypeptide ABCDEF (compound **36**) was adjusted to suitable concentration (ca. 0.1 mg/mL). This solution was poured into the dialysis tubing (MWCO at 8,000 Spectra/Por®) and then dialyzed against the first folding buffer (3 M Gn-HCl, 100 mM Tris-HCl, pH 8.5) containing 4 mM cysteine and 0.5 mM cystine for redox system and left for 12 h at 4ºC. Then, the external buffer solution was replaced to the second folding buffer solution (1 M Gn-HCl, 100 mM Tris-HCl, pH 8.0) and dialysis was performed for 20 h. Finally, the external buffer was discarded and changed again to the third folding buffer solution (10 mM Tris-HCl, pH 7.0). The dialysis was performed for 24 h. The correctly folded EPO **3** and misfolded EPO **4** were purified by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 30 : 70 for 35 min at the flow rate of 1 mL/min). Fractions containing correctly folded EPO **3** and misfolded EPO **4** were collected and then

lyophilized. The purity of correctly folded EPO **3** and misfolded EPO **4** was confirmed by HPLC and ESI-MS spectroscopy. The isolated amounts of correctly folded EPO **3** and misfolded EPO **4** were ca. 64 μg and ca. 53 μg respectively, estimated by the Bradford protein assay. FT-ICR-MS: *m/z* calcd. for $C_{889}H_{1442}N_{235}O_{294}S_5$: $[M+H]^+$ 20285.3948, found for 20285.4012 (deconvoluted).

Synthesis EPO-38

Native chemical ligation (NCL) between segment B (EPO (29-49)-M9-glycopeptide-α-thioester, compound 19) and segment CDEF (EPO (50-166)-peptide, compound 18).

The M9-glycopeptide-α-thioester segment B (compound **19**, 1.60 mg, 0.35 μmol) was dissolved in a ligation buffer (160 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (1.1 mg, 6.5 μmol) and 20 mM TCEP (0.91 mg, 3.3 μmol) at pH 7.0. The peptide segment CDEF (compound **18**, 4.2 mg, 0.32 μmol) was added to the solution and this solution was left for 7 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 75 : 25 to 20 : 80 for 30 min by 1 mL/min flow rate). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 53 : 47 to 35 : 65 for 35 min by 2.5 mL/min flow rate). The product (glycopeptide segment BCDEF, compound **23**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 3.3 mg as white foam. The yield of the forth NCL between segment B (compound **19**) and CDEF (compound **18**) was ca. 53.7% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{759}H_{1228}N_{199}O_{255}S_8$: $[M+H]^+$ 17477.6, found for 17476.0 (deconvoluted).

Desulfurization of segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 23)

M9-glycopeptide BCDEF (compound **23**, 3.0 mg, 0.17 μmol) was dissolved in a 200 mM sodium phosphate buffer (pH 7.0, 244 μl) containing 6 M Gn-HCl. To the solution, 0.5 M TCEP solution (pH = 7.0, 244 μl), 2-methyl-2-propanethiol (61.2 μl), 0.1 M VA-044 (122 μl) was added. The reaction mixture was stirred for 3 h at 37ºC. The reaction was monitored by RP-HPLC, Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 53 : 47 to 20 : 80 for 30 min at the flow rate of 1 mL/min). After completion of the reaction, the reaction mixture was subjected to RP-HPLC

purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 25 : 85 for 35 min by 1 mL/min flow rate). The product (Reduced M9-glycopeptide segment BCDEF, compound **27**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 1.7 mg as white foam. The yield of the reduction of segment BCDEF (compound **23**) was ca. 57.6% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{759}H_{1228}N_{199}O_{255}S_4$: $[M+H]^+$ 17349.4, found for 17348.3 (deconvoluted).

