VIP Glycoprotein Synthesis

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Chemical Synthesis of an Erythropoietin Glycoform Containing a Complex-type Disialyloligosaccharide**

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Chemical synthesis is a powerful approach to investigate how posttranslational modifications of proteins such as glycosylation can affect their functions.[1] These chemical syntheses are achieved by means of sophisticated peptide-coupling reactions such as native chemical ligation (NCL).[2] Native chemical ligation for protein synthesis developed by Kent and co-workers is performed in neutral buffer solutions and involves the reaction of a Cys-peptide with a peptide α-thioester. Thus, the synthesis of peptide α-thioester building blocks is key to chemical protein synthesis. The synthesis of peptide α-thioesters is straightforward by using solid-phase peptide synthesis (SPPS) based on *tert*-butyloxycarbonyl (*tert*-Boc) manipulations.[3] However, conventional *tert*-Boc-based coupling reactions are not applicable to the synthesis of acid-labile peptide α-thioester segments that are essential for the synthesis of proteins containing acid-labile modifications, such as glycoproteins, in which the oligosaccharides are labile under strongly acidic conditions.[4] For this reason, recent developments in the synthesis of peptide α-thioesters have been focused on developing using fluorenylmethoxycarbonyl (Fmoc)-based SPPS. Despite numerous publications,[5] the synthesis of peptide α-thioesters bearing complex glycan moieties is still not straightforward, even by Fmoc SPPS methods.[6]

It is well known that glycosylation is one of the major forms of posttranslational modification of proteins.[7] The asparagine-linked sialyloligosaccharide exhibits essential functions related to protein bioactivity. In particular, cytokines such as erythropoietin require acidic sialic acid residues at the nonreducing terminal of the oligosaccharides.[8] The sialic acid residue **1** is known to be extremely labile under acidic conditions and easily hydrolyzed to generate **2** (Figure 1).[9] Such lability might be attributed to deoxy sugars, which lack a hydroxy group on the carbon adjacent to the anomeric position.

Therefore, to date, modified conditions relying on Fmoc groups have been used for the synthesis of sialylglycopeptide α-thioesters rather than conventional *tert*-Boc-based approach.[6] We thought that there might also be another reason why the sialyl linkages (sialosides) are very labile under acidic conditions. Capon reported that the rate of the acidic hydrolysis of *o*-carboxyphenyl β-D-glucoside is 1000 times greater than that of simple phenyl β-D-glucoside.[10] It was suggested that the carboxylic acid group acts as an intramolecular acid catalyst. Based on this report, we hypothesized that the carboxylic acid group in sialic acid residue **1** might also function as an acid catalyst and facilitate the hydrolysis of the glycosidic bond (Figure 1).[11]

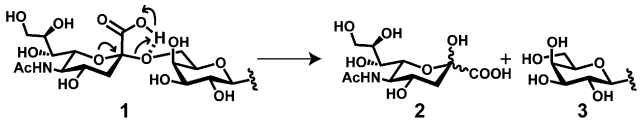

Figure 1. Intramolecular catalyst proposed for the hydrolysis of sialoside.

To elucidate this intramolecular catalytic activity, the carboxylic acid group on a sialic acid residue could be masked with a phenacyl (Pac) group. Phenacyl ester is known to be stable under strongly acidic conditions[12] and therefore is expected to interfere with the catalytic activity. If this masked sialoside were to exhibit such stability under strongly acidic conditions, it would be possible to perform a straightforward synthesis of sialylglycopeptide α-thioesters using the *tert*-Boc SPPS conditions. We envisioned that this would enable us to establish a practical synthetic strategy for the preparation of homogeneous glycoproteins having sialyloligosaccharides. Herein we describe the concise synthesis of acid-labile sialylglycopeptide α-thioesters by improved *tert*-Boc conditions and the chemical synthesis of an erythropoietin[13] glycoform having one sialyloligosaccharide using the improved *tert*-Boc conditions.

