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Glycoprotein Semisynthesis by Chemical Insertion of Glycosyl Asparagine Using a Bifunctional Thioacid-Mediated Strategy

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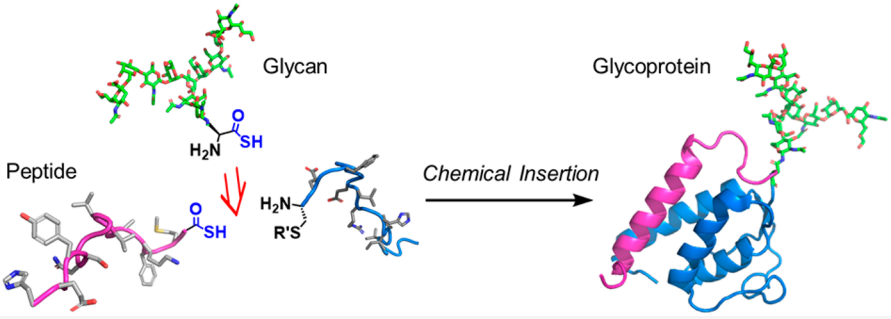

ABSTRACT: Glycosylation is a major modification of secreted and cell surface proteins, and the resultant glycans show considerable heterogeneity in their structures. To understand the biological processes arising from each glycoform, the preparation of homogeneous glycoproteins is essential for extensive biological experiments. To establish a more robust and rapid synthetic route for the synthesis of homogeneous glycoproteins, we studied several key reactions based on amino thioacids. We found that diacyl disulfide coupling (DDC) formed with glycosyl asparagine thioacid and peptide thioacid yielded glycopeptides. This efficient coupling reaction enabled us to develop a new glycoprotein synthesis method, such as the bifunctional thioacid-mediated strategy, which can couple two peptides with the N- and C-termini of glycosyl asparagine thioacid. Previous glycoprotein synthesis methods required valuable glycosyl asparagine in the early stage and subsequent multiple glycoprotein synthesis routes, whereas the developed concept can generate glycoproteins within a few steps from peptide and glycosyl asparagine thioacid. Herein, we report the characterization of the DDC of amino thioacids and the efficient ability of glycosyl asparagine thioacid to be used for robust glycoprotein semisynthesis.

INTRODUCTION

Post- and cotranslational protein modifications are essential for protein function activation.[1] Recent research has revealed that these modifications regulate a wide range of biological processes, including several diseases such as cancer, Alzheimer's disease, and Parkinson's disease.[2−5]

Of these protein modifications, glycosylation is a major modification, whereas the resultant glycans show considerable heterogeneity in their structure.[6] Glycoproteins are found on the cell surface and in body fluids, and these proteins are modified with serine/threonine-linked O-glycans or asparagine-linked N-glycans.[7] However, the biosynthesis of glycans is regulated by the substrate specificity of glycosyltransferases and glycosidases, resulting in considerably diverse glycan structures (glycoforms). However, under these circumstances, we cannot identify which glycans play an important role in sustaining specific biological events.[8−11]

To understand the biological processes arising from each glycoform, the preparation of homogeneous glycoproteins is essential for extensive biological experiments. The major glycoprotein preparation methods are cell expression systems and chemical synthesis. In terms of expression systems, mammalian cells yield heterogeneous glycoproteins, whereas heterogeneous N-glycans can be substituted with homogeneous N-glycans by supplemental enzymatic methods.[12−18] Although the glycan pattern is limited, the yeast system can generate a homogeneous glycoprotein by means of commendable genetic alteration of glycosyltransferases. In addition, efficient methods have been developed to introduce glycan analogs into the dehydroalanine site of the expressed proteins.[19] Other approaches include chemical and semisynthetic methods using native chemical ligation (NCL)[20] and expressed peptide ligation (EPL),[21] respectively. Chemical methods employ both glycopeptides and peptides prepared by solid-phase peptide synthesis (SPPS),[22] and those segments

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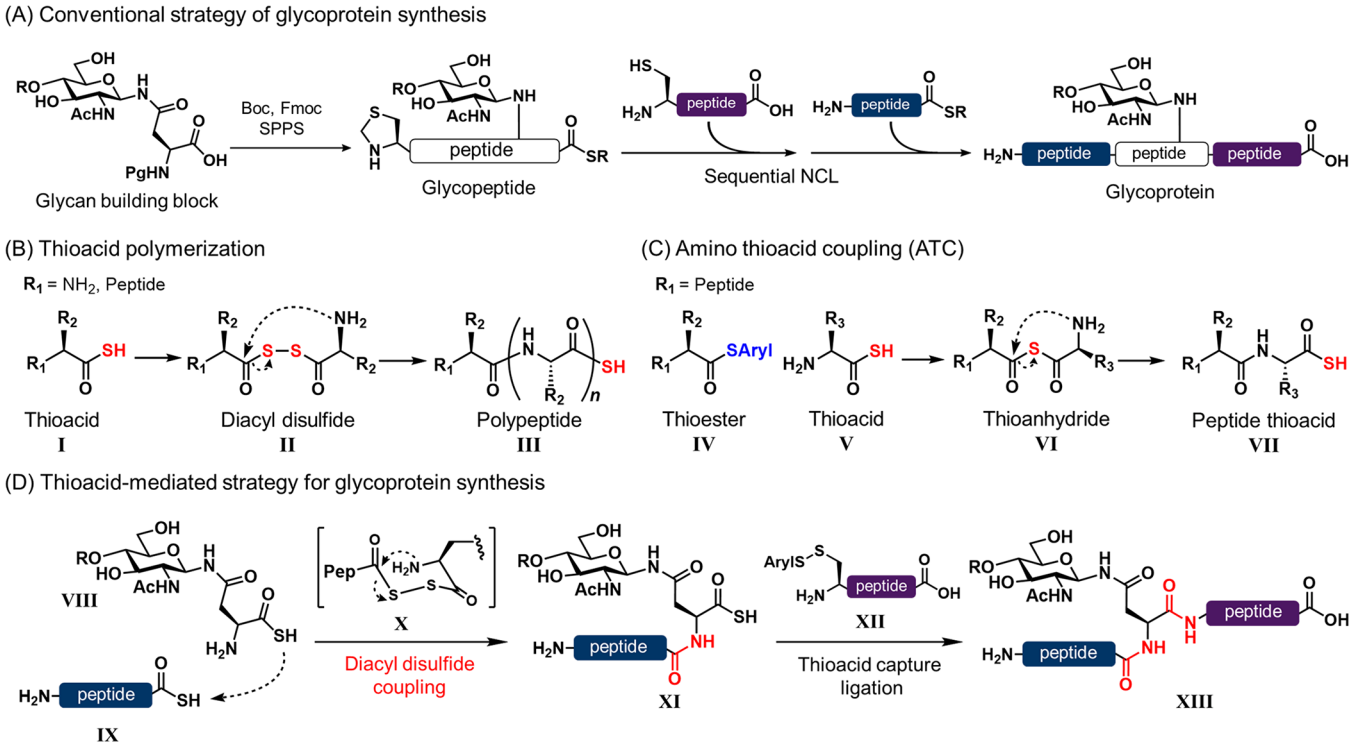