Deprotection of Acetamidomethyl (Acm) group of segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 27)

Above desulfurized M9-glycopeptide BCDEF (compound **27**, 1.7 mg, 0.10 μmol) was dissolved in 90% acetic acid solution (200 μL) containing AgOAc (1.8 mg) and the mixture was stirred for 4 h at ambient temperature. After centrifugation of the reaction mixture, the supernatant was collected in a new tube and was added DTT (3.24 mg). After stirring for 5 min, the resultant precipitate was removed by centrifugation and the supernatant was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 25 : 75 for 35 min at the flow rate of 1 mL/min). The purified M9-glycopeptide BCDEF (compound **31**) was characterized by ESI-MS. This product was lyophilized and afforded 1.3 mg as white foam. The yield of the segment BCDEF (compound **31**), which was deprotected Acm group was ca. 77.3% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{750}H_{1213}N_{196}O_{252}S_4$: $[M+H]^+$ 17136.1, found for 17134.7 (deconvoluted).

Native chemical ligation (NCL) between segment A (EPO (1-28)- peptide-α-thioester, compound 34) and segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 31).

The peptide-α-thioester segment A (compound **34**, ca. 0.57 mg, 0.17 μmol) was dissolved in a ligation buffer (16 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (ca. 0.10 mg, 0.64 μmol) and 40 mM TCEP (ca. 0.18 mg, 0.64 μmol) at pH 7.0. The glycopeptide segment BCDEF (compound **31**, 0.6 mg, 0.035 μmol) was added to the solution and this solution was left for 5.5 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 70 : 30 to 20 : 80 for 30 min by 1 mL/min flow rate). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 30 : 70 for 35 min by 1

mL/min flow rate). The product (EPO glycopeptide segment ABCDEF, compound **37**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded as white foam. ESI-MS: *m/z* calcd. for $C_{889}H_{1446}N_{235}O_{294}S_5$: $[M+H]^+$ 20290.8, found for 20289.2 (deconvoluted).

Folding of EPO M9-glycosylated polypeptide (compound 37)

Folding of EPO M9-glycosylated polypeptide ABCDEF (compound **37**) was performed by using of stepwise dialysis method. EPO M9-glycosylated polypeptide ABCDEF (compound **37**) was dissolved in a Tris-HCl buffer (100 mM, pH 7.5) containing 6 M Gn-HCl. The concentration of the EPO M9-glycosylated polypeptide ABCDEF (compound **37**) was adjusted to suitable concentration (ca. 0.1 mg/mL). This solution was poured into the dialysis tubing (MWCO at 8,000 Spectra/Por®) and then dialyzed against the first folding buffer (3 M Gn-HCl, 100 mM Tris-HCl, pH 8.5) containing 4 mM cysteine and 0.5 mM cystine for redox system and left for 12 h at 4ºC. Then, the external buffer solution was replaced the second folding buffer solution (1 M Gn-HCl, 100 mM Tris-HCl, pH 8.0) and dialysis was performed for 20 h. Finally, the external buffer was discarded and changed again to the third folding buffer solution (10 mM Tris-HCl, pH 7.0). The dialysis was performed for 24 h. The correctly folded EPO **5** and misfolded EPO **6** were purified by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 30 : 70 for 35 min at the flow rate of 1 mL/min). Fractions containing correctly folded EPO **5** and misfolded EPO **6** were collected and then lyophilized. The purity of correctly folded EPO **5** and misfolded EPO **6** was confirmed by HPLC and ESI-MS spectroscopy. The isolated amounts of correctly folded EPO **5** and misfolded EPO **6** were ca. 167.5 μg and ca. 66.4 μg respectively, estimated by the Bradford protein assay. FT-ICR-MS: *m/z* calcd. for $C_{889}H_{1442}N_{235}O_{294}S_5$: $[M+H]^+$ 20285.3948, found for 20285.3562 (deconvoluted).

Synthesis EPO-83

Native chemical ligation (NCL) between segment B (EPO (29-49)- peptide-α-thioester, compound 20) and segment CDEF (EPO (50-166)-M9-glycopeptide, compound 17).