To test our hypothesis, we selected asparaginyl sialyloligosaccharide **4**[14] for the following experiments (Scheme 1). Since selective benzyl esterification of the sialic acid residue over the carboxylic acid of asparagine residue has already been established,[6] selective installation of a phenacyl group was performed using similar conditions. Treatment of **4** with Cs_2CO_3 and then with phenacyl bromide afforded the desired disialyloligosaccharide diphenacyl ester **5** in 82% yield (Scheme 1). In order to study the stability of the phenacyl-esterified sialyloligosaccharide, compounds **4** and **5** were treated with acid (40 mM HCl) and the results are shown in Figure 2a. As we expected, the nonprotected sialyloligosaccharide **4** was easily hydrolyzed, but the phenacyl-esterified sialyloligosaccharide **5** exhibited potent stability. In addition to these experiments, we examined the stability of sialyloligosaccharides under acidic deprotection conditions: trifluoroacetic acid (TFA)/trifluoromethanesulfonic acid (TfOH)/dimethyl sulfide (DMS)/*m*-cresol (5:1:3:1), reagents conventionally used in *tert*-Boc SPPS instead of HF.[15] It

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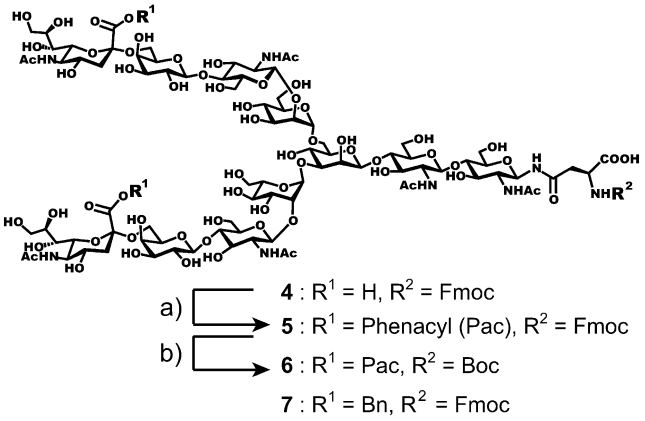


Scheme 1. Preparation of *tert*-Boc-Asn-phenacyl sialyloligosaccharide. a) Cs_2CO_3/H_2O and then PacBr, DMF. b) 1. 1-methylpyrrolidine, hexamethyleneimmine, HOBt, 2. BocOSu, DIPEA, DMF. HOBt = 1-hydroxybenzotriazole, DIPEA = *N*,*N*-diisopropylethylamine.

turned out that sialyloligosaccharide dibenzyl ester **7**[6] underwent cleavage of the sialyl bonds as well as deprotection of the benzyl (Bn) esters (Figure 2b, bottom). In contrast, phenacyl-esterified sialyloligosaccharide **5** exhibited suitable stability (Figure 2b, top). Based on these results, we concluded that the carboxylic acid group might act as an intramolecular catalyst and the sialic acid residues masked with phenacyl group are retained even under strongly acidic conditions.

