"Chemical synthesis of erythropoietin glycoforms for insights into the relationship between glycosylation pattern and bioactivity"

Masumui Murakami, Tatsuto Kiuchi, Mika Nishihara, Katsunari Tezuka, Ryo Okamoto, Masayuki Izumi, and Yasuhiro Kajihara*

The role of sialyloligosaccharides on the surface of secreted glycoproteins is still unclear because of the difficulty in the preparation of sialylglycoproteins in a homogeneous form. We selected erythropoietin (EPO) as a target molecule and designed an efficient synthetic strategy for the chemical synthesis of a homogeneous form of five EPO glycoforms varying in glycosylation position and the number of human-type biantennary sialyloligosaccharides. A segment coupling strategy performed by native chemical ligation using six peptide segments including glycopeptides yielded homogeneous EPO glycopeptides, and folding experiments of these glycopeptides afforded the correctly folded EPO glycoforms. In an in vivo erythropoiesis assay in mice, all of the EPO glycoforms displayed biological activity, in particular the EPO bearing three sialyloligosaccharides, which exhibited the highest activity. Furthermore, we observed that the hydrophilicity and biological activity of the EPO glycoforms varied depending on the glycosylation pattern. This knowledge will pave the way for the development of homogeneous biologics by chemical synthesis.

INTRODUCTION
The chemical synthesis of natural products has made it possible to obtain many world-changing molecules (1). Development of protecting groups, several covalent bond formations, asymmetric synthesis, and structural analysis accelerated the effort to find more bioactive small molecules for making drugs through investigation of the structure-activity relationship.

Over the past two decades, protein drugs that are biologics prepared by cell expression systems have emerged in addition to the above-mentioned small-molecule drugs (2). Many of these biologics require oligosaccharides, a kind of posttranslational modification (3), for stability and solubility and to exert their pharmacokinetic properties in the blood, and these oligosaccharide modifications can be performed in mammalian cell expression systems. Such systems yield several different glycoproteins varying in oligosaccharide number and glycosylation position by mutagenesis, but the glycoproteins obtained exhibit considerable heterogeneity in oligosaccharide structure. Not only does this heterogeneity hinder the elucidations of oligosaccharide structures, but it also makes it difficult to determine which oligosaccharides play important roles in glycoprotein bioactivities.

In the same period, the chemical synthesis of proteins (4) and glycoproteins had emerged (5–7); however, the quantity of glycoprotein synthesized was still small compared to what was needed for further biological studies and clinical applications. The major difficulty in chemical glycoprotein synthesis comes from the time-consuming preparation method of the oligosaccharide building blocks. In principle, the regulation of anomeric configuration, multiple protection, and deprotection steps are required in the multistep organic synthesis. Several reports used these steps and chemically synthesized oligosaccharides for the chemical synthesis of glycoproteins (8), but the practical synthesis of a suitable amount of glycoproteins of high purity and of defined chemical structure remains a formidable challenge.

Bioactive glycoproteins need to have acidic sialyloligosaccharides, especially for the control of the resulting pharmacokinetic properties, where the negative charges of the sialic acid moieties can profoundly affect renal filtration. However, the chemical synthesis of such sialylglycoproteins is challenging because of the unexpected lability of the sialyl linkage under acidic conditions, which is a common problem in organic synthesis (9). Especially, acidic treatment is unavoidable in the chemical synthesis of the sialylglycopeptide-α-thioester that is essential for the chemical synthesis of glycoproteins. The lability of the sialyl linkage was thought to be attributed to the characteristic chemical nature of a deoxy sugar because sialic acid itself is a 3-deoxy sugar. However, our research group discovered that the carboxylic acid group of the sialic acid acts as an acid catalyst to accelerate the hydrolysis of the sialyl linkage (Fig. 1, Band C, and Fig. S1B) (10). This finding enabled us to examine the protection of the carboxylic acid group as a phenacyl ester so that the sialyl linkage would have a high degree of stability under acidic conditions owing to the absence of the catalytic protonation to the glycosidic oxygen (Fig. S1B), and we found that the hydrolysis yield of sialyl linkage was indeed significantly decreased (fig. S1B) (10). Using this protection of the sialyl carboxylic acid, we achieved a practical synthesis of a sialylglycopeptide-α-thioester through butoxycarbonyl (Boc) solid-phase peptide synthesis (Boc SPPS) that yields a suitable amount of peptide-α-thioester. Our optimized Boc protocol accelerated the chemical synthesis of several glycoproteins by means of repetitive native chemical ligation (10, 11).

Here, we describe the efficient chemical synthesis and biological activity of five homogeneous erythropoietin (EPO) glycoforms bearing one, two, and three human type biantennary sialyloligosaccharides at the native positions. These results provide the first insight into the relationship between the number of sialyloligosaccharides as well as glycosylation position and the in vivo bioactivity of EPO.

RESULTS
EPO is a heavily glycosylated protein and is known as a cytokine that induces the maturation of red blood cells (12). EPO has four sialyloligosaccharides on its surface, which are important roles in glycoprotein bioactivities. They are known to affect renal filtration. However, the chemical synthesis of such sialylglycoproteins is challenging because of the unexpected lability of the sialyl linkage under acidic conditions, which is a common problem in organic synthesis (9). Especially, acidic treatment is unavoidable in the chemical synthesis of the sialylglycopeptide-α-thioester that is essential for the chemical synthesis of glycoproteins. The lability of the sialyl linkage was thought to be attributed to the characteristic chemical nature of a deoxy sugar because sialic acid itself is a 3-deoxy sugar. However, our research group discovered that the carboxylic acid group of the sialic acid acts as an acid catalyst to accelerate the hydrolysis of the sialyl linkage (Fig. 1, Band C, and Fig. S1B) (10). This finding enabled us to examine the protection of the carboxylic acid group as a phenacyl ester so that the sialyl linkage would have a high degree of stability under acidic conditions owing to the absence of the catalytic protonation to the glycosidic oxygen (Fig. S1B), and we found that the hydrolysis yield of sialyl linkage was indeed significantly decreased (fig. S1B) (10). Using this protection of the sialyl carboxylic acid, we achieved a practical synthesis of a sialylglycopeptide-α-thioester through butoxycarbonyl (Boc) solid-phase peptide synthesis (Boc SPPS) that yields a suitable amount of peptide-α-thioester. Our optimized Boc protocol accelerated the chemical synthesis of several glycoproteins by means of repetitive native chemical ligation (10, 11).

