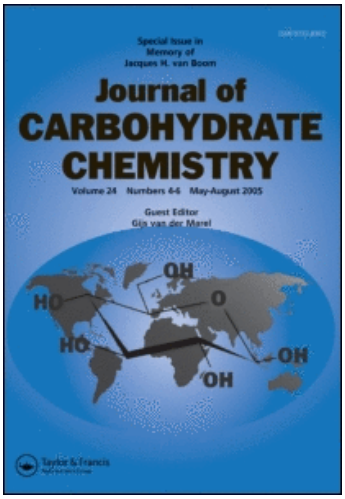


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# Synthesis of Heavily Glycosylated Peptide $^{\alpha}$Thioester

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Erythropoietin (EPO) needs to be heavily glycosylated to exhibit its bioactivity in vivo. In order to synthesize heavily glycosylated EPO analogues, corresponding glycosylated peptide $^{\alpha}$thioesters are essential to prepare glycosylated whole EPO peptide backbones through native chemical ligation. After construction of the peptide $^{\alpha}$thioester corresponding to the 1–32 amino acid sequence in EPO, we aimed to incorporate three complex-type biantennary sialyloligosaccharides to this peptide $^{\alpha}$thioester by the haloacetamide method. The reaction afforded the desired heavily glycosylated peptide $^{\alpha}$thioester.

**Keywords** Erythropoietin; Glycopeptide; Native Chemical Ligation

## INTRODUCTION

Oligosaccharides linked to proteins are concerned with several protein functions such as protein conformation, protein lifetime in the blood, transportation, and antigenicity.[1] Oligosaccharides appear to cause fluctuation and this behavior covers the protein surface. In the case of the glycoprotein hormone erythropoietin (EPO), its sialylated oligosaccharides increase the half-life of EPO in the blood due to the interference of glomerular filtration or interference of the interaction of galactose-binding lectin.[2] Indeed, recombinant EPO, having five complex-type sialyloligosaccharides successfully obtained through a robust mutagenesis process, exhibits a long half-life.[3] Chemical synthesis of glycoproteins and glycopeptides has been developed, and recently synthesis of the two bioactive glycoproteins having complex-type oligosaccharides has

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been reported.[4] We demonstrated the synthesis of the EPO analogue having two complex-type biantennary sialyloligosaccharides by use of native chemical ligation[5] between the combined glycosylated peptide $^{\alpha}$thioester (1–32 amino acid residues) and the peptide (33–166 amino acid residues) prepared from *Escherichia coli* expression. According to this strategy, we examined the synthesis of the additionally glycosylated EPO analogue. We have been interested in whether heavily glycosylated peptides as well as those in their $^{\alpha}$thioester form can be synthesized in order to prepare glycoproteins through a subsequent native chemical ligation and folding process. In this experiment, we employed the haloacetamide method[6] for the additional incorporation of the oligosaccharide to the EPO peptide backbone. EPO fortunately has cysteine residue at the 33 position and this site was used as a ligation junction. Therefore, we examined the synthesis of the same glycosylated peptide $^{\alpha}$thioester, which we prepared before (1–32 amino acid residues),[4] but we aimed to incorporate three complex-type biantennary sialyloligosaccharides. In terms of the glycosylation position, we planned to incorporate the sialyloligosaccharide to the 28 and 32 positions in addition to the native 24 position. As shown in Figure 1,[7] the original cysteine at the 29 position is essential to form a disulfide bond, and the 3D model of the complex of EPO and its receptor suggests that 28 and 32 positions seem to be suitable for the incorporation of the additional two sialyloligosaccharides. In terms of the area between the 24 and 27 positions, oligosaccharide has already been incorporated to the 24 position as in the case of natural EPO and therefore glycosylation at the 25–27 position absolutely causes steric hindrance between each oligosaccharide as well as between the oligosaccharide and the receptor. Therefore, we decided to synthesize the heavily glycosylated peptide $^{\alpha}$thioester (1–32 amino acid residues) having three complex-type biantennary sialyloligosaccharides at the 24, 28, and 32 positions. In this paper, we report the synthesis of the glycosylated peptide $^{\alpha}$thioester having three complex-type biantennary sialyloligosaccharides.