These findings led us to examine the synthesis of sialylglycopeptide α-thioesters by means of *tert*-Boc chemistry. Sialyloligosaccharide diphenacyl ester **5** was converted into its *tert*-Boc-asparagine form **6** for use in *tert*-Boc SPPS (Scheme 1). We employed the poly[acryloyl-bis(aminopropyl)polyethylene glycol] (PEGA) resin bearing mercaptopropionamide,[16] and the peptide was elongated on the resin using *tert*-Boc SPPS in situ neutralization conditions (Scheme 2).[3c] However, the deprotection and the detachment of the product from the solid support were improved, as described in the following paragraphs. Since the thiol linker (β-mercaptopropionic acid) was directly incorporated into the amino group of the resin, the afforded peptide α-thioester was not detached from the solid support by the final acid treatment, but thiolysis released the corresponding peptide α-thioester (Scheme 2).[17] After each coupling step the *tert*-Boc group was removed by using a nonvolatile sulfuric acid, such as 10% sulfuric acid in dioxane,[18] rather than the more volatile TFA. The coupling of *tert*-Boc-asparaginyl- and diphenacyl-esterified sialyloligosaccharide **6** was performed with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) or 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT), and then the subsequent peptide elongation was performed using conventional in situ neutralization conditions employing TFA (or 50% TFA in dichloromethane) for *tert*-Boc deprotection, but not using 10% sulfuric acid in dioxane. Because the glycosyl bonds appears to be unstable in 10% sulfuric acid in dioxane in these experiments, these conditions for *tert*-Boc removal were used only before the coupling of *tert*-Boc-Asn-diphenacyl sialyloligosaccharide **6** or for the synthesis of peptide α-thioesters without oligosaccharides. For *tert*-Boc conditions without HF deprotection, we employed TFA/TfOH/DMS/*m*-cresol (5:1:3:1) under careful monitoring to determine the status of the deprotection step. Conventional acidic deprotection in both Fmoc and *tert*-Boc SPPS is typically performed in a homogeneous acidic solution, but deprotection is sometimes incomplete, depending on the amino acid sequence and the number of amino acids in the peptide. Our simple linker on the solid support is stable during acid treatment and the target peptide α-thioesters are left on the solid support. After repetitive short acid treatment with TFA/TfOH/DMS/*m*-cresol (5:1:3:1) for 20–40 min, the acidic solution was filtered off from the reaction vessel. Then, an aliquot of the resin was treated with a nucleophile or a basic solution (10%

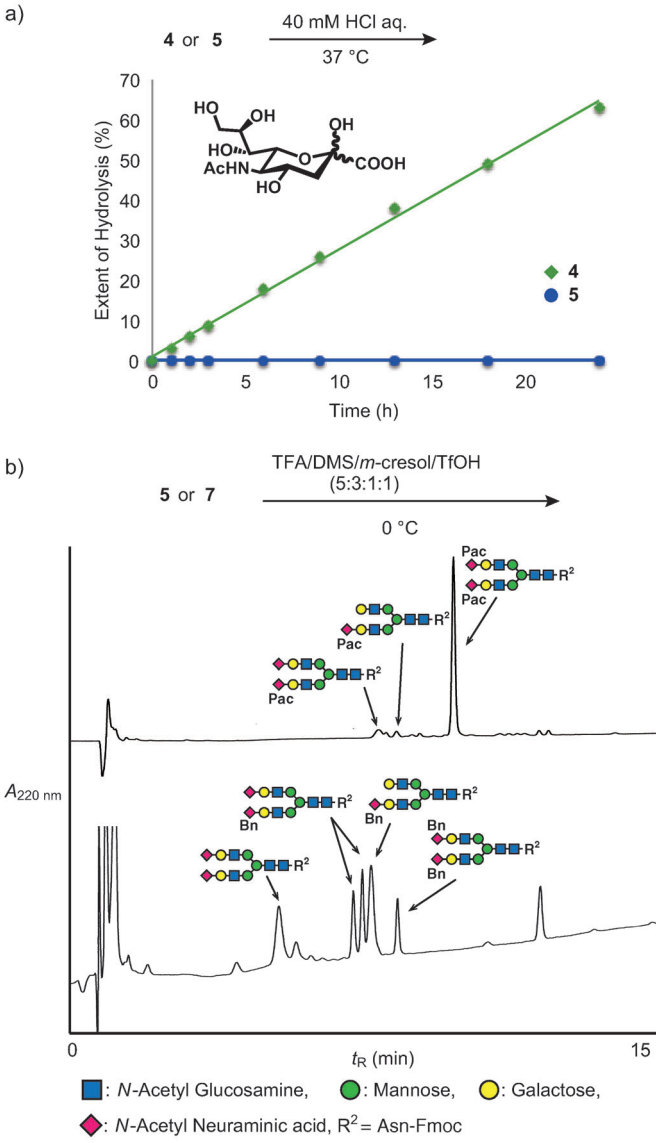
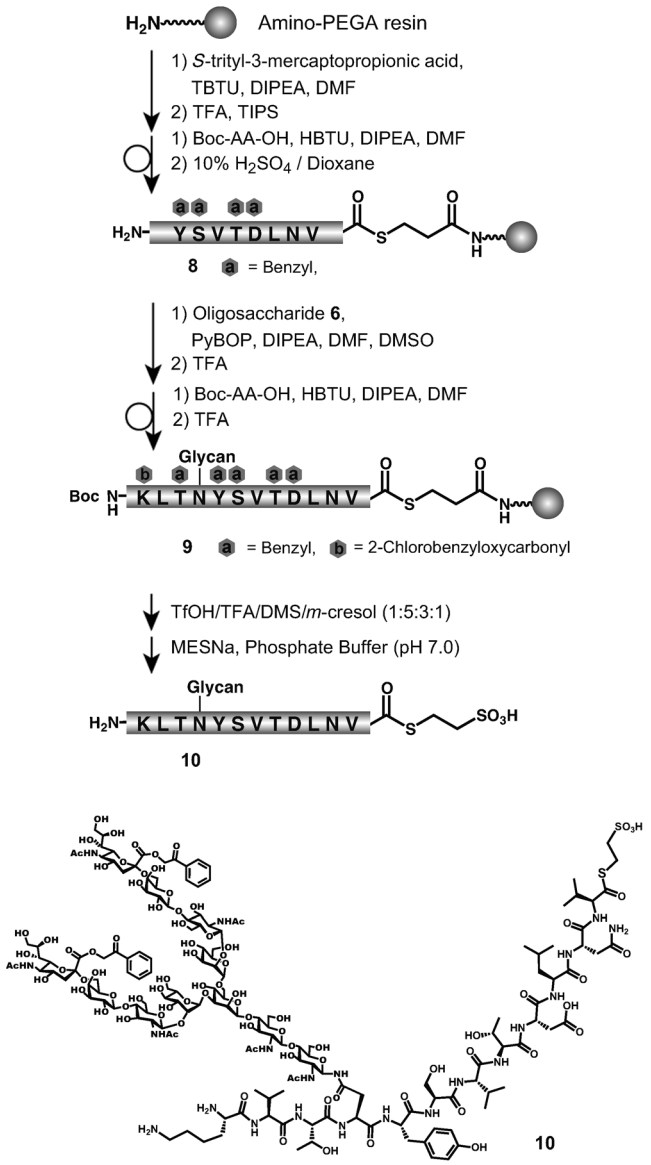