Figure 1. (A) Standard strategy of the synthesis of glycoproteins by SPPS and native chemical ligation (NCL). (B) Thioacid polymerization via oxidation processes in aqueous phase. (C) Amino thioacid coupling (ATC): Chemoselectively couples a peptide thioester with an amino thioacid. (D) Chemical insertion using thioacid-mediated strategy for glycoprotein synthesis: the method assembles a full segment of glycoprotein through two steps.

are sequentially coupled by NCL (Figure 1A). Glycopeptides can also be efficiently prepared by sophisticated convergent methods.[23,24] EPL allows for the synthesis of full-length glycosyl polypeptides by the coupling of glycopeptides prepared by SPPS and long peptides prepared by a bacterial cell-based expression system.[25−29] Applying these chemical methods, many research groups have synthesized a variety of glycoproteins with homogeneous glycans.[6,17,30−37]

However, superior robustness and efficiency need to be developed to synthesize homogeneous glycoproteins and their analogs. Chemical approaches are relatively time-consuming due to requiring multiple synthetic steps and need an appropriate amount of valuable N- or O-glycans because glycopeptide syntheses are set at the early stage of total synthesis (Figure 1A). To accelerate the elucidation studies of glycan functions, we need to solve these synthesis drawbacks.

To set up alternative synthesis methods, we studied several key reactions based on amino thioacids (Figure 1B, C). Thioacids were originally used as acylation functionalities for condensation and ligation reactions with azide, aziridine, thiocarbamate, isocyanide, and sulfonamide.[38−45] Because we were interested in the potent nucleophilicity and low pK_a (∼3) value of thioacids compared with carboxylic acids, our group also studied chemoselective thioacid polymerization reactions with nonprotected amino thioacids (**I**) via oxidation processes in acidic solutions (Figure 1B).[46] The thioacid group is oxidized to form a diacyl disulfide bond (**II**), which is a potent acyl donor. This diacyl disulfide bond then yields polypeptides via intramolecular S−N acyl transfer. The diacyl disulfide formation of thioacids has also been reported by several groups.[47,48] In addition to these reactions, we successfully developed an alternative chemoselective amide bond formation reaction utilizing amino thioacid coupling (ATC: Figure 1C),[49] which couples an α-amino group of an amino thioacid (**V**) to the C-terminus of an unprotected counterpeptide aryl thioester (**IV**). The ATC strategy is an efficient strategy to add an amino thioacid (**V**) to the C-terminus of peptide-thioester (**IV**), but it could not be applied for the synthesis of glycopeptides. The proposed intermediate, glycosyl thioanhydride (**VI**: R3 is a glycan), seemed to be unstable, and therefore many byproducts formed under aqueous conditions.

By studying amino thioacid polymerization (Figure 1B), we could control polymerization reactions and found an efficient amide formation reaction: diacyl disulfide coupling (DDC). DDC could form an amide bond (**XI**) with a glycosyl asparagine thioacid (**VIII**) and a peptide α-thioacid (**IX**) via a disulfide bond intermediate (**X**) without any polymerization reactions.

This DDC enabled us to set up a highly convergent glycoprotein synthesis strategy, the bifunctional thioacid-mediated strategy, that can couple two peptides with the N- and C-termini of glycosyl asparagine thioacid. As shown in Figure 1D, we employed glycosyl asparagine thioacid (**VIII**) as the junction point for the coupling of N- and C-terminal peptides. The first coupling is DDC between a peptide thioacid (**IX**) and glycosyl asparagine thioacid (**VIII**). Because the resultant glycopeptide (**XI**) has a thioacid form at its C-terminus, we could apply thioacid capture ligation (TCL)[50,51] for the coupling of the resultant glycopeptide thioacid (**XI**) and another peptide (**XII**) having a disulfide functional group at its N-terminus to afford the full-length glycoprotein backbone (**XIII**). This route enables us to assemble the entire glycoprotein backbone in just two steps. In addition to this

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efficiency, valuable glycosyl amino thioacids (**VIII**) can be used at the late stage of the synthesis of glycoproteins.

Herein, we report the characterization of diacyl disulfide activation of amino thioacids and the efficient ability of glycosyl asparagine thioacid to be used for robust glycoprotein semisynthesis. To demonstrate our strategy, we performed the total syntheses of two cytokine glycoproteins, namely, chemokine (C−C motif) ligand 1 (**22**, CCL1) and interleukin 3 (**28**, IL3), having homogeneous sialyloligosaccharides. In particular, the synthesis of IL3 could employ a long-chain polypeptide expressed in *E. coli*.

RESULTS

To perform glycopeptide synthesis by DDC, we began this study to understand the characteristic nature of thioacid polymerization under neutral conditions. Our previous coupling reactions with the oxidation method were performed in acidic aqueous solutions, and these reactions generated amino thioacid polymerization.[46] However, acidic aqueous conditions are not suitable for the coupling reaction of glycosyl asparagine thioacid because sialylglycan is an acid-labile molecule. We performed optimization with several amino thioacids to suppress polymerization reactions under neutral conditions. Although we examined the reaction conditions by varying the amino acid sequences of peptide thioacids and solvents (Supporting Information, Table S1), polymerization was the major result, and we did not observe extension of monoamino acids as a major product (Figure 2A).

When we used aqueous conditions for DDC reactions, polymerization or decomposition were major results; therefore, we used organic solvents for subsequent studies. With these optimizations, diacyl disulfide reactions were found to be feasible in DMSO (Figure 2A). In addition, when DTT was added to DMSO, the reactions did not yield products (Supporting Information, Table S1). These results indicated that disulfide bond formation seemed to be an essential process. However, we could not suppress the polymerization reaction for DDC (Figure 2A). We then examined glycosyl asparagine thioacids for DDC.

The synthesis of glycosyl asparagine thioacid **3** is shown in Scheme 1. The substrate Boc-Asn-(sialylglycan)-OH **1** was prepared by the reported method,[52] and the condensation of trityl thiol was performed with PyBOP at −15 °C to give compound **2** (57% yield). Finally, deprotection of both the Boc group and trityl group was performed with trifluoroacetic acid, and triisopropylsilane yielded the desired glycosyl asparagine thioacid **3** (92% yield). The structure was confirmed by nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS).