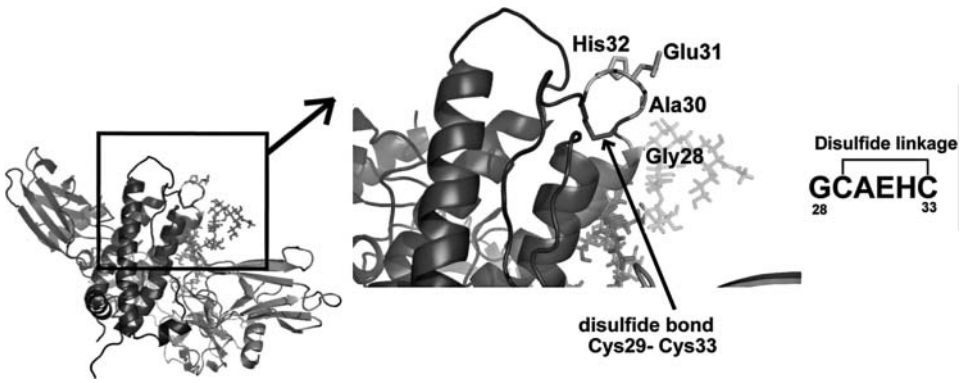


**Figure 1:** 3D model of EPO binding to its receptor and sequence from 28 to 33.

## RESULTS AND DISCUSSION

In order to incorporate oligosaccharides to the 24, 28, and 32 positions, an original EPO amino acid sequence (1–32) was designed to modify with cysteine residues. The peptide was synthesized by the solid-phase peptide synthetic method. Cysteines used to form disulfide bonds at the 7 and 29 positions were protected by Acm groups. Solid-phase synthesis of this peptide employed our previous strategy using 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) and the poly(ethylene glycol)-poly(dimethylacrylamide) copolymer (PEGA) resin.[4] After construction of the desired peptide sequence on the HMPB-PEGA resin, the side chain-protected peptide **1** was detached from the resin by the treatment with AcOH-trifluoroethanol. Toward the C-terminal amino acid, thioesterification was examined at –20°C to suppress undesired epimerization of the C-terminal cysteine.[8] Subsequent deprotection step using 95% TFA and purification using reverse phase HPLC afforded the desired peptide $^{\alpha}$thioester **3** (Fig. 2). Toward this $^{\alpha}$thioester **3**, haloacetamide reaction using bromoacetamidyl sialyloligosaccharide **4** was examined.[6] The peptide $^{\alpha}$thioester **3** was dissolved in a sodium phosphate buffer solution containing DMF and urea and then the bromoacetamidyl sialyloligosaccharide **4** was added to this mixture (Fig. 3). In order to monitor this reaction, an aliquot of this mixture was subjected to reverse phase HPLC and peaks were analyzed by ESI mass spectrometry. As shown in Figure 4, a major product was observed by HPLC after 3 h and mass analysis also supported the desired molecular weight. Due to the steric hindrance of the complex-type sialyloligosaccharide, the incorporation reaction seemed to be inefficient, but this reaction afforded the desired product. After purification, the glycosylated peptide $^{\alpha}$thioester having three complex-type sialyloligosaccharides **5** was obtained in 16% isolated yield. This substrate can be used for the synthesis of heavily glycosylated EPO analogues.

So far, the preparation of such heavy glycosylated proteins in a homogeneous form has been difficult in the biosynthesis. Therefore, our demonstration may contribute to the elucidation of the effect of the heavy glycosylation to the protein surface. Synthesis of heavily glycosylated EPO analogues is in progress and these synthetic results will be reported in due course.

## EXPERIMENTAL SECTION

Fmoc-amino acid derivatives, coupling reagents, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB), the poly(ethylene glycol)–poly (dimethylacrylamide) copolymer (PEGA) resin, 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU), benzotriazole-1-yl-oxytrispyrrolidinophos phonium hexafluorophosphate (PyBOP), and 1-hydroxy-1H-benzotriazole