Figure 2. Stability of sialyloligosaccharides under acidic conditions. a) Acid hydrolysis of sialoside **4** and **5** by 40 mM HCl. b) HPLC analysis of sialoside **5** (top) and **7** (bottom) after treatment with TFA/DMS/*m*-cresol/TfOH. The conditions are given in the Supporting Information.



Scheme 2. Synthesis of sialylglycopeptide α-thioester **10**. AA = amino acid. The arrows with the fused circle represent repeated coupling steps.

hydrazine hydrate) to release the peptide, and the resulting peptides were analyzed by HPLC in order to evaluate the degree of deprotection. By this simple procedure we could monitor the progress of deprotection of the peptide α-thioester on the resin (an example is shown in Figure S5 in the Supporting Information). In addition, it is well known that the benzyl cation generated from the Bn-protected peptide during the acidic deprotection step can react with the peptide on the resin, and this side reaction results in undesired byproducts.[19] Indeed, increasing the reaction scale generated such by-products, even though scavengers such as DMS or thioanisole were present in the reaction mixture. In order to reduce the formation of undesired benzyl adducts, we repeated the short (20–40 min) acid treatment using TFA/TfOH/DMS/*m*-cresol (5:1:3:1) and exchanged the acidic deprotection cocktail. After the completion of deprotection, which was confirmed by the above-mentioned monitoring system, the thiolysis was performed in sodium phosphate buffer (pH 7.0) containing 6 M guanidine-HCl (Gn-HCl) and sodium 2-mercaptoethanesulfonate (MESNa) (5% wt/wt) to obtain the corresponding diphenacyl-esterified sialylglycopeptide α-thioester **10**.