When we employed glycosyl asparagine thioacid **3** and peptide thioacid **4** for DDC, surprisingly, the desired glycopeptide-thioacid formed without any polymerization (Figure 2B). This reaction employed two equivalents of glycosyl asparagine thioacid **3** toward peptide thioacid **4** (one equivalent) with alanine at the C-terminus. The reaction velocity was slow, but the desired monoglycosylated peptide thioacid **11** formed as a major product after 9 h. A longer reaction time did not improve the yield, and the decomposition of glycosyl asparagine thioacid **3** was observed as a major result after 24 h. In terms of this unexpected result, we considered that the steric hindrance of glycans slowed the polymerization reaction.

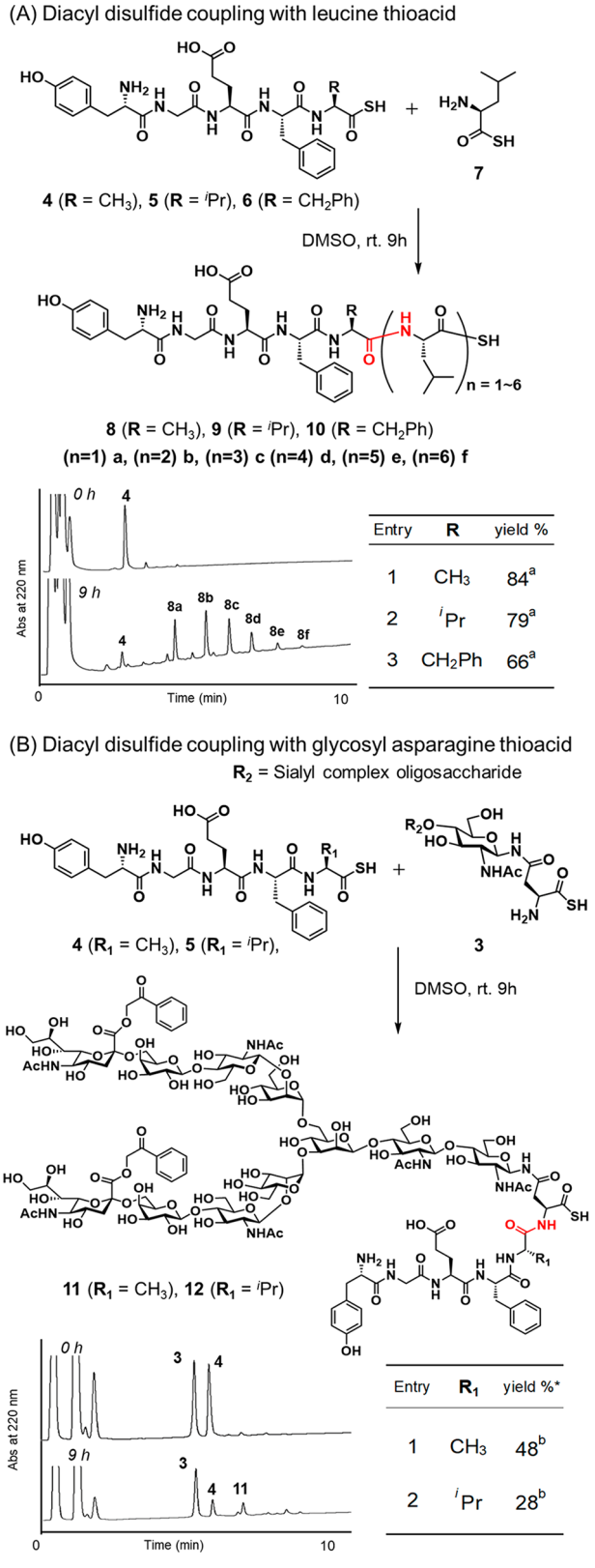

Figure 2. HPLC traces for α-amide formation by amino thioacid. (A) Polymerization of pentapeptide thioacid with leucine thioacid. (B) Coupling of pentapeptide thioacid with glycosyl asparagine thioacid. Reagents and condition: (a) DMSO, peptide thioacid, leucine thioacid (2.0 equiv); (b) DMSO, peptide thioacid, glycosyl asparagine thioacid (2.0 equiv).

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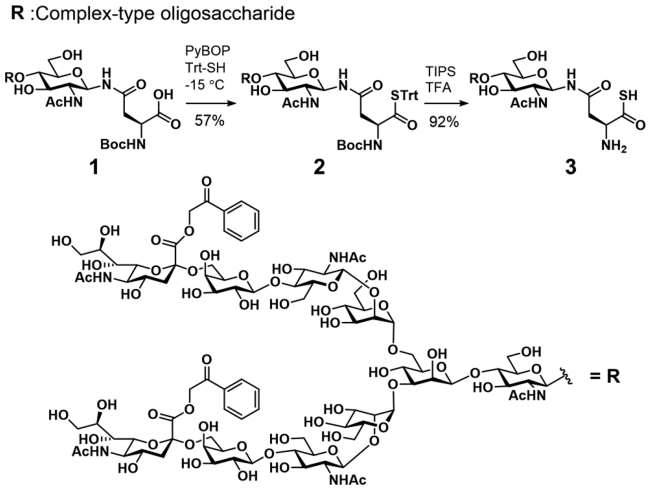
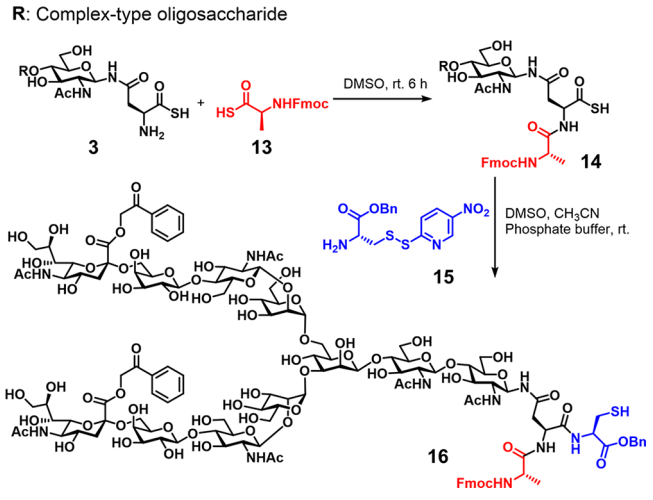
Scheme 1. Synthesis of Glycosyl Asparagine



The coupling reactions of glycosyl asparagine thioacid **3** were then examined with another peptide thioacid **5** having a bulky valine at the C-terminus (Figure 2B). Although the yield was decreased, α-amide formation proceeded with glycosyl asparagine thioacid **3**, and the reaction did not yield undesired polymerization. Extensive optimizations indicated that 2 equiv of glycosyl asparagine thioacid **3** combined with peptide thioacid showed moderate yields.