Here, we describe the efficient chemical synthesis and biological activity of five homogeneous erythropoietin (EPO) glycoforms bearing one, two, and three human type biantennary sialyloligosaccharides at the native positions. These results provide the first insight into the relationship between the number of sialyloligosaccharides as well as glycosylation position and the in vivo bioactivity of EPO. 
ligosaccharides, and three of them are N-linked sialyloligosaccharides at the asparagine 24, 38, and 83 residues (Fig. 1A) (13, 14). These sialyloligosaccharides exhibit a diverse range from bi- to tetraantennary forms and play a critical role in increasing the lifetime of EPO in blood and in enhancing biological activity by interfering with glomerular filtration and galactose-binding lectin interaction (15). The serine at position 126 has a short sialyloligosaccharide that consists of three to four sugars. This short sialyloligosaccharide is not involved in the maturation of red blood cells (16). Therefore, here we focused on elucidating the function of three N-linked sialyloligosaccharides. The Higuchi group (16) reported that the sialyloligosaccharide at the 83 position seemed to be essential for bioactivity in vivo. We have already synthesized EPO bearing a sialyloligosaccharide at the 83 position (10) and found very weak bioactivity in vivo. In addition to this EPO glycoform, we found that our synthesized EPO analogs bearing two or three sialyloligosaccharides around the 24 to 32 positions did not show bioactivity in vivo (17, 18). Therefore, we synthesized an EPO glycoform bearing a sialyloligosaccharide at the 83 position by an efficient new synthetic route and used it in a current assay used to determine the borderline bioactivity in vivo among the three kinds of monoglycosylated EPO. Here, we decided to use a biantennary sialyloligosaccharide because this sialyloligosaccharide, such as 1 (Fig. 1B), can be prepared from egg yolk on a gram scale (10, 19). Preparation of a suitably protected fBoc-Asn-(sialyloligosaccharide diphenacyl ester)-OH 1 for glycopeptide synthesis is briefly summarized in Materials and Methods.

Here, we designed a practical synthetic route for the preparation of five EPO glycoforms by varying the glycosylation number and position. The EPO polypeptide chain consists of 166 amino acids and was divided into six segments (Fig. 2), and each segment was coupled using native chemical ligation (NCL) (4, 20) to yield the full-length glycopolyptide chains. However, EPO does not have a suitable number of cysteines for NCL, so we selected the Cys19 and Ala50, 79, 98, 128 sites for NCL. The Ala50, 79, 98, 128 sites were

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**Fig. 1. Structure of the EPO glycoforms and sialyloligosaccharide.** (A) Primary structure of EPO 2 showing the amino acid sequence, glycosylation sites, ligation sites (red circle), and disulfide bonds (red dotted line). The glutamine at position 78 was substituted with alanine. (B) Structure of the asparaginyl sialyloligosaccharide 1 used for the chemical synthesis of a sialylglycopeptide-α-thioester in Boc SPPS. Sialic acid was protected as a phenacyl (Pac) ester, shown in magenta. (C) Acceleration of hydrolysis by an intramolecular acid catalyst.
prepared as cysteines in the peptide and glycopeptide form, and then these cysteine sites were converted into alanine sites as a native EPO sequence after NCL \((21 - 23)\). Because we had designed a synthetic route for providing several EPO glycoforms varying in sialyloligosaccharide number and glycosylation position, three peptide segments \([1 - 28], [29 - 49], \) and \([79 - 97]\) were each synthesized in glycopeptide and nonglycosylated forms. The nonglycosylated peptide segments \([1 - 28], [29 - 49], [50 - 78], [79 - 97], \) and \([98 - 127]\) were prepared by the Boc SPPS method, and the peptide segment \([128 - 166]\) was prepared by the 9-fluorenyl methoxycarbonyl (Fmoc) SPPS method. Sialylglycopeptide segments \([29 - 49]\) and \([79 - 97]\) were prepared by an improved Boc SPPS method, which uses phenacyl-esterified Boc-Asn(disialyloligosaccharide)-OH \(1\). However, for the glycopeptide-\(\alpha\)-thioester segment \([1 - 28]\), a direct synthesis using the Boc SPPS method was difficult, so a segment coupling strategy on a solid support was used (see Materials and Methods). In the previous synthesis of an EPO glycoform bearing sialyloligosaccharide at the 83 position \((10)\), a mutation at the 21 position was essential because of the setting of the NCL site, but the current segment coupling strategy enabled an examination of the synthesis of a glycopeptide-\(\alpha\)-thioester segment \([1 - 28]\) having a natural amino acid sequence. According to this strategy, the current route needs only a mutation at the 78 position.

Fig. 2. Scheme for the synthesis of the EPO glycoforms by chemical ligation. The suitably glycosylated or nonglycosylated segments of \([1 - 28], [29 - 49] ,\) and \([79 - 97]\) were selected depending on the synthesis of EPO glycoforms \(2\) to \(6\) assembled by peptide ligation reactions. The full-length polypeptides were folded to form a three-dimensional structure through oxidative folding methods. Conditions: (i) NCL; (ii) conversion of thiazolidine into cysteine; (iii) deprotection of the Pac and formyl groups; (iv) deprotection of the Pac group; (v) thioesterification of hydrazide; (vi) desulfurization; (vii) deprotection of the Acm group.
For the folding of the glycopolypeptides thus obtained, we optimized the conditions of the in vitro folding reaction by monitoring the folding intermediates using SDS–polyacrylamide gel electrophoresis (SDS-PAGE), electrospray ionization mass spectrometry (ESI-MS), and the combination method of trypsin digestion and subsequent liquid chromatography (LC)-MS/MS analysis (figs. S16 to S24). The folding experiments were examined with EPO glycopeptide (0.1 mg/ml) based on previous reports (17, 24). Each sialylglycopeptide was completely denatured in a solution containing 8 M guanidine at the beginning of the folding process, and then the guanidine concentration was gradually diluted under dialysis conditions in the presence of cysteine-cystine redox reagents. In the case of EPON24, N83 4 bearing two sialyloligosaccharides at positions 24 and 83, we monitored the sequence of disulfide bond formation steps using peptidase digestion and subsequent LC-MS/MS analysis. When the folding buffer solution was changed from 8 M guanidine to 3 M guanidine containing cysteine-cystine redox reagents, a disulfide bond formed between the Cys29 and Cys33 residues (Fig. 3). However, when the folding buffer solution was changed to buffer containing 1 M guanidine, both oligomeric misfolded EPO and correctly folded EPO formed. Therefore, to avoid the formation of oligomeric misfolded forms, we used a dilute condition in which the initial concentration of the glycopolypeptides was set at 0.01 mg/ml (Table 1), and this condition successfully gave the correctly folded EPO glycoforms in good yields because the protein-protein aggregation was suppressed. Notably, the yields of the in vitro EPO folding were found to be different, depending on the number of sialyloligosaccharides and glycosylation position (Table 1 and figs. S16 to S19). The highest yield was found for EPON38, N83 3 (90% yield), whereas EPON24, N38, N83 2 gave an 86% yield. EPON24, N83 4 and EPON83 6, both lacking the sialyloligosaccharide at asparagine 38, had lower folding yields (66 and 63%, respectively), generating a considerable amount of misfolded products during the folding experiments. This result suggests that the sialyloligosaccharide at asparagine 38 might be important both for the formation of the productive folding intermediate and for the prevention of protein-protein aggregation.

