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Chemical Protein Synthesis

Convergent Chemical Synthesis of [Lysine^{24,38,83}] Human Erythropoietin**

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Erythropoietin (EPO) is a glycoprotein hormone that plays important roles in regulating the production of red blood cells (erythrocytes).[1] Since human EPO was first isolated and purified from urine in 1977,[2] the structure and physiological properties of EPO have been thoroughly studied.[3] Mature human EPO found in nature consists of a polypeptide chain of 165 amino acids with four covalently attached oligosaccharides,[4] one of which is an O-linked oligosaccharide at Ser^{126}, and the other three are N-linked oligosaccharides at residues Asn^{24,38,83}.[5] EPO is used as a therapeutic agent to treat anemia caused by chronic kidney disease.[6] Commercial EPO is prepared using recombinant DNA technology. The carbohydrate moieties of both native and recombinant EPO[7] are heterogeneous and composed of multiple glycans with different lengths and composition at each glycosylation site.[5a,8] This heterogeneity makes it difficult to evaluate the effects of carbohydrate moieties on EPO's pharmacokinetic properties and, more importantly, complicates the understanding of mechanisms of EPO's action at the molecular level. For these reasons, it is important to develop an alternative strategy to prepare homogeneous EPO.

The production of homogeneous, glycosylated EPO by total synthesis is a major current objective of the organic synthesis community.[9] Recent developments in chemical synthesis of proteins[10] and glycoproteins[11] have made it possible in principle to prepare homogeneous EPO by total synthesis, thus offering the prospect of complete control of the covalent structure in order to perform systematic structure–activity studies. An early synthetic achievement was the preparation of a series of homogeneous polymer-modified EPO analogues of defined covalent structure and full biological activity.[12] These glycoprotein mimics contained the full-length 166-residue polypeptide chain encoded by the EPO gene and were prepared by sequential assembly of four synthetic peptide segments using thioester-mediated native chemical ligation.[13] This linear strategy of sequentially assembling multiple peptide segments involves repetitive ligation and purification, which results in low yields of final products.[14] More recently, a semisynthesis of an EPO analogue containing thioether-linked complex glycan moieties has been reported.[15] Finally, the Danishefsky group has set as a target the total synthesis of both the complex glycan moieties and the full-length polypeptide chain of EPO; progress in this endeavor has been recently summarized.[16] Ultimately, a practical total synthesis of EPO will necessitate the fully convergent synthesis of the 165-residue mature polypeptide chain. None of the synthetic studies reported to date have successfully addressed this objective.

A primary function of the attached oligosaccharides is to extend EPO's circulation time in vivo. Although the carbohydrate moiety accounts for about 35% of the weight of the EPO molecule,[1,4] the carbohydrate is not required for activity in vitro. The folded 165 amino acid polypeptide, with two native disulfide bonds, is responsible for binding to and activating the EPO receptor.[1b,3] Therefore, nonglycosylated EPO analogues are expected to be important for the study of structure–activity relationships. [Lys^{24,38,83}]EPO produced by recombinant means has previously been used to determine the structure of the EPO protein molecule by NMR spectroscopy [17] and the structure of EPO bound to its receptor by X-ray crystallography.[18] In that work, lysine residues were placed at the N-glycosylation sites to increase the protein's isoelectric point and to thus prevent aggregation of nonglycosylated EPO at neutral or acidic pH values. This previous research[17,18] showed that replacement of the three N-glycosylation sites with lysine residues does not affect the structure of EPO and its receptor binding activity.

We report herein a convergent total chemical synthesis of [Lys^{24,38,83}]EPO, containing two disulfide bonds, which is composed of a 165 amino acid residue polypeptide with the three native N-glycosylation residues Asn^{24,38,83} mutated to Lys^{24,38,83}. The synthetic protein is biologically active. This work is an important guide to the effective total synthesis of natural EPO and will enable the systematic preparation of neoglycoprotein analogues of EPO for the systematic dissection of the effect of glycan structure on the properties of the EPO molecule.