Because glycosyl asparagine thioacid **3** can be coupled with peptide thioacids, we then examined the second thioacid-mediated reaction with cysteine-activated *p*-nitropyridyl disulfide (Npys) derivative **15** (Scheme 2) according to the strategy shown in Figure 1D (**XI** to **XIII**). In this case, we used monoamino acid coupling at both the N- and C-termini of glycosyl asparagine thioacid **3** for feasible structural analysis. The coupling of glycosyl asparagine thioacid **3** and Fmoc-Ala-thioacid **13** was performed to give glycosyl peptide thioacid **14**. Then, we examined disulfide coupling with cysteine-activated *p*-nitropyridyl disulfide derivative **15**. The original disulfide coupling was reported by Tam's group.[50] The coupling efficiently proceeded and gave desired glycopeptide **16**, as shown in Scheme 2.

Scheme 2. Synthesis of Fmoc-Ala-Asn(glycan)-Cys-OBn 16 by the Thioacid Mediated Strategy



We next confirmed the structure of glycopeptide **16** synthesized with glycosyl asparagine thioacid **3** by extensive NMR analyses. We compared the structure of glycopeptide **16** with the authentic glycopeptide synthesized by a conventional protocol with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP). After isolation of both products, ^{1}H NMR, HSQC, HMBC, NOESY, and DIPSI experiments were conducted. The basic assignments were performed by HMBC connectivity (Figure 3A: blue arrows).

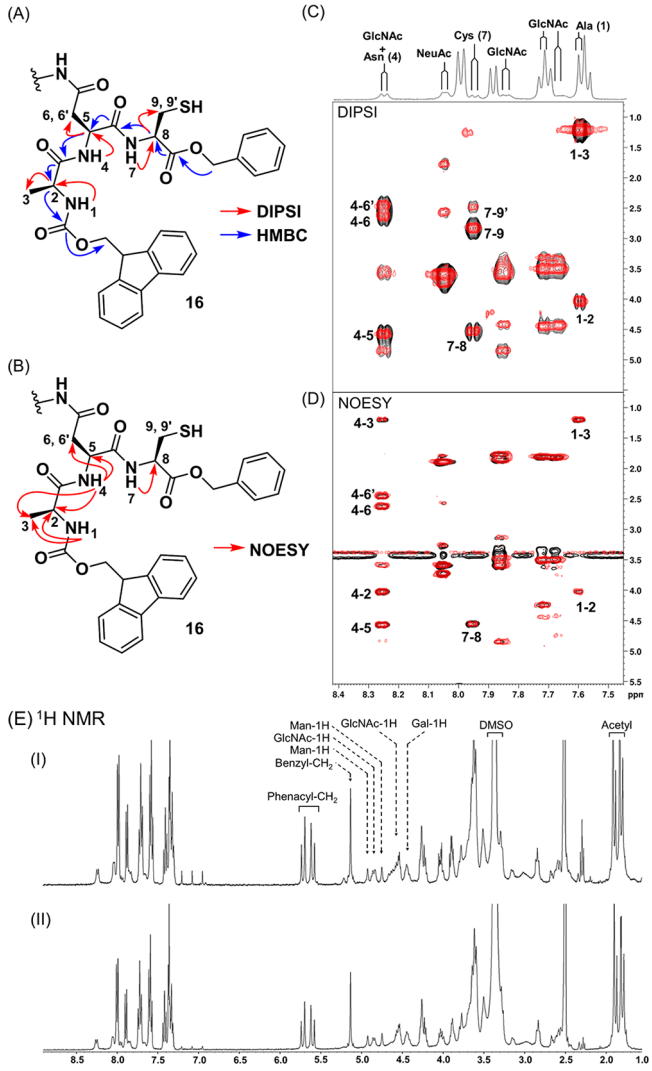


Figure 3. NMR analyses of Fmoc-Ala-Asn(glycan)-Cys-OBn **16**. (A) DIPSI (red arrows) and HMBC (blue arrows) connectivity. (B) Observed NOESY correlations are shown by red arrows. (C) DIPSI spectrum by 700 MHz NMR. Black color contour plot indicates authentic **16** and red color contour plot indicates **16** prepared by thioacid-mediated strategy. (D) NOESY spectrum by 700 MHz NMR. (E) ^{1}H NMR spectra of Fmoc-Ala-Asn(glycan)-Cys-OBn **16** (I): synthesized by the thioacid-mediated strategy, (II): synthesized by PyBOP strategy.

The representative cross peak between benzyl methylene protons and the carbonyl carbon of cysteine was the starting point, and subsequent connectivity was successfully observed from the cysteines to Fmoc-protected alanine, as shown in Figure 3A (blue arrows). In addition to HMBC, assignments of NOESY (Figure 3B: red arrows) and DIPSI (Figure 3A: red arrows) signals supported the structure of both glycopeptides

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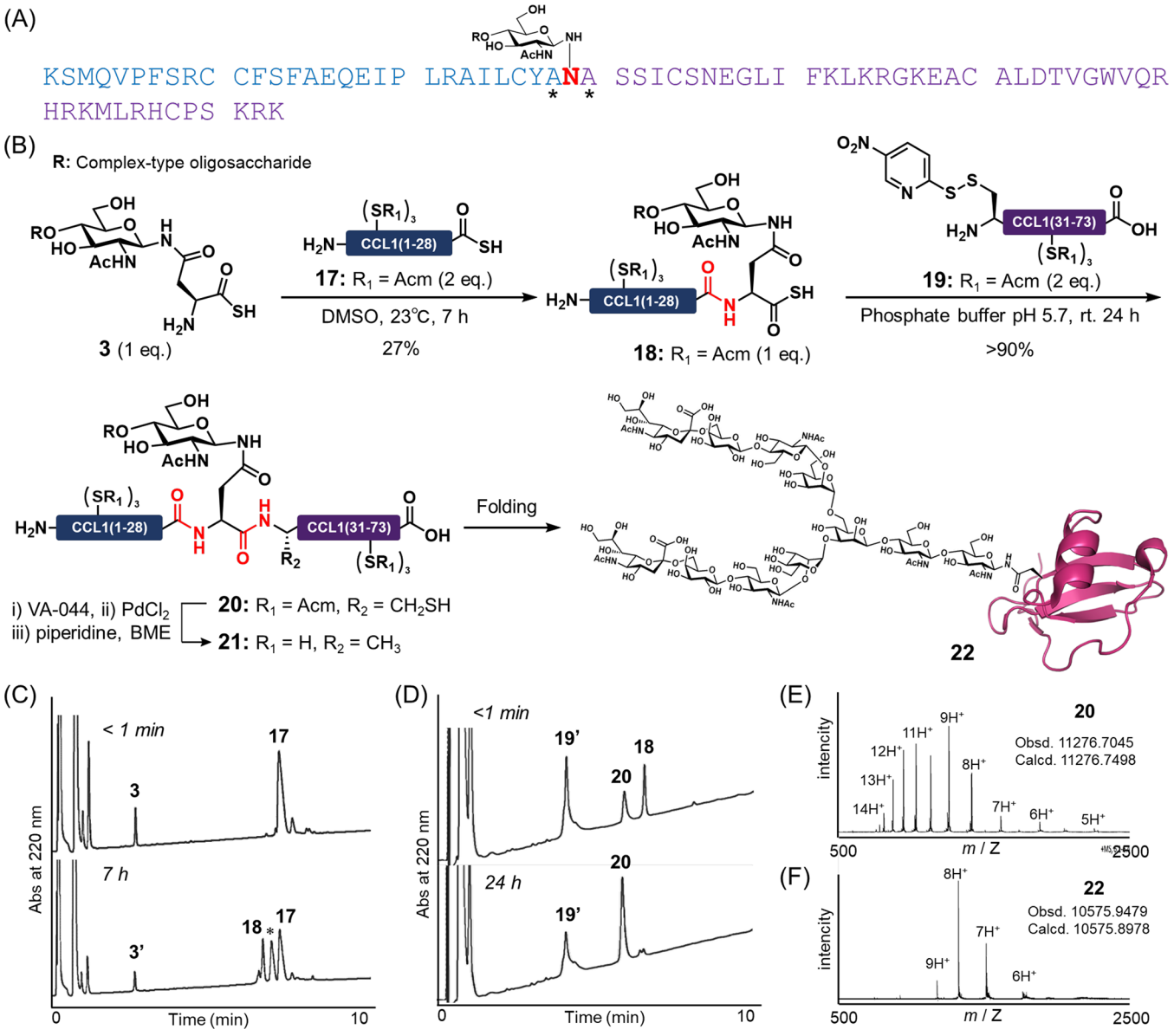