Compound **10** was characterized by ^{1}H NMR spectroscopy and electrospray ionization mass spectrometry (Figure 3a and Figure S3 in the Supporting Information). These data clearly indicate that the resulting glycopeptide α-thioester is homogeneous and that the improved *tert*-Boc SPPS protocol did provide acid-labile sialylglycopeptide α-thioesters more effectively compared with the previous minimum-protection-*tert*-Boc conditions.[17b] The conventional protocol using TFA/TfOH/DMS/*m*-cresol sometimes results in considerable problems. The nonvolatile TfOH may remain in the products even after precipitation and washing with chilled ether, and this results in the decomposition of the product.[20] In the improved protocol, the acid solution was removed by a simple filtration and the remaining trace amounts of acid can be neutralized by a subsequent thiolysis step which employs a neutral buffer solution. The phenacyl group can be removed during the subsequent NCL or by treatment with a high concentration of alkylthiol during thiolysis.

This reaction scheme was also applied to the synthesis of the sialylglycopeptide α-thioesters **11** and **12**, asialoglycopeptide α-thioester **13**, and the simple peptide α-thioesters **14** and **15**. As shown in Figure 3, the crude materials after SPPS exhibit excellent purity, and subsequent purification by preparative HPLC afforded homogeneous glycopeptide α-thioesters as well as peptide α-thioesters. We also examined the synthesis of longer peptide α-thioesters consisting of 30 and 40 amino acid residues by using the same improved *tert*-Boc conditions. These conditions combined with the simple monitoring system during the final deprotection step also resulting in the efficient deprotection of the longer peptide α-thioesters (an example is shown in Figure S5 in the Supporting Information).

We also examined the syntheses of peptide α-phenyl thioester **17** and peptide α-hydrazide **18** by using conditions similar to those used for the generation of alkylthioester **16** (Figure 4). It is known that the peptide α-hydrazide can be successfully applied to peptide condensation reactions.[21] Kent and co-workers showed that peptide α-phenyl thioester is useful for kinetically controlled ligation.[22] As shown in Figure 4, substitution reactions with thiophenol or hydrazine efficiently afforded C-terminal-activated peptide derivatives which can be used in subsequent NCL (80–90% yield).

Using these improved *tert*-Boc conditions along with conventional Fmoc conditions, we examined the synthesis of a human erythropoietin (EPO) glycoform, which consists of 166 amino acid residues[23–25] and has a single complex-type *N*-linked oligosaccharide at the 83 position (Figure 5a). It is known that an increased amount of acidic sialic acids at the nonreducing ends of the oligosaccharides in EPO enhances its hematopoietic activity in vivo.[8a] However, homogeneous EPO analogues having two or three complex-type disialyloligosaccharides (at the 24, 30, and 32 positions) did not exhibit suitable hematopoietic activity in vivo,[24b,c] although