Our convergent synthetic strategy for the preparation of [Lys^{24,38,83}] human EPO is shown in Scheme 1. The combination of native chemical ligation[13] with kinetically controlled

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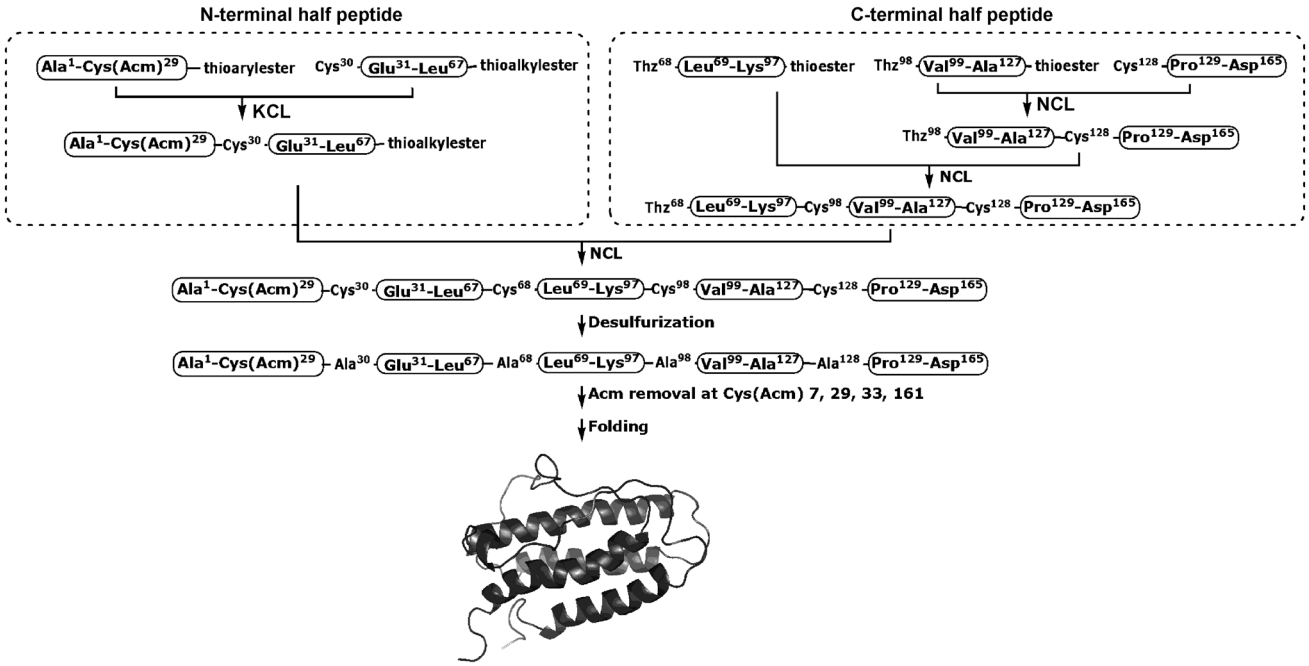

Scheme 1. Convergent strategy for the total chemical synthesis of [Lys[24,38,83]] human EPO. NCL = native chemical ligation, KCL = kinetically controlled ligation, Acm = acetamidomethyl, Thz = (*R*)-1,3-Thiazolidine-4-carboxylic acid.

ligation strategy[19] makes it possible to efficiently assemble multiple peptide segments in a convergent fashion. Kinetically controlled ligation is based on the reaction of a peptide1-α-thioarylester with a second Cys-peptide2-α-thioalkylester to give a unique peptide1-peptide2-α-thioalkylester product. Both native chemical ligation and kinetically controlled ligation use Xaa-Cys ligation sites (Xaa = any amino acid). One challenge of EPO chemical synthesis is the limited number and uneven distribution of the natural cysteine residues (at amino acid positions 7, 29, 33, and 161) in the target polypeptide chain. To extend the application of native chemical ligation in the synthesis of cysteine-free proteins, Dawson and co-workers introduced the combination of native chemical ligation with subsequent desulfurization of cysteine residues to alanine residues.[20] Recently, two different protocols for selective desulfurization of cysteine residues in the presence of acetamidomethyl (Acm)-protected cysteine and methionine residues have been reported: one uses Raney nickel as catalyst;[21] the other is a phosphine-induced radical reduction.[22] Thus, Xaa-Ala sequences can be used as potential ligation sites in EPO synthesis, in the presence of suitably protected native Cys residues.