Figure 4. Synthesis of CCL1 having a human complex-type sialyloligosaccharide by the thioacid-mediated strategy. (A) Amino acid sequence of the target CCL1. The asterisks indicate mutation sites, R28A, T30A. (B) Scheme of the synthesis of CCL1. (C) HPLC monitoring of diacyl disulfide coupling between glycosyl asparagine thioacid **3** and peptide thioacid **17**. Compound **3′** indicates an aspartimide form of glycosyl asparagine thioacid **3**. The asterisk indicates peptide-COOH generated by hydrolysis of peptide-thioacid **17**. (D) HPLC monitoring of thioacid capture ligation between peptide **19** and glycosyl peptide thioacid **18**; **19′** indicates reductive form (having no disulfide at Cys^{30}) of peptide **19**. (E) HRMS of full length of CCL1 **20**. (F) HRMS of folded CCL1 **22**.

synthesized by the thioacid-mediated strategy and conventional coupling strategy. All DIPSI, NOESY, and ^{1}H NMR spectra perfectly overlapped between glycopeptide **16** (red contour plot) and its authentic sample (black contour plot) (Figure 3C–E). As a result, the structures of both glycopeptides were completely identical in NMR analyses. When we analyzed the signal pattern, byproduct signals corresponding to the epimerized peptides were not observed (Figure S12).

Furthermore, we investigated the epimerization amount of amino thioacids on α-amide formation in detail. We examined the condensation reactions of the L and D forms of leucine thioacid (Leu^L-thioacid, Leu^D-thioacid) with peptide thioacids having either the L form or D form of phenylalanine at the C-terminus (TyrGlyGlyPhe^L-thioacid or TyrGlyGlyPhe^D-thioacid). Consequently, we prepared all patterns of isomers (TyrGlyGlyPhe^{L}Leu^L-thioacid, TyrGlyGlyPhe^{L}Leu^D-thioacid, TyrGlyGlyPhe^{D}Leu^L-thioacid, and TyrGlyGlyPhe^{D}Leu^D-thioacid) by DDC reactions and compared the retention time of the product with reversed-phase HPLC. All four peptide thioacids were well separated by HPLC analysis (Figure S9). Based on these results, we confirmed that the individual DDC reaction did not yield any of the isomers (no epimerization, Figure S14).

To confirm the epimerization amount under potential oxazolone formation, we further examined the coupling of dipeptide-thioacid with glycosyl asparagine thioacid and the subsequent TCL reaction. The structure of the resultant product (Fmoc-Tyr-Ala-Asn(glycan)-Cys-OBn, Figures S15–S17) was also confirmed by extensive NMR analyses. All

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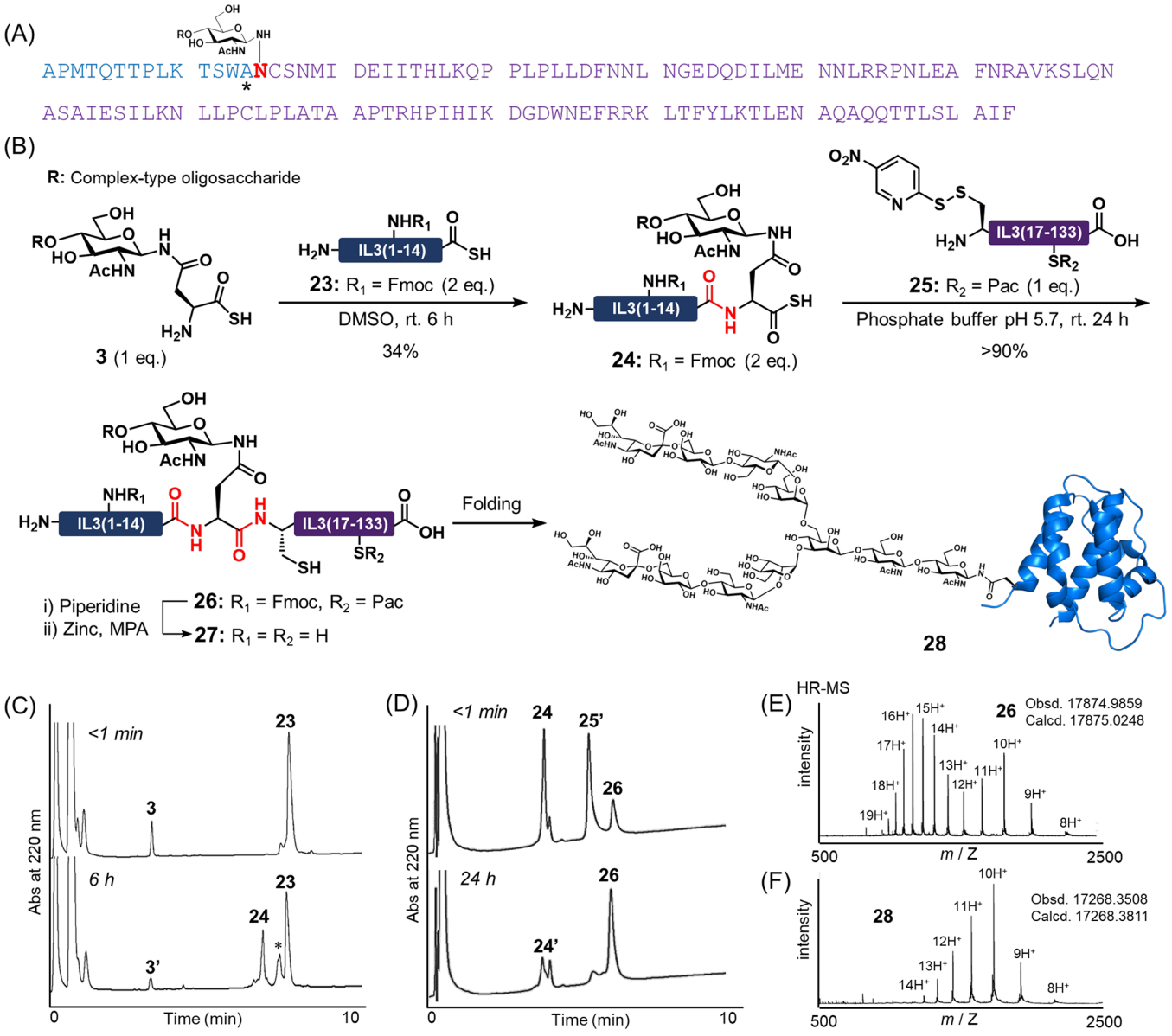