All of the folded EPO glycoforms were purified by reversed-phase high-performance liquid chromatography (RP-HPLC), and their molecular weight and disulfide bond positions were analyzed with ESI-MS (Fig. 4, A and B, and figs. S20 to S24). The molecular weights of all of the folded EPO glycoforms were identical as compared with the theoretical monoisotopic mass value based on high-resolution ESI mass spectra (figs. S26 to S30). To confirm the disulfide bond positions, all the purified EPO glycoforms were digested with trypsin.

(Q→A) to avoid an undesired side-chain cyclization during NCL, and the other sequence is prepared as a human-type EPO amino acid sequence. After preparation of all of the peptide segments as well as glycopeptide segments, sequential segment coupling was performed according to the convergent synthetic strategy shown in Fig. 2. Segment coupling and deprotection of the Acm groups as well as phenacyl groups were performed in good yield. In our previous synthesis of EPO bearing a sialyloligosaccharide at asparagine 83 (17, 24), the desulfurization and deprotection of the Acm groups were performed in the final step. Previously, when we had used a related strategy for the synthesis of the EPO bearing three sialyloligosaccharides 2, we found that silver acetate used for the deprotection of the Acm groups seemed to induce the absorption of the hydrophobic EPO polypeptide into silver acetate. This also seemed to accelerate the aggregation of the EPO polypeptide. After extensive investigation, we determined that it is essential to perform desulfurization and deprotection of the Acm group before the final NCL step (Fig. 2). This new synthetic route enabled us to obtain each of the five glycosylated EPO polypeptides in sufficient yield (figs. S11 to S15).

For the folding of the glycopolypeptides thus obtained, we optimized the conditions of the in vitro folding reaction by monitoring the folding intermediates using SDS–polyacrylamide gel electrophoresis (SDS-PAGE), electrospray ionization mass spectrometry (ESI-MS), and the combination method of trypsin digestion and subsequent liquid chromatography (LC)-MS/MS analysis (figs. S16 to S24). The folding experiments were examined with EPO glycopeptide (0.1 mg/ml) based on previous reports (17, 24). Each sialylglycopeptide was completely denatured in a solution containing 8 M guanidine at the beginning of the folding process, and then the guanidine concentration was gradually diluted under dialysis conditions in the presence of cysteine-cystine redox reagents. In the case of EPON24, N83 4 bearing two sialyloligosaccharides at positions 24 and 83, we monitored the sequence of disulfide bond formation steps using peptidase digestion and subsequent LC-MS/MS analysis. When the folding buffer solution was changed from 8 M guanidine to 3 M guanidine containing cysteine-cystine redox reagents, a disulfide bond formed between the Cys29 and Cys33 residues (Fig. 3). However, when the folding buffer solution was changed to buffer containing 1 M guanidine, both oligomeric misfolded EPO and correctly folded EPO formed. Therefore, to avoid the formation of oligomeric misfolded forms, we used a dilute condition in which the initial concentration of the glycopolypeptides was set at 0.01 mg/ml (Table 1), and this condition successfully gave the correctly folded EPO glycoforms in good yields because the protein-protein aggregation was suppressed. Notably, the yields of the in vitro EPO folding were found to be different, depending on the number of sialyloligosaccharides and glycosylation position (Table 1 and figs. S16 to S19). The highest yield was found for EPON38, N83 3 (90% yield), whereas EPON24, N38, N83 2 gave an 86% yield. EPON24, N83 4 and EPON83 6, both lacking the sialyloligosaccharide at asparagine 38, had lower folding yields (66 and 63%, respectively), generating a considerable amount of misfolded products during the folding experiments. This result suggests that the sialyloligosaccharide at asparagine 38 might be important both for the formation of the productive folding intermediate and for the prevention of protein-protein aggregation.

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Table 1. Yields of in vitro folding. Yields (%) were estimated by RP-HPLC area.

| Concentration | EPON24, N38, N83 2 | EPON38, N83 3 | EPON24, N83 4 | EPON24, N38 5 | EPON83 6 |
|---------------|--------------------|---------------|---------------|---------------|----------|
| 0.1 mg/ml     | 68 (±1)            | 58 (±5)       | 43 (±3)       | 49 (±5)       | 37 (±3)  |
| 0.01 mg/ml    | 86 (±6)            | 90 (±6)       | 66 (±2)       | 76 (±11)      | 63 (±2)  |

Fig. 3. Proposed EPO in vitro folding process. All folding intermediates were analyzed by trypsin digestion and subsequent MS/MS analyses. The analysis revealed that the disulfide bond Cys29–Cys33 formed first under redox conditions.
followed by LC-MS/MS analysis. These analyses clearly indicated that the synthesized EPO glycoforms have correct disulfide bond patterns, which are Cys29-Cys33 and Cys7-Cys161.

The circular dichroism (CD) spectra of all the EPO glycoforms were also measured and showed the same spectral profiles (Fig. 4C), which were identical with those of the reported EPO (25), indicating that all of the EPO glycoforms used the same suitable protein secondary structure. According to these results, the differences in sialyloligosaccharide number and glycosylation position do not affect the protein’s folded structure.