The peptide-α-thioester segment B (compound **20**, 1.4 mg, 0.52 μmol) was dissolved in a ligation buffer (120 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (0.81 mg, 4.8

μmol) and 40 mM TCEP (1.37 mg, 4.8 μmol) at pH 7.0. The M9-glycopeptide segment CDEF (compound **17**, 3.6 mg, 0.24 μmol) was added to the solution and this solution was left for 4.5 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 60 : 40 to 30 : 70 for 30 min by 1 mL/min flow rate). 0.2 M MESNa (3.9 mg, 23.7 μmol) was added to the reaction mixture to remove the oxidized and branched by-products. After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 35 : 65 for 35 min by 2.5 mL/min flow rate). The product (glycopeptide segment BCDEF, compound **24**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 2.4 mg as white foam. The yield of the forth NCL between segment B (compound **20**) and CDEF (compound **17**) was ca. 57.1% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{759}H_{1228}N_{199}O_{255}S_8$: $[M+H]^+$ 17477.6, found for 17478.9 (deconvoluted).

Desulfurization of segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 24)

M9-glycopeptide BCDEF (compound **24**, 1.2 mg, 0.069 μmol) was dissolved in a 200 mM sodium phosphate buffer (pH 7.0, 220 μl) containing 7.5 M Gn-HCl and 0.25 M TCEP. To the solution, 2-methyl-2-propanethiol (19.8 μl), 0.1 M VA-044 (39.8 μl) was added. The reaction mixture was stirred for 3 h at 37 ºC. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 53 : 47 to 30 : 70 for 30 min at the flow rate of 1 mL/min). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 25 : 85 for 35 min by 1 mL/min flow rate). The product (Reduced M9-glycopeptide segment BCDEF, compound **28**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 0.8 mg as white foam. The yield of the reduction of segment BCDEF (compound **24**) was ca. 67.2% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{759}H_{1228}N_{199}O_{255}S_4$: $[M+H]^+$ 17349.4, found for 17349.7 (deconvoluted).

Deprotection of Acetamidomethyl (Acm) group of segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 28)

Above desulfurized M9-glycopeptide BCDEF (compound **28**, 0.8 mg, 0.046 μmol) was dissolved in 90% acetic acid solution (141 μL) containing AgOAc (0.82 mg) and the mixture was stirred for 4 h at ambient temperature. After centrifugation of the reaction mixture, the supernatant was collected in a new tube and was added DTT (0.95 mg). After stirring for 5 min, the resultant precipitate was removed by centrifugation and the supernatant was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 58 : 42 to 30 : 70 for 35 min at the flow rate of 1 mL/min). The purified M9-glycopeptide BCDEF (compound **32**) was characterized by ESI-MS. This product was lyophilized and afforded 0.6 mg as white foam. The yield of the segment BCDEF (compound **32**), which was deprotected Acm group was ca. 75.9% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{750}H_{1213}N_{196}O_{252}S_4$: $[M+H]^+$ 17136.1, found for 17136.5 (deconvoluted).

Native chemical ligation (NCL) between segment A (EPO (1-28)- peptide-α-thioester, compound 34) and segment BCDEF (EPO (29-166)-M9-glycopeptide, compound 32).

The peptide-α-thioester segment A (compound **34**, ca. 1.1 mg, 0.33 μmol) was dissolved in a ligation buffer (16 μL) containing 6 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (ca. 0.10 mg, 0.64 μmol) and 40 mM TCEP (ca. 0.18 mg, 0.64 μmol) at pH 7.0. The glycopeptide segment BCDEF (compound **32**, 0.6 mg, 0.035 μmol) was added to the solution and this solution was left for 10 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 70 : 30 to 20 : 80 for 30 min by 1 mL/min flow rate). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 55 : 45 to 40 : 60 for 35 min by 1.0 mL/min flow rate). The product (EPO glycopeptide segment ABCDEF, compound **38**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded as white foam. ESI-MS: *m/z* calcd. for $C_{889}H_{1446}N_{235}O_{294}S_5$: $[M+H]^+$ 20290.8, found for 20290.9 (deconvoluted).