Figure 5. Synthesis of IL3 having a human complex-type sialyloligosaccharide by the thioacid mediated strategy. (A) Amino acid sequence of the target IL3. The asterisk indicates a mutation site, V14A. (B) Scheme of the synthesis of glycosyl IL3. (C) HPLC monitoring of diacyl disulfide coupling between glycosyl asparagine thioacid **3** and peptide thioacid **23**. **3′** indicates an aspartimide form of glycosyl asparagine thioacid **3**. The asterisk indicates peptide-COOH generated by hydrolysis of peptide-thioacid **23**. (D) HPLC monitoring of thioacid capture ligation between peptide **25** and glycosyl peptide thioacid **24**. **24′** indicates the hydrolyzed form of glycosyl peptide thioacid **24**. **25′** indicates reductive form (having no disulfide at Cys[16]) of peptide **25**. (E) HRMS of full-length glycosyl IL3 **26**. (F) HRMS of folded glycosyl IL3 **28**.

signals of the DDC/TCL product were found to be identical to those of the authentic product synthesized by a conventional protocol with PyBOP. We observed a single HSQC cross peak between the α-proton and its carbon signal of all amino acids, but we could not exclude the possibility of the complete overlap of the signals caused by epimerization (Figure S17C). Therefore, we further examined the monitoring of epimerization under DDC reaction conditions. We synthesized Fmoc-L-Tyr-L-Ala-thioacid and Fmoc-L-Tyr-D-Ala-thioacid and left both substrates in DDC conditions. We found the best conditions for the separation of both HPLC profiles (Figure S14), and these data clearly indicated no epimerization. Combined data (Figures S14–S17) with Figure S9 supported that DDC was less prone to cause epimerization by oxazolone formation.

After we studied the optimization of a new α-amide formation reaction employing a peptide thioacid and glycosyl asparagine thioacid **3**, we examined glycoprotein synthesis by means of the thioacid-mediated strategy shown in Figure 1D. Figures 4 and 5 show the synthesis of the chemokines CCL-1 **22** and interleukin-3 **28** with a homogeneous glycan by the bifunctional thioacid-mediated strategy.

We examined the synthesis of the cytokine glycoproteins CCL1 and IL3, which consist of 73 and 133 amino acid residues, respectively (Figures 4 and 5).

First, we synthesized CCL1 with a sialyloligosaccharide. CCL1 has a complex-type, N-linked sialyloligosaccharide at the 29th position (Figure 4A).[53,54] Previous CCL1 synthesis with a homogeneous glycan employed SPPS of the glycopeptide segment.[55,56] In this experiment, we examined the synthesis of

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CCL1 by a thioacid-mediated strategy using two peptide segments (Lys[1]-Ala[28]-COSH **17** and Cys[30](Npys)-Lys[73] **19**) prepared by SPPS. Although the native CCL1 sequence contains Thr at the 30th position, we used a cysteine at the 30th position for TCL and a subsequent desulfurization protocol to convert cysteine to alanine. We considered that mutation of Thr to the resultant Ala may not have a negative effect on folding processes because the N(glycan)-Thr-Ser sequence is located near the flexible loop position.[57]

According to the developed strategy, segments **17** and **19** were prepared by the Boc[58] and Fmoc[59] SPPS conditions (average yield = 5%), respectively, and then the thioacid-mediated strategy was examined. All internal peptide cysteines were protected by the acetamidomethyl (Acm) protecting group because their thiol groups can prevent essential disulfide bond formation in DDC and TCL. Lys[1]-Asn[29](glycan)-COSH **18** was obtained by the DDC of 2 equiv of peptide thioacid **17** and glycosyl asparagine thioacid **3** (15 mM), with a 27% isolated yield. The reaction was monitored by LC-MS, and the resultant product was confirmed by LC-MS without any aggregation of peptide **17** or **18** in DMSO. The DMSO experiments did not need denaturation conditions for short peptide substrates. After isolation of **18**, Lys[1]-Asn[29](glycan)-COSH **18** and 2 equiv of peptide **19** (1.0 mM) were coupled by TCL in a buffer solution (0.2 M sodium phosphate, pH 5.7) containing 6 M guanidine-HCl to yield the protected full-length CCL1 peptide Lys[1]-Asn[29](glycan)-Lys[73] **20** (>90% isolated yield). Desulfurization of the 30th cysteine with a radical initiator[60] and subsequent deprotection of the Acm-protecting groups of cysteines with $PdCl_2$[61,62] and the phenacyl-protecting group of sialyloligosaccharide with piperidine and 2-mercaptoethanol (BME) were performed to yield glycosyl CCL1 polypeptide **21**. Finally, oxidative folding under redox conditions[55,56] at pH 8.0 yielded CCL1 **22** with a sialyloligosaccharide at the 29th position. After isolation of the folded CCL1, enzyme digestion was performed to analyze disulfide bond positions (Figure S26). The circular dichroism (CD) spectrum and the high-resolution mass (10575.9479, average isotopes) supported the correctly folded structure of glycosyl CCL1 **22**. These results indicated that the feasibility of synthetic processes dramatically improved compared with that of previous synthesis.[55]