The hydrophobicity of each EPO glycoform was also evaluated with a provisional method based on the profile of RP-HPLC, and the results revealed that the most hydrophilic EPO was EPON24, N38, N83 bearing three sialyloligosaccharides, whereas the most hydrophobic one was, surprisingly, EPON24, N38, N83, N162 bearing two sialyloligosaccharides (Fig. 5A). Although the addition of sialyloligosaccharide was expected to increase the hydrophilicity of the EPO glycoform, an interesting finding was that EPON24, N38, N83 exhibited greater hydrophobicity than EPO, N38, N83 bearing one sialyloligosaccharide. The sialyloligosaccharide at position 83 might be important to cover the hydrophobic protein surface (the orange over the yellow hydrophilic surface) around glycosylation position 83 (Fig. 5C) to increase the solubility of EPO. The Dubé group (26) reported that an EPO mutant lacking glycosylation at asparagine 38 and 83 resulted in a low-yield secretion and EPO might be metabolized as a nonnatural glycoprotein because of its highly hydrophobic nature.

The in vitro bioactivity of EPO, N38, N83, N162, based on the cell proliferation activity resulting from the binding of the synthetic EPO to the EPO receptor on the TF-1 cell (27), was confirmed to be slightly higher than that of commercially available EPO [EPOGIN expressed by CHO (Chinese hamster ovary) cell lines] (Fig. 5B). This might arise from a difference between the oligosaccharide structures of the two EPO samples. The synthesized EPO, N38, N83, N162 has biantennary sialyloligosaccharides, whereas EPOGIN has three sialyloligosaccharides in which the structure has a variety of bi- to tetra-antennary forms. EPO bearing highly branched oligosaccharides has been reported to result in lower cell proliferation activity in vitro (16). Thus, the results of our biological assay were found to be consistent with previous reports, and these results clearly support the finding that EPO, N38, N83, N162 has a correct three-dimensional structure in its protein portion.

To assess the biological activity of the synthetic EPO glycoforms, we evaluated the in vivo hematopoietic activity (hematocrit) using mice (Fig. 5D). Because EPOGIN at 4.4 μM showed a saturation of hematopoietic activity, the data of EPOGIN at 1.4 μM were used for the comparison of synthetic EPO glycoforms 2 to 6. All of the synthesized EPO displayed hematopoietic activity, but the activity varied depending on the glycosylation pattern, as expected. Among the synthesized EPO

![Fig. 4. Characterization of EPO glycoforms 2 to 6.](image)

(A) (a to e) ESI mass spectra of EPO glycoforms 2 to 6. All mass spectra are not of the ESI-MS data derived from the top area of the HPLC profile. All mass spectra were measured with total solution of individual EPO glycoforms isolated. (B) RP-HPLC chromatogram and ESI-MS spectrum of folded 2. (C) CD spectra of EPO glycoforms 2 to 6 in 0.1% TFA aq.
glycoforms, the most potent bioactive compound was found to be EPO^{N24, N38, N83} \text{2} (1.4 \mu M), although it displayed a lower hematopoietic activity than EPOGIN (1.4 \mu M) (day 2: 60\%, day 5: 68\%, day 7: 83\% versus day 7 EPOGIN: 100\%). Additionally, EPO bearing two biantennary sialyloligosaccharides (3, 4, and 5) resulted in a lower activity compared with 2, and the hematopoietic values of these glycoforms were different depending on glycosylation position. A remarkable finding is that the sialyloligosaccharides at asparagine 38 and 83 are much more important than the sialyloligosaccharide at asparagine 24 in terms of higher hematopoietic activity. The Yamaguchi group (28) also reported a relationship between glycosylation position and hematopoietic activity, although their EPO mutants, prepared by a cell expression system, showed considerable heterogeneity in the oligosaccharide structure. Therefore, our result is the first assay of hematopoietic activity performed using homogeneous EPO glycoforms and provides insight into the relationship between glycosylation pattern and hematopoietic activity in vivo.

In addition to our initial assays of each homogeneous EPO glycoform, we also carried out a unique assay using a solution that consisted of a mixture of equimolar amounts of EPO glycoforms 2 to 6 to investigate whether homogeneous EPO would exhibit more potent hematopoietic activity than the heterogeneous mixture of the glycoforms, which resembles commercially available EPO biologics. Recently, glycosylated biologics and their biosimilars have come out, and it remains to be determined whether homogeneous oligosaccharide is essential for their bioactivity (29). Therefore, we thought that this unique assay, which can be performed with just chemical synthesis, is interesting. As expected, the mixture of glycoforms 8 (individual EPO glycoform concentration was set at 0.28 \mu M, but total EPO protein concentration was 1.4 \mu M). Blue bar, 0 days; red bar, 2 days; green bar, 5 days; purple bar, 7 days.

Fig. 5. Characterization of the EPO glycoforms. (A) RP-HPLC chromatogram of a mixture of EPO glycoforms 2 to 6 to obtain insight into their hydrophobicity assessed by the elution time. Compounds \text{2}, \text{4}, \text{5}, and \text{6} (3.0 \mu g each) and \text{3} (4.5 \mu g) were mixed, and the resultant solution was injected in RP-HPLC. Retention time: \text{2}, 33.38 min; \text{3}, 33.99 min; \text{4}, 34.52 min; \text{5}, 38.64 min; \text{6}, 35.35 min. (B) Cell proliferation assay. Orange circle, synthetic EPO^{N24, N38, N83} \text{2}; gray triangle, EPOGIN. (C) EPO protein surface, with the three N-glycosylation sites highlighted in purple. The hydrophilic amino acids are shown in yellow, and the hydrophobic amino acids are shown in orange [the model was created from the NMR structure of human EPO (Protein Data Bank: 1BUY)]. (D) In vivo hematopoietic activity of the synthesized EPO glycoforms and EPOGIN. The concentrations of 2 to 7 were set at 1.4 \mu M. Sample 7 was a misfolded form of EPO 4. Sample 8 was a mixture of 2 to 6 (individual EPO glycoform concentration was set at 0.28 \mu M, but total EPO protein concentration was 1.4 \mu M).
activity, although weaker than that of its folded EPO\textsuperscript{N24, N83} 4. From the result of the trypsin digestion, it was confirmed that the misfolded EPO 7 has one disulfide bond at Cys\textsuperscript{29}–Cys\textsuperscript{33}, but we could not observe the disulfide bond at Cys\textsuperscript{7}–Cys\textsuperscript{61}. In addition, SDS-PAGE showed that EPO 7 was a mixture of a dimer, trimer, and multimer, which was easily reduced to a monomer by the reducing agents tris(2-carboxyethyl)phosphine hydrochloride (TCEP) or d,L-dithiothreitol (DTT). This misfolded EPO 7 displayed a quite similar CD profile as the folded EPO\textsuperscript{N24, N83} 4 (fig. S25). From these results, we hypothesized that both the misfolded EPO\textsuperscript{N24, N83} 7 and its native EPO\textsuperscript{N24, N83} 4 have similar protein structures, but the misfolded one may form weak aggregates of oligomeric form. The misfolded EPO 7 was stable enough to be isolated by HPLC purification, suggesting that it would be stable under physiological conditions. However, we could not confirm which form of EPO binds to the EPO receptor in mice so as to have hematopoietic activity: the monomeric form produced by refolding in blood or the oligomeric form itself. We speculate that even oligomeric misfolded EPO\textsuperscript{N24, N83} 7, which may be formed by disulfide bonds and hydrophobic interactions, has a correctly folded protein structure to be able to induce weak hematopoietic activity.