Based on the distribution of potential Xaa-Ala ligation sites in the EPO polypeptide chain, five synthetic peptide segments, Ala^{1}–Cys^{29}(29 aa), Ala^{30}–Leu^{67}(38 aa), Ala^{68}–Lys^{97}(30 aa), Ala^{98}–Ala^{127}(30 aa), Ala^{128}–Asp^{165}(38 aa), of comparable lengths were selected as building blocks for assembling the full-length EPO polypeptide chain. Full details of the preparation of these synthetic peptide segments are given in the Supporting Information. The 165 amino acid polypeptide chain can be furnished by a final native chemical ligation condensation of two large peptide segments (Scheme 1): Ala^{1}–Leu^{67}-α-thioalkylester and Cys^{68}–Asp^{165}. The N-terminal segment Ala^{1}–Leu^{67}-α-thioalkylester can be prepared by kinetically controlled ligation of peptide segments Ala^{1}–Cys^{29}-α-thioarylester and Cys^{30}–Leu^{67}-α-thioalkylester. The C-terminal segment Cys^{68}–Asp^{165} can be prepared by native chemical ligation of Thz^{98}–Ala^{127}-α-thioester and Cys^{128}–Asp^{165} followed by conversion of Thz to Cys and a subsequent native chemical ligation with peptide segment Thz^{68}–Lys^{97}-α-thioester.

Thz was employed as a protected form of N-terminal L-cysteine, to avoid cyclization or oligomerization of Cys-peptide-α-thioester segments during native chemical ligation.[23] The side-chain thiol groups of the four native cysteine residues (at amino acid positions 7, 29, 33, and 161) were protected with the Acm group during the solid-phase synthesis of peptide segments Ala^{1}–Cys^{29}, Cys^{30}–Leu^{67}, and Cys^{128}–Asp^{165}. Four cysteine residues (at amino acid positions 30, 68, 98, and 128) were introduced as ligation sites and can subsequently be reduced to alanine residues using the selective desulfurization protocol[21,22] after the full-length polypeptide chain is assembled. After removal of the Acm groups from native Cys^{7,29,33,161} residues, the 165-residue polypeptide chain is folded with concomitant formation of two disulfide bonds to give synthetic [Lys^{24,38,83}]EPO.

Kinetically controlled ligation of peptide segments Ala^{1}–Cys(Acm)^{29}-$COSC_6H_4CH_2COOH$ and Cys^{30}–Leu^{67}-α-thioalkylester was performed at pH 6.8 without the use of added thiol catalyst (Figure 1). The starting peptide segments were essentially consumed after one hour. In addition to the formation of ligation product Ala^{1}–Cys^{30}–Leu^{67}-α-thioalkylester, a significant amount of branched thioester byproduct Ala^{1}–Cys^{30}(Ala^{1}–Cys(Acm)^{29})–Leu^{67}-α-thioalkylester (Fig-