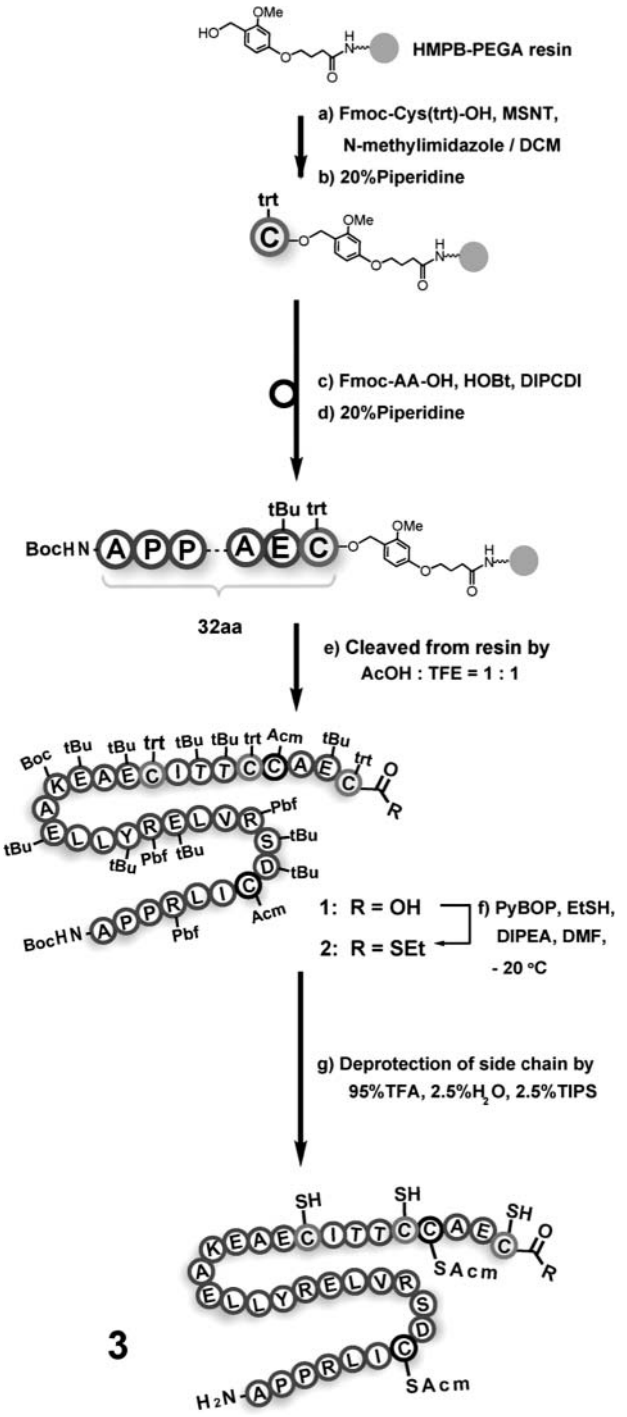


**Figure 2:** Synthesis of peptide $^{\alpha}$thioester **3**.

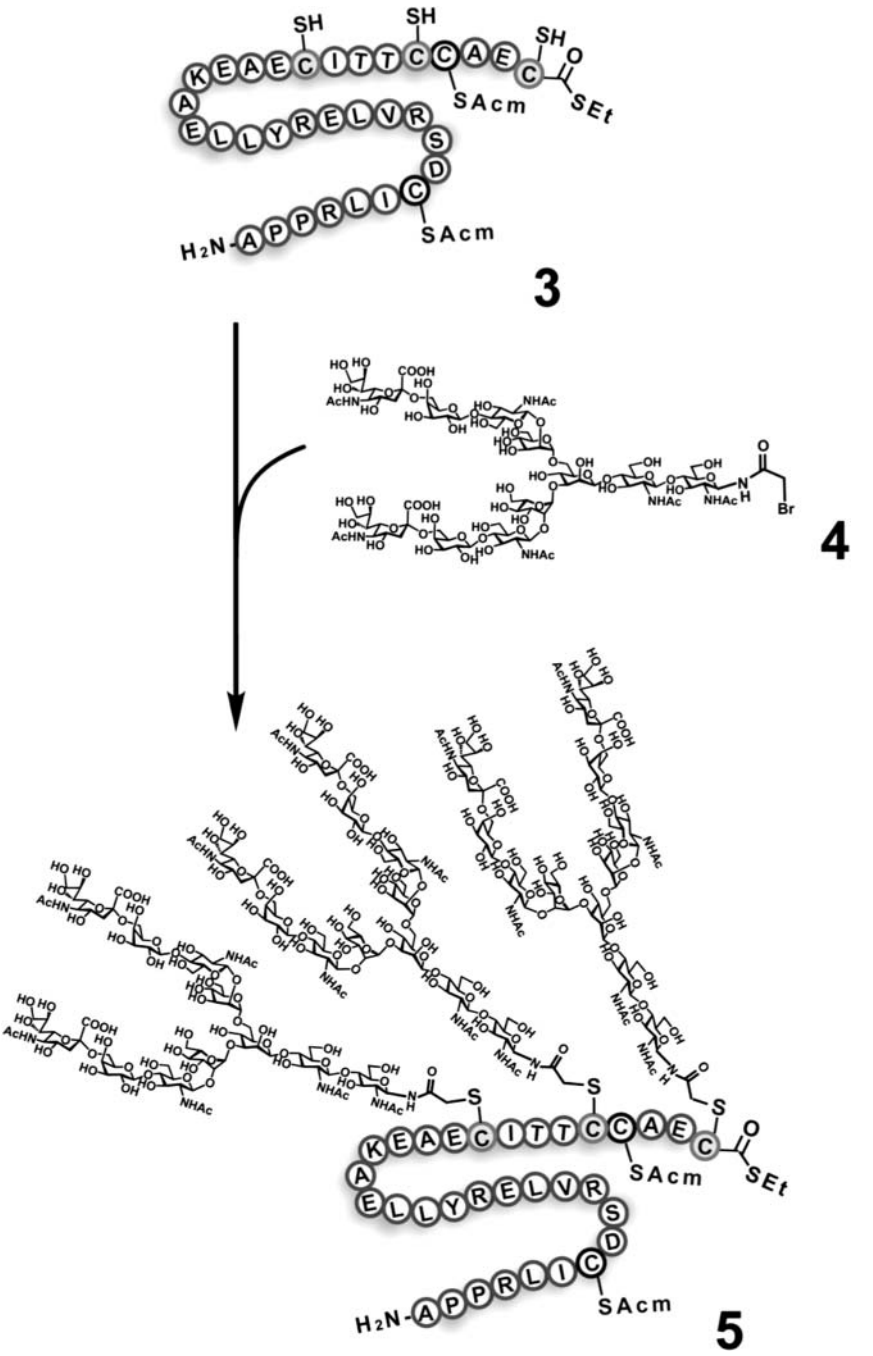


**Figure 3:** Synthesis of glycosylated peptide $^{\alpha}$thioester **5** by use of haloacetamide method (bromoacetamidyl undecasaccharide **4**, 30 mM urea, 100 mM sodium phosphate:DMF (2:1), pH = 7.5, RT. 3 h, 16%).

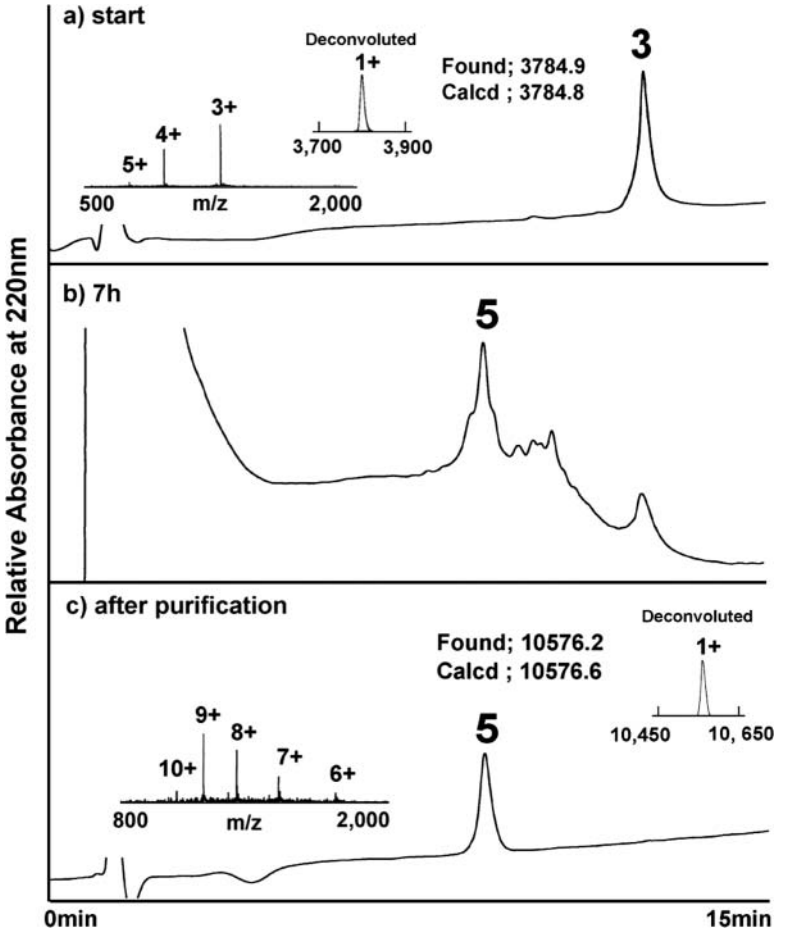


**Figure 4:** Synthesis of glycosylated peptide EPO (1–32) $^{\alpha}$thioester **5** by use of haloacetamide method monitored by RP-HPLC and ESI-MS analysis.