Folding of EPO M9-glycosylated polypeptide (compound 38)

Folding of EPO M9-glycosylated polypeptide ABCDEF (compound **38**) was performed by using of stepwise dialysis method. EPO M9-glycosylated polypeptide ABCDEF (compound **38**) was dissolved

in a Tris-HCl buffer (100 mM, pH 7.5) containing 6 M Gn-HCl. The concentration of the EPO M9-glycosylated polypeptide ABCDEF (compound **38**) was adjusted to suitable concentration (ca. 0.1 mg/mL). This solution was poured into the dialysis tubing (MWCO at 8,000 Spectra/Por®) and then dialyzed against the first folding buffer (3 M Gn-HCl, 100 mM Tris-HCl, pH 8.5) containing 4 mM cysteine and 0.5 mM cystine for redox system and left for 12 h at 4ºC. Then, the external buffer solution was replaced the second folding buffer solution (1 M Gn-HCl, 100 mM Tris-HCl, pH 8.0) and dialysis was performed for 20 h. Finally, the external buffer was discarded and changed again to the third folding buffer solution (10 mM Tris-HCl, pH 7.0). The dialysis was performed for 24 h. The correctly folded EPO **7** and misfolded EPO **8** were purified by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 60 : 40 to 30 : 70 for 35 min at the flow rate of 1 mL/min). Fractions containing correctly folded EPO **7** and misfolded EPO **8** were collected and then lyophilized. The purity of correctly folded EPO **7** and misfolded EPO **8** was confirmed by HPLC and ESI-MS spectroscopy. The isolated amounts of correctly folded EPO **7** and misfolded EPO **8** were ca. 78.7 μg and ca. 45 μg respectively, estimated by the Bradford protein assay. FT-ICR-MS: *m/z* calcd. for $C_{889}H_{1442}N_{235}O_{294}S_5$: $[M+H]^+$ 20285.3948, found for 20285.3514 (deconvoluted).

Synthesis of INF-β

Synthesis of INF-β glycopeptide segments B (compound 39)

Synthesis of glycopeptide-α-thioester B (compound **39**) was performed by the same manner described above. The analytical results are shown in Figure 3-7.

Native chemical ligation (NCL) between segment B (INF-β (68-88)- M9-glycopeptide-α-thioester, compound 39) and segment C (INF-β (89-166)-peptide, compound 40).

The M9-glycopeptide-α-thioester segment B (compound **39**, 1.4 mg, 0.317 μmol) was dissolved in a ligation buffer (148 μL) containing 8 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (0.99 mg, 5.9 μmol) and 20 mM TCEP (0.84 mg, 3.0 μmol) at pH 6.9. The peptide segment C (compound **40**, 2.5 mg, 0.26 μmol) was added to the solution and this solution was left for 26 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA :

0.1% TFA in 90% MeCN = 65 : 35 to 30 : 70 for 30 min by 1 mL/min flow rate). After completion of the reaction, *N*-terminal thiazolidine (Thz) moiety of the product was converted into Cys residue followed with the same manner as mentioned above. After 2 h, the reaction mixture was subjected to RP-HPLC purification (Vydac C4 Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 59: 41 to 40 : 60 for 35 min by 2.5 mL/min flow rate). The product (INF-β glycopeptide segment BC, compound **42**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 1.6 mg as white foam. The yield of the first NCL between segment B (compound **39**) and C (compound **40**) was ca. 44.4% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{615}H_{965}N_{154}O_{204}S_4$: $[M+H]^+$ 13908.4, found for 13905.9 (deconvoluted).

Native chemical ligation (NCL) between segment A (INF-β (1-67)- peptide-α-thioester, compound 41) and segment BC (INF-β (68-166)-M9-glycopeptide, compound 42).