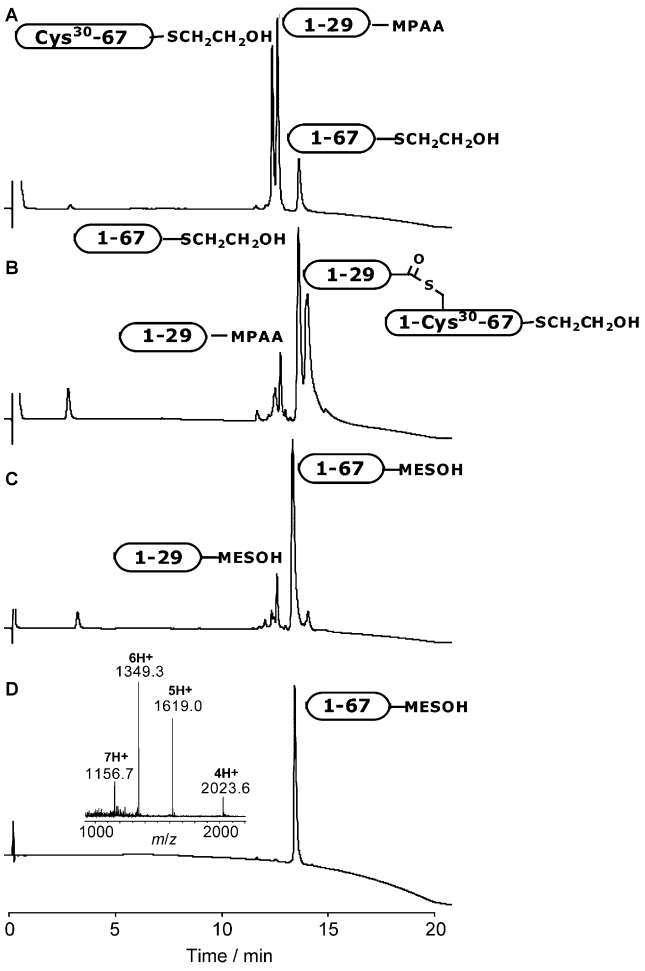

Figure 1. Kinetically controlled ligation of peptide segments Ala[1]–Cys-(Acm)[29]-α-$COSC_6H_4CH_2COOH$ and Cys[30]–Leu[67]-α-$COSCH_2CH_2OH$. A) $t<1$ min. B) $t=1$ h. C) After adding MESNa for two hours. D) After HPLC purification. ESI-MS (inset) gave an observed mass of (8090.0 ± 0.2) Da (calcd 8089.1 Da, average isotopes). Analytical HPLC traces (detected at $\lambda=214$ nm) are shown. MESOH = mercaptoethanesulfonic acid.

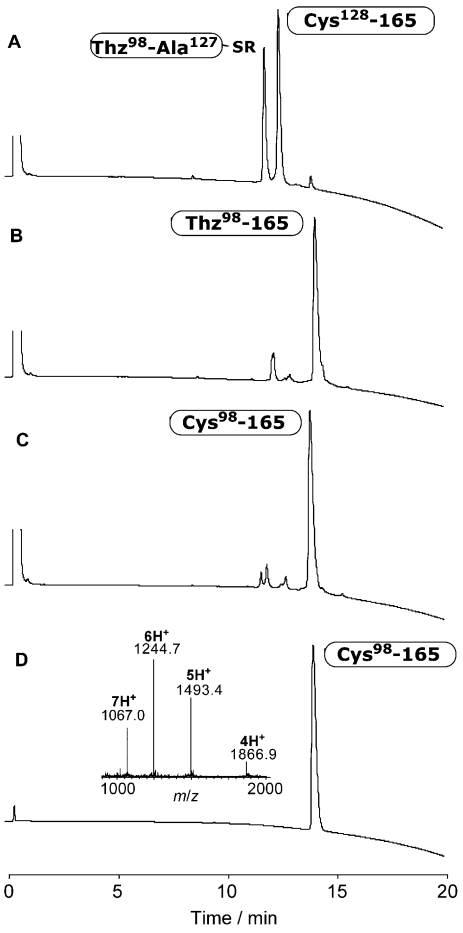

Figure 2. Native chemical ligation of peptide segments Thz[98]–Ala[127]-α-$COSCH_2CH_2COLeu$ and Cys[128]–Asp[165]. A) $t<1$ min. B) $t=5$ h. C) After adding $MeONH_2{\cdot}HCl$ for 14 h at pH 4.0, to convert Thz-peptide to Cys-peptide. D) After HPLC purification. ESI-MS (inset) gave an observed mass of (7462.5 ± 0.7) Da (calcd 7462.7 Da, average isotopes).

ure 1B) was generated. Addition of an excess of sodium 2-mercaptoethanesulfonate (MESNa) regenerated the desired Ala[1]–Leu[67]–α-thioalkylester (Figure 1C), which was purified by reversed-phase HPLC (Figure 1D).