After successful synthesis of glycosyl CCL1 **22**, we set out to synthesize IL3, a cytokine produced by T cells as a regulator of hematopoiesis.[63−65] Glycosylated IL3 is an ideal target to apply our strategy, as there are no synthetic examples to date. Normally, the IL3 protein is known to have a complex-type, N-linked sialyloligosaccharide at the 15th asparagine[63−65] (Figure 5A). Although the native IL3 sequence contains Val at the 14th position, we used alanine at the 14th position to improve DDC yield after observing a low yield (Figure 2B, entry 2: compound **12**). For the synthesis of IL3, we employed glycosyl asparagine thioacid **3** and two peptides (Ala[1]-Ala[15]-COSH **23** and Cys[17](Npys)-Phe[133] **25**), as shown in Figure 5B. Peptide **25** was prepared in >40% yield (total 4 steps) by an *E. coli* expression system and several modification steps, including pyridyl disulfide formation of the N-terminal thiol (Supporting Information S36). The internal cysteine of **25** was protected by the phenacyl (Pac) protecting group because the thiol group can prevent essential disulfide bond formation in DDC and TCL. A short segment **23** was synthesized using safe Boc SPPS conditions (Figure S28).[58] The DDC of 2 equiv of Ala[1]-Ala[15]-COSH **23** with glycosyl asparagine thioacid **3** yielded glycopeptide-thioacid **24** in 34% (isolated yield). Then, the second ligation was performed with 2 equiv of Ala[1]-Asn[16](glycan)-COSH **24** and peptide **25** (1.0 mM) by TCL in a buffer solution (0.2 M sodium phosphate, pH 5.7) containing 6 M guanidine-HCl to obtain full-length IL3 peptide **26** (>90% isolated yield). The full-length glycosyl IL3 Ala[1]-Asn[16](glycan)-Phe[133] **26** was subjected to deprotection of the formyl protecting group and Fmoc protecting group with piperidine, and then the removal of the internal phenacyl group with zinc reduction[66] afforded glycosyl IL3 polypeptide **27**. The oxidative folding of **27** employed stepwise dialysis conditions under 6, 3, and 1 M guanidine-HCl solution and a final 10 mM Tris-HCl buffer solution, where a 3 M guanidine-HCl solution contained a mixture of 4.0 mM cysteine and 0.5 mM cystine for disulfide bond formation.[52,67] The folding processes were monitored by LC-MS, and this condition successfully afforded folded glycosyl IL3 **28**. After isolation of the folded glycosyl IL3, CD spectra and a high-resolution mass (17268.3508, average isotopes) were obtained.

The *in vitro* bioassay with glycosyl IL3 **28** was performed based on the cell proliferation of TF-1 cell,[65] and the activity of **28** was confirmed to be similar to that of commercially available nonglycosylated IL3 expressed in *E. coli* (Figure 6). The biological assay and analytical data, such as the CD spectrum and mass spectrum, supported that synthetic glycosyl IL3 **28** employed the native folded structure.

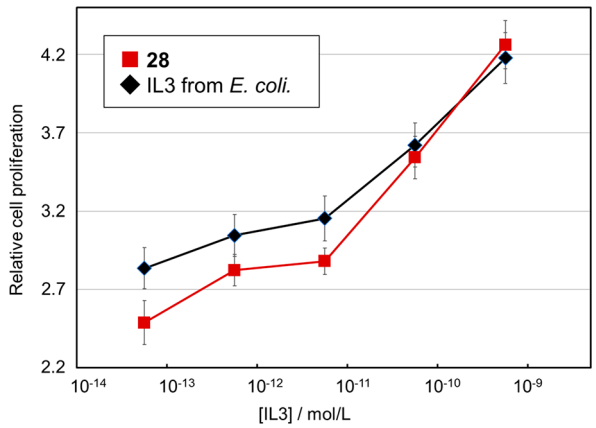

Figure 6. TF-1 Cell proliferation assay. TF-1 cells were cultured for 72 h. Red and black color shows the activity of synthetic glycosylated IL3 **28** and IL3 expressed in *E. coli.*, respectively. Error bars are SD (n = 6). Relative proliferation was calculated by luminescence.

DISCUSSION

The crucial finding in developing this new chemoselective convergent synthesis of glycoproteins was how to combine glycosyl asparagine thioacid **3** with a peptide thioacid without polymerization. We found that glycosyl asparagine thioacid **3** unexpectedly did not yield polymerization but afforded the desired glycopeptide thioacid. This phenomenon was also observed with asparagine thioacid with mono GlcNAc. Even the monosaccharide did not yield any polymerization of glycosyl asparagine thioacid. Therefore, the inhibition of polymerization seemed to be due to the steric repulsion between monosaccharides or oligosaccharides at the stage of either the S−N acyl shift from the diacyl disulfide intermediate **31**−**32** or the formation of the diacyl disulfide intermediate **30** itself (Figure 7). In addition, we considered that fluctuation in

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the glycan contributed to the reduction in polymerization due to steric hindrance.

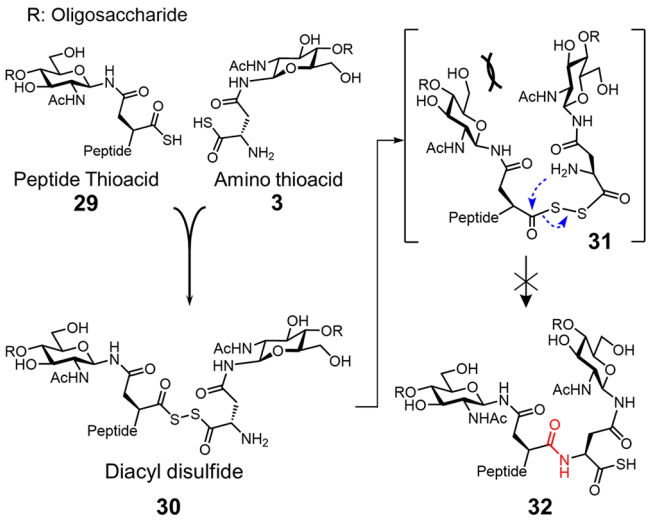

Figure 7. Mechanism of the inhibition of polymerization by glycans of glycosyl asparagine thioacid.

The diacyl disulfide intermediate consisting of peptide-thioacid and glycosyl asparagine thioacid **3** can form, and then a subsequent S−N acyl shift occurs to yield glycopeptide-thioacid. As shown in Figure 2B, the peptide thioacid with valine at the C-terminus suppressed the yield compared with those with alanine at the same C-terminus. These results supported our hypothesis that steric hindrance interferes with the formation of the diacyl disulfide intermediate and S−N acyl shift to yield glycopeptide-thioacid.