The peptide-α-thioester segment A (compound **41**, 1.44 mg, 0.17 μmol) was dissolved in a ligation buffer (100 μL) containing 8 M Gn-HCl, 200 mM sodium phosphate, 40 mM MPAA (ca 0.67 mg, 4 μmol) and 40 mM TCEP (ca 1.1 mg, 4 μmol) at pH 7.0. The glycopeptide segment BC (compound **42**, 1.6 mg, 0.12 μmol) was added to the solution and this solution was left for 19 h at ambient temperature. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 55 : 45 to 25 : 75 for 30 min by 1 mL/min flow rate). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 51 : 49 to 35 : 65 for 35 min by 2.5 mL/min flow rate). The product (INF-β glycopeptide segment ABC, compound **43**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 1.4 mg as white foam. The yield of the final NCL between segment A (compound **41**) and BC (compound **42**) was ca. 54.9% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{987}H_{1540}N_{251}O_{310}S_7$: $[M+H]^+$ 22170.9, found for 22168.4 (deconvoluted).

Desulfurization of segment ABC (INF-β (1-166)-M9-glycopeptide, compound 43)

M9-glycopeptide ABC (compound **43**, 1.4 mg, 0.063 μmol) was dissolved in a 200 mM sodium phosphate buffer (pH 7.0, 256 μl) containing 8 M Gn-HCl and 0.25 M TCEP. To the solution, 2-methyl-2-propanethiol (23.1 μl), 0.1 M VA-044 (46.3 μl) was added. The reaction mixture was stirred for 3 h at 37ºC. The reaction was monitored by RP-HPLC (Proteonavi Φ4.6 × 250 mm, 0.1%

TFA : 0.1% TFA in 90% MeCN = 52 : 48 to 25 : 75 for 30 min at the flow rate of 1 mL/min). After completion of the reaction, the reaction mixture was subjected to RP-HPLC purification (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 51 : 49 to 35 : 65 for 35 min by 2.5 mL/min flow rate). The product (Reduced INF-β M9-glycopeptide segment ABC, compound **44**) thus obtained was characterized by ESI-MS. This product was lyophilized and afforded 0.6 mg as white foam. The yield of the reduction of segment ABC (compound **43**) was ca. 42.9% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{987}H_{1540}N_{251}O_{310}S_7$: $[M+H]^+$ 22106.8, found for 22104.6 (deconvoluted).

Deprotection of Acetamidomethyl (Acm) group of segment ABC (INF-β (1-166)-M9-glycopeptide, compound 44)

Above desulfurized M9-glycopeptide ABC (compound **44**, 2.2 mg, 0.1 μmol) was dissolved in 90% acetic acid solution (388 μL) containing AgOAc (2.3 mg) and the mixture was stirred for 4 h at ambient temperature. After centrifugation of the reaction mixture, the supernatant was collected in a new tube and was added DTT (4.2 mg). After stirring for 5 min, the resultant precipitate was removed by centrifugation and the supernatant was subjected to RP-HPLC purification (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 51 : 49 to 35 : 65 for 35 min at the flow rate of 2.5 mL/min). The purified M9-glycopeptide ABC (compound **45**) was characterized by ESI-MS. This product was lyophilized and afforded 1.2 mg as white foam. The yield of the INF-β segment ABC (compound **45**), which was deprotected Acm group was ca. 54.5% as the isolated yield. ESI-MS: *m/z* calcd. for $C_{978}H_{1525}N_{248}O_{307}S_7$: $[M+H]^+$ 21893.6, found for 21890.1 (deconvoluted).