**DISCUSSION**

On the basis of the results shown in Fig. 5D, the relationship between glycosylation number as well as glycosylation position and hematopoietic activity was evaluated. Hematopoietic activity increased with increasing number of sialyloligosaccharides and a prolonged half-life of EPO in the blood. In a previous report, the sialyloligosaccharide at asparagine 24 of both urinary human EPO (30) and recombinant human EPO (16) was shown to exhibit diversity in a range from a bi- to a tetraantennary pattern, whereas the sialyloligosaccharides at asparagine 38 and 83 were shown to exist mainly in a tetraantennary form. We hypothesized that there is a hydrophobic protein surface (Fig. 5C: orange color) around asparagine 38 and 83, and this surface may decrease the solubility of EPO into the body fluid or perhaps accelerate aggregation. Therefore, our results in conjunction with previous reports (10, 17, 18) suggest that the sialyloligosaccharides at asparagine 38 and 83 are necessary to cover the hydrophobic protein surface with tetraantennary sialyloligosaccharide, which is needed to retain their solubility in the blood and to prevent protein-protein aggregation. A chemically synthesized, homogeneous polymer-modified EPO derivative of defined chemical structure (25, 31), which has branched polymer moieties bearing four negatively charged carboxylates at positions 24 and 126, where N- and O-glycosylation respectively occur, was previously reported to exhibit the most potent hematopoietic activity thus far. Because this negatively charged polymer modification enabled the EPO derivative to increase its solubility and to have reduced protein-protein aggregation as well as improved glomerular filtration, the half-life of EPO derivative in vivo was prolonged. On the other hand, an EPO derivative in which the asparagine residues of the three N-glycosylation sites were mutated to lysine displayed suitable solubility in comparison with that of nonglycosylated EPO with the native amino acid sequence (24, 32). This substitution might change the isoelectric point of the EPO analog protein and thus interfere with protein-protein aggregation, although the hematopoietic activity of this EPO derivative was not confirmed in vivo. Considering these effects of protein modifications, we hypothesized that the human body uses glycosylation as a posttranslational modification in order for the secreted protein to regulate its hydrophilicity and protect it from protein-protein aggregation, as well as to regulate renal filtration in the case of EPO, because the oligosaccharide exhibits highly hydrophilic and low antigenic properties in the body.

It has been a long-challenged goal of organic chemists to reveal the relationship between glycosylation and the biological activity of EPO. This is the first work to give an insight into the relationship between glycosylation pattern and hematopoietic activity based on the chemical synthesis of EPO glycoforms bearing biantennary sialyloligosaccharides with a natural linkage between oligosaccharide and protein. Removing any one of the three sialyloligosaccharides at the native positions resulted in a decrease in hematopoietic activity, whereas our previous study demonstrated that EPO bearing the same three sialyloligosaccharides at nonnative positions did not display any hematopoietic activity in vivo (17, 18). This means that glycosylation position and glycosylation pattern are critical for the bioactivity of glycoproteins. Glycosylation pattern might be dependent on the chemical property of the changing protein surface in the course of biological evolution. This knowledge and method developed for the chemical synthesis of glycoproteins will open a path to studying how glycosylation is determined at gene-coded positions and to the making of homogeneous biologics by chemical synthesis.
Br-Z: 2-bromo benzyloxy carbonyl; Thz: 1-4-thiazolidine carboxylic acid. The Fmoc-amino acids used were Gly, Ala, Val, Leu, Ile, Pro, Ser(tBu), Thr(tBu), Asp(OrBu), Glu(OrBu), Asn(Trr), Gln(Trr), Lys(Boc), Arg(Pbf), Phe, Tyr(OrBu), and Trp(Boc) (where tBu: tert-butyl; Try: trityl; Pbf: 2,2,4,6,7-pentamethyldihydrazofuran-5-sulfonyl).

RP-HPLC analyses were performed using Vydac C18, C8 (GRACE Co.), Cadenza CD-C18 (Intakt), or Proteonavi (Shiseido) for analytical and semipreparative HPLC. Proteonavi (Shiseido) was used in LC/MS analysis. LC/MS and direct infusion ESI-MS spectra were recorded on a Bruker Daltonics amaZon mass spectrometer, respectively. High-resolution mass spectra (direct infusion) were measured on a Bruker Daltonics Esquire 3000 mass spectrometer. The Fmoc group was removed by treatment with 9-fluorenylmethylchloroformate in H2O (30 ml) was adjusted to pH 3.6 with aq. Cs2CO3 (2.5 mg/ml) and then lyophilized. To a solution of this residue in DMF (4 ml), phenacyl bromide (5.7 mg, 0.03 mmol) was added to obtain Fmoc-Asn-(sialyloigosaccharide)-COOH.

A solution of homogeneous Fmoc-Asn-(sialyloigosaccharide)-OH (for example, 20.0 mg, 8.3 μmol) in cold H2O (4°C) was passed through a short cation exchange resin [Dowex-50WX8 (H+)] to remove salt. After lyophilization of the eluent, a solution of this residue in H2O (30 ml) was adjusted to pH 3.6 withaq. Cs2CO3 (2.5 mg/ml) and then lyophilized. To a solution of this residue in dry DMF (4 ml), phenacyl bromide (5.7 mg, 0.03 mmol) was added to obtain Fmoc-Asn-(sialyloigosaccharide)-COOH. Purification was performed by preparative C18 RP-HPLC (10).