On the other hand, the anhydride form of ATC[49] between glycosyl asparagine thioacid **3** and peptide-thioester **33** did not give the desired glycopeptide thioacid (data not shown). In this case, steric hindrance might interfere with S-to-N migration via a five-membered ring intermediate **34**. The retardation of S-to-N migration by steric hindrance or sugar moiety fluctuation led to the resultant anhydride remaining in a solution or organic solvents, and it might have immediately decomposed. Therefore, the anhydride activation method (Figure 8A) might not give the desired glycopeptide thioacid. On the other hand, the six-membered ring **36** formed by a disulfide bond seemed to be suitable for S-to-N migration (Figure 8B) since the three covalent bonds C−S−S−C showed a specific angle and flexibility.[68]

In terms of the preparation of glycosyl asparagine thioacid **3**, lyophilized glycosyl asparagine thioacid **3** was not a suitable substrate for the thioacid-mediated strategy. Residual water in the lyophilized material might easily attack diacyl disulfide intermediate **36** and the resultant glycopeptide-thioacid, and these processes caused low yields. Because oligosaccharides can uptake water molecules around the interface of two glycan branches by the hydrogen bonding network, the glycan releases water during the DDC in DMSO; therefore, this condition accelerates the hydrolysis of the intermediate and the resultant glycopeptide-thioacid. Even asparagine thioacid with a monosaccharide, namely, GlcNAc, the lyophilized substrate suppressed the coupling yield. These data indicated that sugars and oligosaccharides easily form hydration shells.[69,70] Our group also suggested that glycans readily retain water molecules and then form a kind of hydration shell.[37]

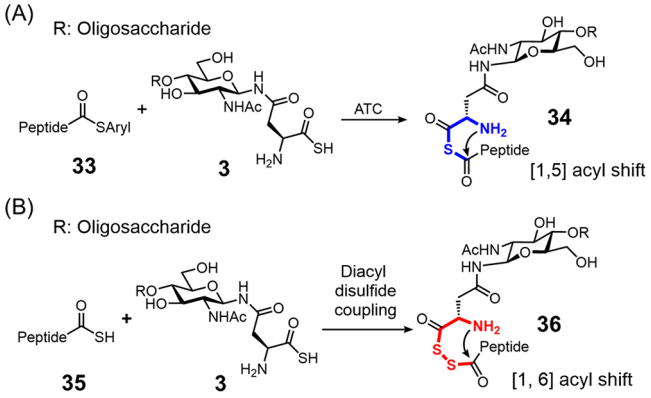

Figure 8. (A) Anhydride formation **34** of ATC with peptide-thioester **33** and glycosyl asparagine thioacid **3**. A subsequent [1,5]-acyl shift yields an amide bond. (B) Diacyl disulfide formation with peptide-thioacid **35** and glycosyl asparagine thioacid **3** by DDC. Subsequent [1,6]-acyl shift yields an amide bond.

Therefore, glycosyl asparagine thioacid was dried under high vacuum conditions for the thioacid-mediated strategy.

For the preparation of glycosyl asparagine thioacid **3**, asparagine with a homogeneous complex-type biantennary sialyloligosaccharide can be isolated from egg yolk or egg powder on a gram scale.[71−74] These glycans are now easily converted into diverse structures by glycosidases and glycosyltransferases.[75,76] In particular, the Boons group developed sophisticated enzymatic synthesis of many asymmetric N-glycans.[77] These methodologies will serve to prepare many glycoforms.

In terms of the preparation of peptide thioacids, the current experiments described here used SPPS. Because several groups have reported the preparation of peptide thioacids from peptide thioester[78] and peptide hydrazide,[79] we can use longer peptide thioacids that can be prepared from peptide thioesters by an Intein system[80,81] or chemical thioesterification method[82−84] with *E. coli* expression methods. However, dependent on peptide sequence and length, the combination of DDC with NCL and EPL[21] is more practical for the preparation of the N-terminal long peptide of glycoproteins.

The thioacid-mediated strategy can yield glycopeptides without epimerization at the N-terminal amino acid of peptide-thioacid and glycosyl asparagine itself. It is known that peptide-thioester generates epimerization under basic conditions, whereas peptide-thioacid has a different chemical characteristic nature. Because the diacyl disulfide bond does not show a potent electron-withdrawing effect in DDC under neutral or acidic conditions, we considered that epimerization can be considerably reduced.

In terms of cell proliferation activity, we focused on glycosyl IL3. Glycosyl IL3 showed a steep slope at a high concentration compared with that of nonglycosyl IL3. This interesting profile was reproducible. The native *N*-glycosylation position we implemented was located at the interface between IL3 and its receptor. Therefore, the *N*-glycan appeared to change some chemical properties of IL3 rather than interfering with the formation of the IL3/IL3 receptor complex (PDB: 5UV8). We hypothesized that hydration shells of *N*-glycans that can absorb water molecules such as sweet sugar syrup can exclude water molecules between the IL3/IL3 receptors to accelerate the formation of the complex. In other words, we assumed that water molecules at the interface between the IL3/IL3 receptor

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were allowed to be adsorbed by the hydration property of adjacent N-glycans. This unique water flux may accelerate the formation of the IL3/IL3 receptor complex.

This hydration shell hypothesis is applicable to erythropoietin and its receptor (PDB: 1EER), and we have noted the function of *N*-glycan hydration shells.[37] Intriguingly, the IL3 receptor also has two *N*-glycans at the interface with IL3, and these *N*-glycans may also be able to absorb water molecules and exclude water molecules from the interface. Therefore, we hypothesized that there is only a slight difference in the biological activity of glycosylated and nonglycosylated IL3. In the case of the IL7/IL7 receptor complex, two *N*-glycans of the receptor located at the interface with IL7 dramatically changed the binding affinity (PDB: 3DI3, 3DI2).[85] We have originally found many examples in which *N*-glycosylation determined by genetic sequence analysis (Asn(glycan)-X-Ser/Thr: X is any amino acid except for Pro) is often located at the interface between the IL/IL receptor (PDB: 1P9M, 6LFM, 1F45, 4GS7, 3TGX, etc.). However, the functions of these *N*-glycans are still unclear. Research to prove this hypothesis is in progress in our laboratory using synthetic homogeneous glycoproteins.

CONCLUSION

In conclusion, we developed a novel and efficient method for synthesizing glycoproteins. We also found that glycosyl asparagine thioacid did not afford a polymerization reaction. This unexpected finding led us to develop a chemoselective DDC reaction and an efficient strategy for synthesizing glycoproteins. The bifunctional thioacid-mediated strategy based on chemical insertion of glycans allows for the synthesis of the full-length glycosyl polypeptide of the target glycoprotein with short steps. These results indicated that our thioacid-mediated strategy can be used as an additional strategy along with existing NCL/EPL strategies. Because peptides can be prepared by the *E. coli*. expression system, we will synthesize homogeneous glycoproteins easily. The development of an efficient method for glycoprotein synthesis with N- and O-glycans and other posttranslational modifications (PTMs) is in progress.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c02601.

Data on all experiments, HPLC and MS profiles (PDF)

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Notes

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