Folding of INF-β M9-glycosylated polypeptide (compound 45)

Folding of INF-β M9-glycosylated polypeptide ABC (compound **45**) was performed by using of stepwise dialysis method. INF-β M9-glycosylated polypeptide ABC (compound **45**) was dissolved in a Tris-HCl buffer (100 mM, pH 8.5) containing 8 M Gn-HCl. The concentration of the INF-β M9-glycosylated polypeptide ABC (compound **45**) was adjusted to suitable concentration (ca. 0.1 mg/mL). This solution was poured into the dialysis tubing (MWCO at 8,000 Spectra/Por®) and then dialyzed against the first folding buffer (4 M Gn-HCl, 100 mM Tris-HCl, pH 8.5) containing 8 mM glutathione and 1 mM glutathione-disulfide for redox system and left for 11 h at 4ºC. Then, the external buffer solution was replaced the second folding buffer solution (1 M Gn-HCl, 100 mM

Tris-HCl, pH 8.0) and dialysis was performed for 20 h. Finally, the external buffer was discarded and changed again to the third folding buffer solution (10 mM AcOH, pH 3.4). The dialysis was performed for 24 h. The correctly folded INF-β **46** was purified by RP-HPLC (Proteonavi Φ10 × 250 mm, 0.1% TFA : 0.1% TFA in 90% MeCN = 100 : 0 to 51 : 49 to 35 : 65 for 35 min at the flow rate of 2.5 mL/min). Fractions containing correctly folded INF-β **46** were collected and then lyophilized. The purity of correctly folded INF-β **46** was confirmed by HPLC and ESI-MS spectroscopy. The isolated amount of correctly folded INF-β **46** was ca. 44 μg, estimated by the Bradford protein assay. Misfolded INF-β was not obtained during the folding procedure. FT-ICR-MS: *m/z* calcd. for $C_{978}H_{1523}N_{248}O_{307}S_7$: $[M+H]^+$ 21889.9489, found for 21889.9801 (deconvoluted).

Characterization of correctly folded and misfolded EPO analogs

Disulfide mapping of correctly folded and misfolded EPO analogs (compound 1-8)

In order to determine the position of disulfide bonds, we employed a standard strategy using trypsin digestion. The correctly folded or misfolded EPO analogs (compound **1-8**, ca. 1.4 μg) were dissolved in phosphate buffer (pH 7.0, 100 mM, 50 μL), and then an appropriate amount of trypsin (ca. 0.1 μg) was added. After 11 h, the resultant fragments were analyzed by LC-MS. Sequentially, this solution was treated by 20 mM TCEP for 1 h.

Measurement of Circular dichroism (CD) spectra of correctly folded and misfolded EPO analogs (compound 1-8)

Far-UV CD spectra of correctly folded and misfolded EPO analogs (compound **1-8**) were measured with a JASCO-j820 CD spectropolarimeter (Fig. 2-52 and Fig. 2-53). The concentrations of EPO analogs were ca. 1.7 μM.

Biological Activity of correctly folded EPO-38 (compound 5) and EPO-24, 38, 83 (compound 1)

The biological activities of correctly folded EPO-38 (compound **5**) and EPO-24, 38, 83 (compound **1**) were evaluated. A cell proliferation assay using TF-1 cells, which were established from marrow cell, was examined. Commercially available EPO and only the cell culture medium were used as a positive

control and negative control, respectively. The plates containing TF-1 cells and EPO were incubated under 5% CO_2 atmosphere at 37ºC. After 72 h, cell growth was estimated by using of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-8). WST-8 was added to each well of the plate and incubated for 2 h. Absorbance of WST-8 in each well was measured at 450 nm and 630 nm. These data are shown in Figure 2-54 and Figure 2-55.

Denaturalization of misfolded EPO-38 (compound 6)

Misfolded EPO-38 (compound **6**, ca. 0.5 μg) was dissolved in 2 μl water. TCEP solution (40 mM, pH 7.3, 2 μl) was added to the solution and this solution was incubated for 1 h at ambient temperature. The solution which containing 6 M Gn-HCl (pH 7.0, 16 μl) was added to the reaction solution. After 1h, this solution was analyzed by LC-MS.