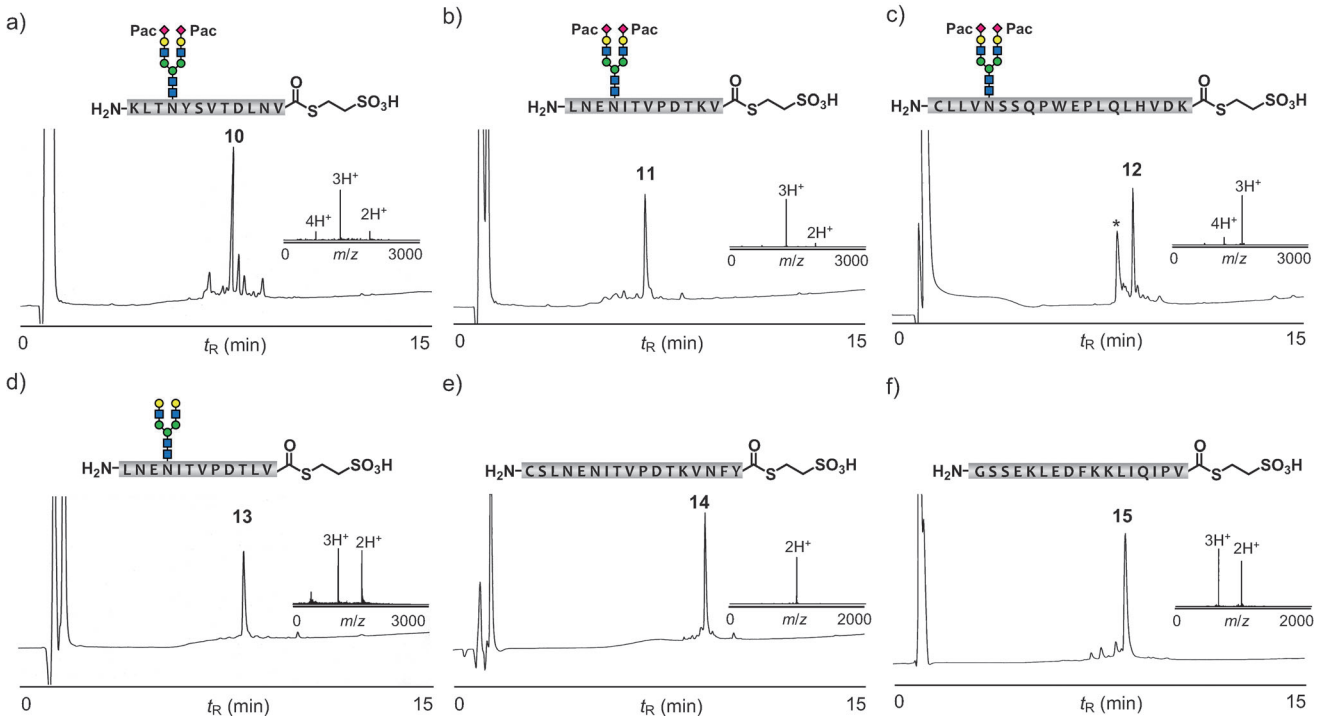


Figure 3. HPLC profiles of phenacyl-protected sialylglycopeptide α-thioester analogues of a) human INF-γ (94–105), b) human EPO (35–46), and c) human EPO (79–97; an impurity from the reagents is marked with an asterisk), and of d) human EPO (35–46) asialoglycopeptide α-thioester and e) human EPO (33–49) and f) bovine INF-γ (84–100) peptide α-thioesters. Only the EPO segment (79–97) shown in (c) was used for the synthesis of sialyl-EPO **19**. The mass values observed (a–f) and the HPLC profiles of purified glycopeptide α-thioesters and peptide α-thioesters are shown in Figure S4 in the Supporting Information.

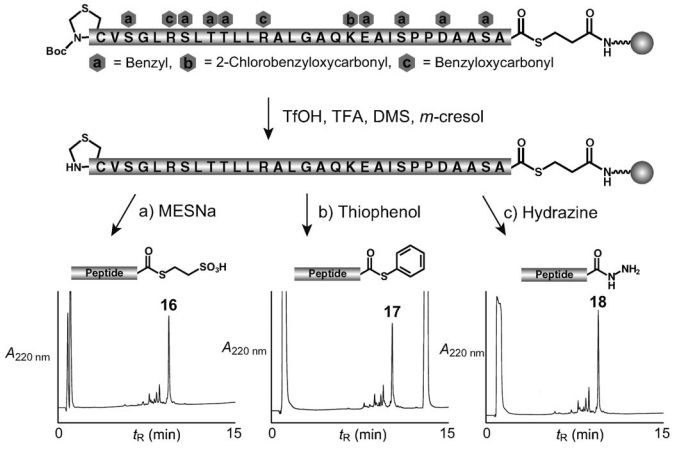


Figure 4. Deprotection and cleavage of EPO segment (98–127) from the resin using different nucleophiles: HPLC profile of crude peptide a) thioester, b) phenyl thioester, c) hydrazide.

the expressed EPO glycoform having complex-type biantennary sialyloligosaccharides at the 24, 38, and 83 positions did exhibit suitable in vivo activity.[26] It is also known that the sialyloligosaccharide at the 83 position is essential for EPO hematopoietic activity in vivo.[8c] However, this has been investigated by using the expressed EPO which displays heterogeneity in the sialyloligosaccharide structure at the 83 position. Under these circumstances, we examined the synthesis of homogeneous EPO glycoform having biantennary complex-type disialyloligosaccharide at the 83 position using our improved *tert*-Boc conditions and NCL (Figure 5a).