Native chemical ligation of peptide segments Thz[98]–Ala[127]-α-thioalkylester and Cys[128]–Asp[165] was performed at pH 6.8 with the use of the aryl thiol $HSC_6H_4CH_2COOH$ (MPAA) as catalyst (Figure 2). Reaction was essentially complete after five hours and gave the desired ligation product Thz[98]–Asp[165], which was converted to Cys[98]–Asp[165] by overnight reaction with methoxyamine hydrochloride at pH 4.0 (Figure 2C).

The dinitrophenyl (Dnp) group was removed from residue His[94] by treating peptide segment Thz[68]–His(Dnp)[94]–Lys[97]-α-thioalkylester with an excess of MPAA at pH 6.8, prior to the native chemical ligation of peptide segments Thz[68]–Lys[97]-α-thioester and Cys[98]–Asp[165]. The aryl thiol reagent MPAA not only cleaved Dnp from histidine residues by thiolysis, it also generated a more reactive α-thioarylester Thz[68]–His[94]–Lys[97]-α-$COSC_6H_4CH_2COOH$ through transthioesterification (see the Supporting Information).[24] Native chemical ligation of segments Thz[68]–Lys[97]-α-$COSC_6H_4CH_2COOH$ and Cys[98]–Asp[165] was performed at pH 6.8 for eight hours (Figure 3A,B). The reaction mixture was then treated with methoxyamine hydrochloride at pH 4.0 (Figure 3C) to furnish the desired C-terminal half peptide Cys[68]–Asp[165] (Figure 3D) to be used in the final ligation to form the full-length EPO polypeptide chain.

The final ligation of peptide Ala[1]–Leu[67]- α-$COSCH_2CH_2SO_3^-$ and peptide Cys[68]–Asp[165] was performed at pH 6.8 with the use of MPAA as catalyst. This ligation, at a less reactive Leu-Cys site,[25] took about 15 h to complete (Figure 4B). An unusually large amount of hydrolyzed Ala[1]–Leu[67]-α-thioester byproduct, Ala[1]–Leu[67]-OH (Figure 4B), was formed during the reaction. The origin of this side reaction is uncertain; adjusting the pH value of the reaction with more diluted NaOH solution did not reduce the amount of hydrolyzed byproduct. After ligation was complete, the three N^{In}-formyl protecting groups were removed from

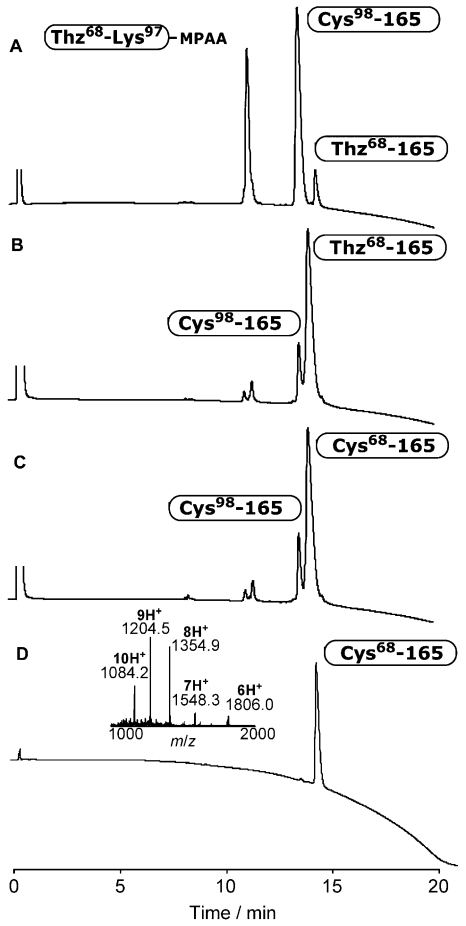

Figure 3. Native chemical ligation of peptide segments Thz[68]–Lys[97]-α-$COSC_6H_4CH_2COOH$ and Cys[98]–Asp[165]. A) $t<1$ min. B) Ligation after four hours, and subsequent treatment with MPAA for four hours. C) After adding $MeONH_2$·HCl for 15 h to convert Thz- to Cys-peptide. D) After HPLC purification. ESI-MS (inset) gave an observed mass of (10831.2 ± 0.7) Da (calcd 10831.6 Da, average isotopes).