Recombinant UGGT assay for correctly folded EPO analogs (compound 1, 3, 5, 7) and INF-β (compound 46)

UGGT assay were carried out in 50 mM Tris-HCl at pH 7.5 containing 5 mM $CaCl_2$, 0.5 mM UDP-Glc, 5 uM of folded EPO or INF-β and 3.9 ng/μL recombinant UGGT. Recombinant human UGGT was expressed by yeast. The mixture was incubated at 37ºC. This reaction was monitored by LC-MS.

Recombinant UGGT assay for misfolded EPO analogs (compound 2, 4, 6, 8)

UGGT assay with misfolded EPO analogs are difficult to evaluate due to the disordered MS spectra of misfolded EPO analogs. Therefore, trypsin digestion was applied to analyze whether the misfolded EPO analogs were glucosylated or not. After the reaction with misfolded EPO analogs and UGGT as following above, the solutions were heated up to 100ºC to deactivate UGGT. Then, trypsin was added to this solution for the digestion of misfolded EPO to peptide and glycopeptide fragments. Finally, this solution was analyzed by LC-MS. Observing glycopeptide fragment reveals the result of UGGT assay.

UGGT assay for correctly folded EPO-24, 38, 83 (compound 1) with Glu-C

Glu-C digestion was applied to estimate the position dependency of glucosylation by UGGT. After the

UGGT assay as following above, the solution was heated up to 100ºC to deactivate UGGT. Then, Glu-C was added to this solution for digestion of EPO-24, 38, 83 to peptide and glycopeptide fragments. Finally, this solution was analyzed by LC-MS.

ANS assay

Binding of ANS was measured with FP-6500 (JASCO). Correctly folded EPO analogs (compound **1**, **3**, **5**, **7**, 10 μM) or misfolded EPO analogs (compound **2**, **4**, **6**, **8**, 10 μM) or correctly folded INF-β (compound **46**, 10 μM) were incubated with ANS (50 μM) at 37ºC for 10 min. The excitation wavelength was set at 369 nm. The emission was scanned from 400 nm to 600 nm in the suitable spectrum mode. All measurements were carried out in 50 mM Tris-HCl at pH 7.5.

Isolation of rough ER

Rough ER lysate was isolated from rat liver by sucrose density gradient centrifugation. Rat liver 33 g was cut into small pieces and the fibrous were filtered with grind on ice. The extract was homogenized by a homogenizer. The homogenate was centrifuged at 10000 g for 10 min at 4ºC. The supernatant was recovered and centrifuged at 10500 g for 60 min at 4ºC again. The precipitate was suspended by 2.1 M sucrose containing Tris-HCl, $MgCl_2$ and KCl. This suspension was filtered and applied to sucrose density gradient (0.3 M, 0.8 M and 1.15 M). This gradient was centrifuged at 150000 g for 90 min at 4ºC. The bottom-most layer was recovered and applied to next sucrose density gradient (0.8 M, rough ER containing layer (1.35 M) and 2.1M). This gradient was centrifuged at 300000 g for 60 min at 4ºC. The layer between 2.1 M and 1.35 M was recovered. The amount of proteins was estimated by the Bradford protein assay.

ER assay

ER assay were carried out in 50 mM HEPES at pH 7.5 containing 5 mM $CaCl_2$, 2 mM UDP-Glc, 5 uM of correctly folded EPO or INF- β and 290 ug/ml ER lysate. 1-deoxynojirimycin (5 mM) and kifunensine (1 mM) were used as inhibitors of glucosidases and mannosidases, respectively. The mixture was incubated at 37ºC. This reaction was monitored by LC-MS.

ER assay with antibodies

ER lysate (290 ug/ml) and antibodies (6.7 ug/ml) were incubated in 50 mM HEPES and 5 mM $CaCl_2$ for 4 h at 4ºC. To this mixture 5 uM EPO and 2 mM UDP-Glc were added. This mixture was incubated at 37ºC. This reaction was monitored by LC-MS.

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