Removing the Fmoc group of Fmoc-Asn-(sialyloigosaccharide) dipeptide ester-OH (for example, 20.0 mg, 7.2 μmol) in DMF (2.0 ml) was performed with 1-methylpyrroldine (75.0 μmol, 1.77, μmol), and glycopeptide dipeptide ester-OH treated with BocOSu (7.7 mg, 36 μmol) in DMF (1.0 ml). After 3 hours, diethyl ether (10 ml) was added to this mixture, and then the precipitate was purified by preparative C18 RP-HPLC to obtain tert-Boc-Asn-(sialyloigosaccharide dipeptide ester)-OH (13 mg, 68%) (10).

Synthesis of tert-Boc-Asn-(sialyloigosaccharide dipe面貌）ester-OH 1

Preparation of the target compound has been already reported (10). In brief, hexapeptidyl-sialyloigosaccharide was purified from egg yolk, and then the peptide portion was digested with peptidase (Actinase-E, Kaken Pharmaceutical Co. Ltd.) to have a crude H2N-Asn-(sialyloigosaccharide)-COOH. The resulting H2N-Asn-(sialyloigosaccharide)-COOH was protected with an Fmoc group by treatment with 9-fluorenylmethyl-N-succinimidylcarbonate (3 equiv.) and NaHCO3 (4 equiv.) in H2O-acetone. Purification of the product was performed with RP-HPLC (C18 column, isocratic 50 mM NH2OAc-MeCN) to obtain Fmoc-Asn-(sialyloigosaccharide)-COOH.

A typical procedure of Boc SPPS was used for the synthesis of peptide-α-thioesters and glycospeteptide-α-thioesters (10). The peptide-α-thioesters and glycopeptide-α-thioesters were prepared by an improved Boc SPPS method based on the original in situ neutralization Boc SPPS protocol established by the Schünzler group (33). A HSC2H2CONH-PEGA resin (PEG resin: poly[(acryloyl-bis(aminopropyl)polyethylene glycol)] was used for the synthesis of the sialyglycopeptide-α-thioesters H-[Ala1-Gly28]-α-thioester (fig. S3), H-[Cys29-Ala49]-α-thioester (fig. S4), H-[Cys37-Lys97]-α-thioester (fig. S6), and H-[Cys38-Ala127]-α-thioester (fig. S8), and a HSC2H2CONH-NovaPEG resin was used for the synthesis of the sialyglycopeptide-α-thioesters H-[Cys29-Asn83(glycan)-Ala87]-α-thioester (fig. S7). Both resins, bearing a linker, were prepared as follows. S-Trityl-3-mercaptopropionic acid (80.0 mg, 0.2 mmol) was dissolved in DMF (1.0 ml), and the solution was stirred for 1 min for activation. This solution was added to resin (ca. 50 μmol) to incorporate mercaptopropionic acid into the amino resin to make an amide-form linker. The trityl group was then removed by 1-min treatment with 5% TIPS in TFA twice. The coupling of amino acids according to the peptide sequence was carried out by using Boc-amino acids (0.4 mmol), HBTU (72.1 mg, 0.19 mmol), and DIPEA (70.0 μl, 1.15 mmol), with a preactivation for 1 min and coupling for 15 min at ambient temperature. After coupling of the Boc-amino acid, the Boc group was removed by 3-min neat TFA treatment twice. After synthesis of the desired peptides or glycopeptides on the resin, the complete deprotection of the side chain protection was performed on the solid support, and then thiolysis was performed to detach the peptide-α-thioester or glycospeteptide-α-thioester from the resin. The peptides were deprotected using a low-TfOH cocktail (TFA/DMS/m-cresol/TIOH 5:3:1:1) and a high-TfOH cocktail (TFA/thioanisole/ethanediol/TIOH 20:2:1:1) according to standard Boc-chemistry procedures, and the sialyglycopeptides were deprotected using the low-TfOH cocktail. The resulting peptides and glycopeptides on the resins were washed with DCM and DMF; then to this resin, 5% (w/v) MESNa/sodium phosphate buffer (200 mM, pH 6.0 to 6.8) containing 6 M Gn-HCl was added to detach the peptide-α-thioesters and glycopeptide-α-thioesters. After the above described deprotection steps, almost all of protecting groups were removed except for the formyl group of Trp by the treatment with 2% hydrazine-monohydrate for 30 min at room temperature; then the pH of the solution was adjusted to pH 4.2 with methoxyamine hydrochloride to remove the Thz group. The Thz group was removed by treatment for 2 hours at room temperature (fig. S9).
Synthesis of H-[Cys\textsuperscript{128}-Arg\textsuperscript{166}]-OH
The conventional Fmoc-based SPPS protocol was used for the preparation of H-[Cys\textsuperscript{128}-Arg\textsuperscript{166}]-OH (10). The first amino acid, Fmoc-Arg(pbf)-OH (353 mg, 1.0 mmol), was attached to the 4-(hydroxymethyl)phenoxyacetic acid linker–PEGA resin (HMPA Linker PEGA resin, 215 mg, 200 μmol) using MSNT (296 mg, 1.0 mmol) and N-methylimidazole (60 μl, 750 μmol) in DCM (4.0 ml). The resin was treated with 20% piperidine in DMF for 15 min at ambient temperature for the removal of the Fmoc group. Peptide coupling was carried out using a microwave-assisted protocol as reported in the literature (34). Fmoc–amino acids (0.25 mmol) were preactivated by DIC (38.7 μl, 0.25 mmol) and HOBr (33.8 mg, 0.25 mmol) in DMF (1.0 ml) for 5 to 10 min, and the solution was added to the resin. The Fmoc–amino acids used were Gly, Ala, Val, Leu, Ile, Pro, Ser(\textit{tBu}), Thr(\textit{tBu}), Asp(\textit{tBu}), Glu(\textit{tBu}), Asn(Trt), Gln(Trt), Lys(Boc), Arg(Pbf), Phe, Tyr(\textit{tBu}), and Trp(Boc). Coupling of Fmoc-Cys(Trt), Fmoc-Cys(Acm), and Fmoc-His(Trt) was carried out at ambient temperature for 60 min without any microwave. After coupling of the individual Fmoc–amino acid, the Fmoc group was removed by 20% piperidine/DMF (1.5 ml) for 20 min. After construction of the peptide, H-[Cys\textsuperscript{128}-Arg\textsuperscript{166}]-OH was released from the resin with a solution of TFA/TIPS/H\textsubscript{2}O (3:1:1). To this solution was added Et\textsubscript{3}O to afford the precipitate of the peptide. The precipitate was collected by centrifugation. Purification of the residue by RP-HPLC (Vydac C18 \textPhi \times 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 70:30 to 20:80 over 30 min at a flow rate of 4 ml/min) afforded H-[Cys\textsuperscript{128}-Arg\textsuperscript{166}]-OH. ESI-MS: mass/charge ratio (m/z) calc. for C\textsubscript{206}H\textsubscript{334}N\textsubscript{62}O\textsubscript{56}S\textsubscript{2}:[ M + H ]\textsuperscript{+} 4640.4, found: 4639.6 (deconvoluted).