Chemical syntheses of EPO derivatives have been performed by several groups[23,24,27,28] and we also employed the convergent synthetic strategy shown in Figure 5b. The native EPO sequence contains only four Cys residues (Figure 5a), but this number and the positions are not sufficient for the use of NCL for the synthesis of the full-length EPO polypeptide chain. In particular, there is no Cys residue between 34 and 159 positions. Therefore we decided to use the metal-free desulfurization protocol developed by Danishefsky and Wan.[29–31] Cysteine residues can be easily converted into alanine residues using this protocol. Our synthetic strategy makes use of six segments (1Ala–21Ala **A**, 22Cys–49Tyr **B**, 50Cys–78Ala **C**, 79Cys–97Lys **D**, 98Cys–127Ala **E**, and 128Cys–166Arg **F**; mutation sites: Glu21Ala, Gln78Ala) and the cysteine residue at positions 22, 50, 79, 98, and 128 are converted into alanine after NCL. Each segment was prepared by application of the improved *tert*-Boc conditions (segments **C**, **D**, and **E**) or the Fmoc conditions (segements **A**, **B** and **F**). After the synthesis of each segment **A**–**F**, we carried out the NCL reactions sequentially from the C terminus and performed desulfurization to obtain the glycosylpolypeptide chain of EPO (see the Supporting Information for the details of all experiments). The obtained EPO glycosylpolypeptide chain was treated with silver acetate to remove the acetamidomethyl (Acm) protecting groups at the 7Cys, 29Cys, 33Cys and 161Cys positions. Phenacyl groups were removed after NCL between segment **D** and segment **EF** by treatment with