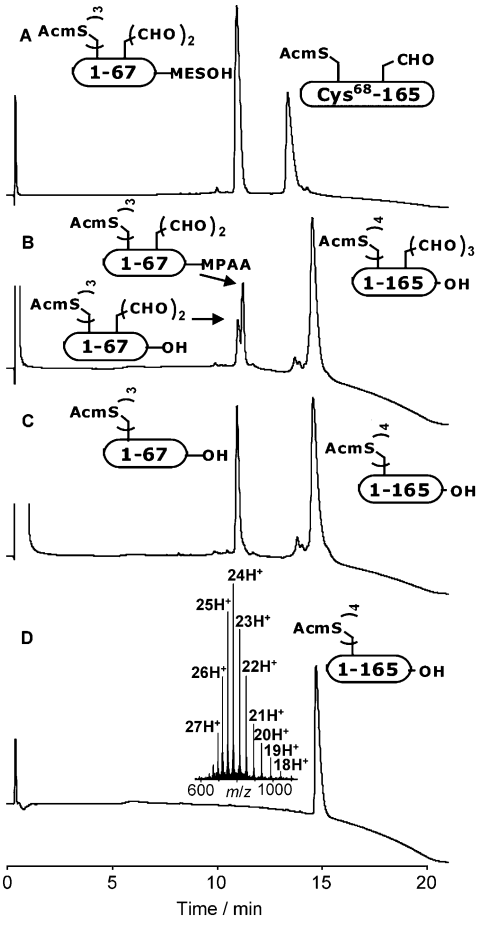

Figure 4. Native chemical ligation of peptide segments Ala[1]–Leu[67]-α-$COSCH_2CH_2SO_3^-$ and Cys[68]–Asp[165]. A) $t<1$ min. B) $t=15$ h. C) After adding 2-mercaptoethanol and piperidine for 0.5 h to remove the N^{In}-formyl protecting groups from Trp residues. D) After HPLC purification. ESI-MS (inset) gave an observed mass of (18696.5 ± 2.2) Da (calcd 18694.7 Da, average isotopes).

tryptophan (Trp) residues at amino acid positions 51, 64, and 88 by adding 2-mercaptoethanol and piperidine to the reaction mixture to final concentrations of 37 and 25% (v/v), respectively.[19c] Formyl groups were quantitatively cleaved from the full-length EPO polypeptide after 0.5 h (Figure 4C).

The full-length synthetic EPO polypeptide chain was subject to selective desulfurization to convert $Cys^{30,68,98,128}$ to $Ala^{30,68,98,128}$, while keeping four Acm-protected cysteine residues (at amino acid positions 7, 29, 33, 161) intact. Initial trials of selective desulfurization were performed using Raney nickel catalyst as described in published procedures.[21,26] Although success has been shown in the selective desulfurization of the hormone amylin and a small model protein EETI-II in the presence of Acm-protected cysteine residues,[21] we found that under these conditions the differentiation in desulfurization rates between free cysteine residues and Acm-protected cysteine residues in the full-length EPO polypeptide was not sufficient for selective desulfurization. A recently reported free-radical-based desulfurization protocol[22] has been proven to be a mild and highly selective method. In this protocol, a water-soluble radical initiator 2-2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044), and tris(2-carboxyethyl)phosphine (TCEP) were used for reducing cysteine residues. The full-length EPO polypeptide Ala^1–$Cys(Acm)^{7,29,33,161}$-$Cys^{30,68,98,128}$–Asp^{165} was exposed to a mixture of TCEP, EtSH, *t*BuSH, and VA-044 in guanidine hydrochloride and phosphate buffer. EtSH and *t*BuSH are used as additional thiol radical sources. A guanidine buffer was used to improve the solubility of full-length EPO peptide. The desulfurization was performed at 37 °C and monitored by LC–MS. After five hours, ESI-MS analysis showed near-quantitative conversion of free cysteine residues to alanine residues, although no obvious change in retention time was observed between starting peptide and desulfurized product. The analytical HPLC trace and ESI-MS spectrum of the purified desulfurization product Ala^1–$Cys(Acm)^{7,29,33,161}$–Asp^{165} are shown in Figure 5A (mass: obsd (18566.2 ± 0.7) Da; calcd 18566.4 Da, average isotopes).