Typical procedure for the synthesis of EPO derivatives: Synthesis of EPO\textsubscript{N24, N38, N83} polypeptide bearing three N-glycans
First ligation. The first NCL was previously reported (10). The NCL of H-[Cys\textsuperscript{89}(Thz)-Ala\textsuperscript{257}]-α-thioester (2.5 mg, 0.80 μmol) and H-[Cys\textsuperscript{128},Cys\textsuperscript{161}(Acm)-Arg\textsuperscript{166}]-OH (3.7 mg, 0.80 μmol) was performed in a sodium phosphate buffer solution (0.2 M, pH 6.8, 0.4 ml) containing 6 M Gm-HCl, 40 mM MPAA (2.7 mg, 16 μmol), and 40 mM TCEP. The ligation reaction was completed within 3 hours. After completion of the reaction, the N-terminal Thz moiety of the product was converted into a Cys residue as a one-pot reaction by adjusting the pH to 4.0 with methoxyamine hydrochloride. After 2 hours, the reaction mixture was subjected to RP-HPLC (Vydac C18 \textPhi \times 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 75:25 to 25:75 for 30 min at a flow rate of 4 ml/min). Fractions containing the desired product were collected and lyophilized to give H-[Cys\textsuperscript{89},Cys\textsuperscript{161}(Acm)-Arg\textsuperscript{166}]-OH (yield: ca. 92% based on HPLC analysis). HPLC analytical yield was estimated based on HPLC area: 100 × [area of product/(area of product + peptide-thioester + peptide-MPAA-thioester)].

Second ligation. The second NCL was previously reported (10). The NCL of H-[Cys\textsuperscript{89}(Thz)-Asn\textsuperscript{183}(glycan)-Lys\textsuperscript{374}]-α-thioester (3.8 mg, 0.8 μmol) and H-[Cys\textsuperscript{38},Cys\textsuperscript{83}(Acm)-Arg\textsuperscript{166}]-OH (6.0 mg, 0.8 μmol) was performed in a sodium phosphate buffer solution (0.2 M, pH 8.0, 60 μl) containing piperidine (5.0 μl) and 2-mercaptoethanol (3.0 μl) with added to this ligation solution to remove the phenacyl group of the sialic acid and the formyl group of Trp. After 2 hours, the solution was neutralized with HCl, and then Thz was converted into Cys as a one-pot reaction by the addition of methoxyamine hydrochloride (the pH was adjusted to 4.0). After 3 hours of incubation, the product was purified by RP-HPLC (Vydac C18 \textPhi \times 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 75:25 to 25:75 for 30 min at a flow rate of 4 ml/min) to yield H-[Cys\textsuperscript{89}-Asn\textsuperscript{38}(glycan)-Cys\textsuperscript{161}(Acm)-Arg\textsuperscript{166}]-OH. The purity of the product was confirmed by HPLC and ES-MS analysis. The yield of the second NCL was ca. 97% based on HPLC analysis. HPLC analytical yield was estimated based on HPLC area: 100 × [area of product/[area of product + peptide-thioester + peptide-MPAA-thioester]]

Third ligation: Synthesis of H-[Cys\textsuperscript{29, 33}(Acm)-Asn\textsuperscript{38}(glycan)-Ala\textsuperscript{78}]-α-hydrazide (fig. S11). The NCL of H-[Cys\textsuperscript{29, 33}(Acm)-Asn\textsuperscript{38}(glycan)-Ala\textsuperscript{78}]-α-hydrazide (3.8 mg, 1.2 μmol) was performed in a sodium phosphate buffer solution (0.2 M, pH 6.8, 0.478 ml) containing 6 M Gm-HCl, 40 mM MPAA (2.7 mg, 16 μmol), and 40 mM TCEP. The ligation reaction was completed within 3 hours. After completion of the reaction, the solution was adjusted to pH 9.5 by 3 M NaOH aq (ca. 10 μl), and the white foam (fig. S10)
solution was left at room temperature for 2 hours to remove the phenacyl group. The reaction was monitored by HPLC (Proteonavi C4 4.6 × 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 80:20 to 15:85 over 25 min at 1 ml/min). The solution was treated with 10 mM TCEP for 5 min to reduce undesired intermolecular disulfide bonds, and then the product was purified by preparative HPLC (Proteonavi C4 4.6 × 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 95:5 over 5 min, then 55:45 to 20:80 over 30 min at 2.5 ml/min). Fractions containing the desired product were collected and lyophilized to give H-[Cys29, 33, 161 (Acm)-Asn38, 83 (glycan)-2-Arg166]-OH (1.4 mg, 70% isolated yield) as a white foam. ESI-MS: m/z calcd. for C683H737N338O322S6 [M+H]+ 19824.6, found: 19823.9 (deconvoluted).

Deprotection of the Acm group of H-[Cys29, 33, 161 (Acm)-Asn38, 83 (glycan)-2-Arg166]-OH (fig. S14). H-[Cys29, 33, 161 (Acm)-Asn38, 83 (glycan)-2-Arg166]-OH (ca. 0.8 mg, 0.04 µmol) was dissolved in 90% acetic acid solution (81 µl) containing AgOAc (ca. 1.01 mg, 6.0 µmol), and the mixture was stirred for 2 hours at ambient temperature. After centrifugation of the reaction mixture, the supernatant was collected in a new tube, and 90% acetic acid solution (161 µl) containing DTT (24.9 mg, 160 µmol) was added. After stirring for 5 min, the resultant precipitate was removed by centrifugation, and the supernatant was subjected to preparative HPLC (Proteonavi C4 4.6 × 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 95:5 over 5 min, then 55:45 to 20:80 over 30 min at 2.5 ml/min). Fractions containing the desired product were collected and lyophilized to give H-[Cys29, 33, 161 (Acm)-Asn38, 83 (glycan)-2-Arg166]-OH (0.7 mg, 89% isolated yield) as a white foam. ESI-MS: m/z calcd. for C683H737N338O322S6 [M+H]+ 19861.3, found: 19611.3 (deconvoluted).