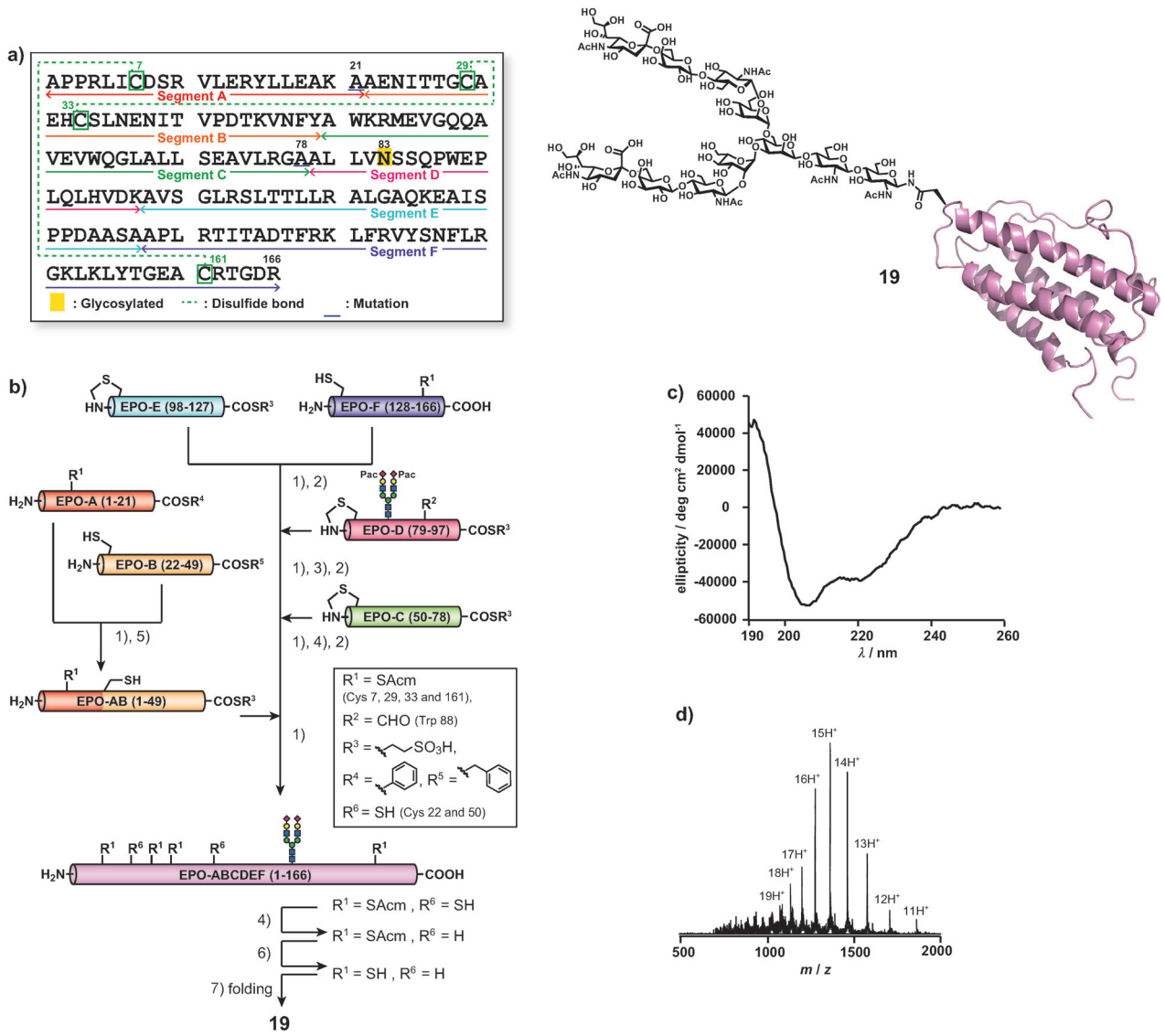


Figure 5. Synthesis of erythropoietin. a) Amino acid sequence and complete structure of the target EPO **19**. b) The synthesis of EPO with a human complex-type disialyloligosaccharide by chemical ligation and selective desulfurization: 1) ligation, 2) conversion of Thz into Cys with methoxyamine-HCl, 3) deprotection of formyl group of Trp and Pac group with piperidine, 4) desulfurization of cysteine, 5) thioester exchange with MESNa, 6) removal of Acm groups with AgOAc, 7) folding experiments under cysteine–cystine redox conditions. c) CD spectrum of folded EPO **19**. d) Mass spectrum of folded EPO **19**.

piperidine. The oxidative folding under the combined dialysis and redox conditions[23a] afforded suitably folded EPO having sialyloligosaccharide at the 83 position. These synthetic details are summarized in Figures S6–S20 in the Supporting Information. As shown here, the improved *tert*-Boc conditions along with the existing Fmoc conditions enabled the efficient preparation of a practical amount of the sialylglycopeptide α-thioester as well as peptide α-thioesters and a relatively large (166 amino acids) homogeneous glycoprotein.

In conclusion, we have discovered the reason for the acid lability of sialoside and this finding enabled us to synthesize sialyloligosaccharylpeptide α-thioesters using an improved version of the *tert*-Boc SPPS protocol. In addition, with these optimized conditions we could perform *tert*-Boc SPPS to prepare longer peptide α-thioesters without HF, which is not a conveniently available reagent in our laboratory. The preparation of a practical amount of peptide α-thioester segments and sialylglycopeptide α-thioester segments has proven to be difficult with Fmoc conditions, which require several conversion steps. The simple and efficient method described here enabled us to solve these problems and to achieve the chemical synthesis of homogeneous monoglycosyl-EPO glycoform. The bioassay of **19** and synthesis of other homogeneous EPO glycoforms are in progress.

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