(HOBt) were purchased from NovaBioChem. RP-HPLC analysis was performed on a Waters HPLC system equipped with a photodiode array detector (Waters 2996) using a Cadenza column (Imtakt Corp., 3 $\mu$m, 75 $\times$ 4.6 mm) for analytical HPLC, and a Vydac column C-18 (5 $\mu$m, 250 $\times$ 10 mm) for semipreparative HPLC. ESI mass measurement was carried out on a Bruker Daltonics/Esquire3000 plus.

## Glycosylated Peptide Synthesis

*Synthesis of EPO (1–32) Peptide $^{\alpha}$Thioester **3***

The peptide $H_2N$-APPRLIC(Acm)DSRVLERYLLEAKEAECITTCC(Acm) AEC- SEt **3** was synthesized on HMPB-PEGA resin (50 $\mu$mol scale) by Fmoc

solid-phase peptide synthesis.[9] The first amino acid (5.0 equiv.) was coupled to the resin with MSNT (5.0 equiv.) and *N*-methylimidazole (3.5 equiv.) in $CH_2Cl_2$ (Fmoc-amino acid concentration was adjusted to 330 mM) for 1 h. This coupling was conducted twice. The peptide chain was elongated by a standard Fmoc-based protocol. The coupling conditions were as follows: Fmoc amino acid (5.0 equiv.), HBTU (5.0 equiv.), HOBt (5.0 equiv.), and DIPEA (10.0 equiv.) in DMF (Fmoc-amino acid concentration was adjusted to 250 mM). Peptide coupling was allowed to continue for 1 h except for cysteine. Each amino acid was double coupled. All cysteine residues (Cys7, Cys24, Cys28, Cys29, Cys32) were coupled by use of DIPCDI (5.0 equiv.) and HOBt (5.0 equiv.) for 1 h. Boc-Ala-OH was used for the coupling of the N-terminal alanine residue. Deprotection of Fmoc groups was performed with 20% piperidine in DMF for 20 min. After all coupling steps were completed, the fully protected peptide **1** was released from the resin by overnight exposure to acetic acid/trifluoroethanol (1:1, 5 mL). The solution was filtered and the filtrate was concentrated in vacuo; then the residue was redissolved in benzene and residual segments were coevaporated with benzene for three times. This crude peptide was used for the thioesterification of the C-terminus without further purification. To a solution of this peptide **1** (ca 7.6 $\mu$mol estimated by HPLC) in DMF (1 mL) was added ethanethiol (16 $\mu$L, 30 equiv.). After cooling to –20°C, PyBOP (20 mg, 5.0 equiv.) and DIPEA (6.6 $\mu$L, 5.0 equiv.) were added to this mixture and stirring was continued at –20°C for 2 h, avoiding epimerization at the C-terminal.[8] The reaction mixture was then poured into ice-cold $Et_2O$, forming a precipitate of the peptide $^{\alpha}$thioester **2**. The resulting white precipitate was collected and then dissolved in a solution containing 95% TFA, 2.5% triisopropylsilane (TIPS), and 2.5% $H_2O$ in order to remove acid labile protecting groups. After 3 h, this solution was poured into ice-cold $Et_2O$. The resulting white precipitate was collected again. This precipitate containing peptide **3** was purified by RP-HPLC using a linear water/acetonitrile gradient and characterized by ESI-MS analysis. Yield of peptide thioesterification was to be ca 10% to 20% (estimation by HPLC) and isolated yield was 5%.



*Synthesis of glycosylated Peptide $^{\alpha}$Thioester **5***

Coupling reaction[6] of the peptide **3** (2.0 mg) with bromoacetamidyl sialyloligosaccharide **4** (1.5 equiv. toward each sulfhydryl group) was performed in phosphate buffer (100 mM, pH 7.5, 500 $\mu$L) containing 30 mM urea:DMF = 2:1. Monitoring of this reaction was performed by RP-HPLC (Cadenza C-18 column at a flow rate of 1 mL/min linear gradient of 18% to 54% $CH_3CN$ containing 0.09% TFA in 0.1% TFA solution over 15 min). After 3 h, direct HPLC purification afforded product **5** in moderate yield (16%). The product was characterized by ESI-MS analysis as shown in Figure 4.

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