Fifth ligation: Synthesis of H-[Ala1-Cys7, 29, 33, 161 (Acm)-Asn24, 38, 83 (glycan)-2-Arg166]-OH (fig. S15). A ligation buffer solution (0.2 M sodium phosphate, pH 6.5) containing 6 M Gn-HCl, 100 mM MPAA, and 50 mM TCEP was freshly prepared by bubbling with argon gas for 2 min. H-[Ala1-Asn24 (glycan)- Gly7]-α-thioester (0.5 mg, 0.089 µmol) and H-[Cys29, 33, 161 (Acm)-Asn38, 83 (glycan)-2-Arg166]-OH (0.7 mg, 0.036 µmol) were dissolved in the ligation buffer (17.8 µl), and the solution was left at room temperature for 3 hours. To remove the phenacyl group of sialyloligosaccharide at the 24 position, the solution was adjusted to pH 9.3 by the addition of 5 M NaOH aq. (ca. 2 to 5 µl) under monitoring with a micro pH meter and was left at room temperature for 2 hours. The reaction was monitored by HPLC (Proteonavi C4 4.6 × 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 70:30 to 20:80 over 20 min at 1 ml/min). The solution was treated with 10 mM TCEP for 5 min to reduce undesired disulfide bonds and was then purified by preparative HPLC (Proteonavi C4 4.6 × 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 95:5 over 5 min, then 55:45 to 20:80 over 30 min at 2.5 ml/min). Fractions containing the desired product were collected and lyophilized to give full-length H-[Ala1-Cys7, 29, 33, 161 (Acm)-Asn24, 38, 83 (glycan)-2-Arg166]-OH polypeptide (ca. 0.6 mg, 67% isolated yield) as a white foam. ESI-MS: m/z calcd. for C1065H1722N250O423S5 [M+H]+ 24957.9, found: 24957.0 (deconvoluted).

Folding and formation of disulfide bonds in the glycosylated EPO polypeptide H-[Ala1-Cys7, 29, 33, 161 (Acm)-Asn24, 38, 83 (glycan)-2-Arg166]-OH (fig. S16). Folding of the glycosylated EPO polypeptide H-[Ala1-Cys7, 29, 33, 161 (Acm)-Asn24, 38, 83 (glycan)-2-Arg166]-OH was performed by using a stepwise dialysis method. The EPO polypeptide was dissolved in a tris-HCl buffer (100 mM, pH 7.5) containing 8 M Gn-HCl, and the EPO polypeptide concentration was adjusted to a suitable concentration (0.1 or 0.01 mg/ml). This solution was placed at room temperature for 1 hour and then poured into the dialysis tube (molecular weight cutoff at 8000, Spectra/Por). Then, the solution was dialyzed against the first folding buffer (3.0 M Gn-HCl, 100 mM tris-HCl, pH 8.5) containing 4 mM cysteine and 0.5 mM cystine for the redox system and left for 24 hours at 4°C. The external buffer solution was replaced with the second folding buffer solution (1.0 M Gn-HCl, 100 mM tris-HCl, pH 8.0), and dialysis was performed for 16 hours. Finally, the external buffer solution was discarded.
and replaced with the third folding buffer solution (10 mM tris-HCl, pH 7.0), and this final dialysis was performed for 16 hours. The folded sialyl-EPO was purified by RP-HPLC (Proteanavi C4, 5 μm, 4.6 × 250 mm, 0.1% TFA:0.1% TFA in 90% MeCN = 60:40 to 25:75 for 30 min at a flow rate of 1 ml/min). Fractions containing the folded sialyl-EPO were collected and then lyophilized. The folding yield was estimated by HPLC analysis and shown in Table 1. The purity of the folded sialyl-EPO was confirmed by HPLC and ESI-MS. HPLC analytical yield was estimated based on HPLC area: 100 × [area of folded EPO/(area of folded EPO + misfolded EPO)].

Confirmation of the disulfide bond positions
To confirm the position of the disulfide bonds that formed, the folded EPO derivatives were individually digested with a suitable amount of trypsin (incubated for 12 hours), and then the resultant peptide fragments were analyzed with an HPLC-MS instrument. To identify which fragments included disulfide bonds, treatment with TCEP was performed to reduce the disulfide bonds, and then all of the peptide fragments were analyzed by HPLC-MS to compare the HPLC profiles before and after TCEP treatment. The disulfide bond positions were determined based on this HPLC-MS analysis.

Biological assay of the synthesized EPO derivatives: Cell proliferation assay (in vitro assay)
The in vitro cell proliferation assay (27) was performed using a TF-1 cell line in the presence of EPON24, N38, N83 2 or commercially available EPOGIN (recombinant human EPO). The concentrations of EPON24, N38, N83 2 and EPOGIN were varied from 10 pM to 100 nM. For a negative control, only the cell culture medium, instead of EPO, was used. The plates containing TF-1 cells and EPO were incubated in a 5% CO2 atmosphere at 37°C. Cell growth was monitored every 24 hours by microscopy. After 72 hours, cell growth was estimated using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-8) (purchased from DOJIN) (38). WST-8 was added to each well of the plate, and the cells were incubated for 2 hours. The absorbance of WST-8 in each well was measured at 450 and 630 nm. Cell proliferation was determined based on this HPLC-MS analysis.

In vivo hematopoietic activity
Groups of three mice (7 weeks old, BALB/c) were dosed intravenously three times on days 0, 2, and 5 with the indicated protein concentrations (1.41 μM, 100 μl per day). Blood samples were collected on days 0, 2, 5, and 7 for the evaluation of the effect on hematologic parameters. The hematocrit was determined by measurement of the packed cell volume for each time point immediately after blood collection. The hematocrit was estimated and is shown in Fig. 5B. All animal experiments were performed under the strict regulation established by Osaka University (permission number 26-03-0).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/1/e1500678/DC1

Fig. 51. Acid stability of sialyloligosaccharide phencyl ester.

Fig. 52. General scheme of the synthesis of a sialylglycopeptide-o-thioester by an improved Boc SPPS method.

Fig. 53. HPLC profile and ESI mass spectrum of H-[Ala1-Gly30]-o-thioester.

Fig. 54. HPLC profile and ESI mass spectrum of H-[Cys29,31-(Acym-Acm)-Tyr5]-o-thioester.

Fig. 55. HPLC profile and ESI mass spectrum of H-[Cys29,31-(Acym-Acm)-Gly30(Try5)]-o-thioester.

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