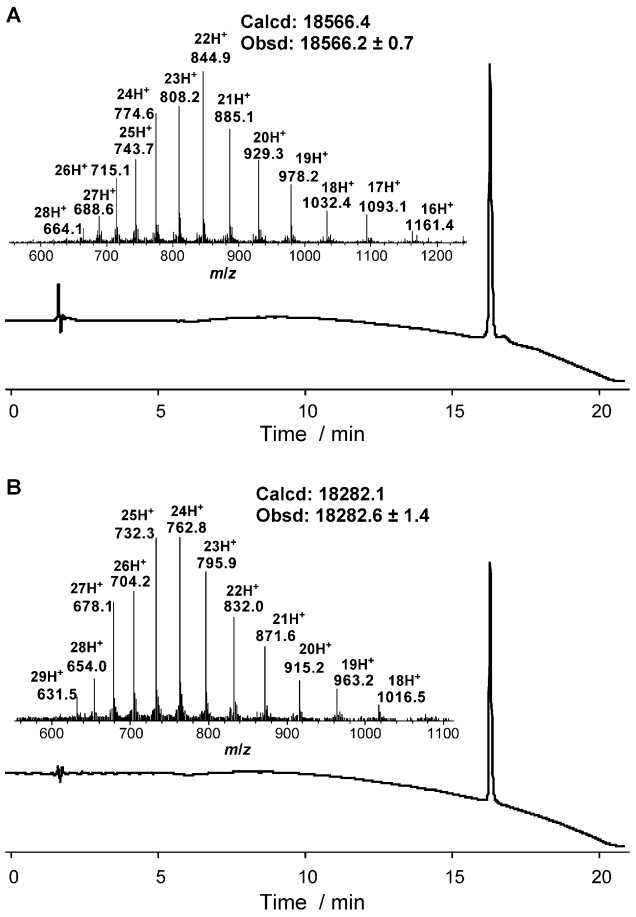

Figure 5. A) Purified selective desulfurization product Ala[1]–Cys-(Acm)[7,29,33,161]-Ala[30,68,98,128]–Asp[165] and B) full-length polypeptide product Ala[1]–Cys[7,29,33,161]-Ala[30,68,98,128]–Asp[165] after Acm removal. Analytical HPLC traces ($\lambda = 214$ nm) and ESI-MS spectra are shown.

The four Acm protecting groups were removed by treating EPO polypeptide with AgOAc in 1:1 AcOH/H_2O. Reaction progress was monitored by LC–MS. Removal of the Acm groups from the EPO polypeptide chain gave no change in retention time on HPLC analysis, but on-line LC–MS showed that reaction was completed after six hours. Then, a solution of 1,4-dithiothreitol (0.2 M, DTT) in guanidine hydrochloride (6 M) was added to quench excess AgOAc and to convert peptide with silver thiolates to peptide with free thiols. The analytical HPLC trace and ESI-MS spectrum of purified Acm-removal product Ala[1]–Cys[7,29,33,161]-Ala[30,68,98,128]–Asp[165] are shown in Figure 5B (mass: obsd (18282.6 ± 1.4) Da; calcd 18282.1 Da, average isotopes).

Folding of the synthetic 165-residue EPO polypeptide chain was performed according to the published procedure.[12] The reduced form of the polypeptide was first dissolved in guanidine hydrochloride solution (6 M). Then, the polypeptide solution underwent a three-step dialysis process sequentially against guanidine hydrochloride (3 M) with L-cysteine (4 mM) and L-cystine (0.5 mM) (pH 8.5), guanidine hydrochloride (1 M, pH 8.0), and finally tris(hydroxymethyl)aminomethane hydrochloride (Tris, 10 mM, pH 7.0) solutions, to give folded protein containing two disulfide bonds. The synthetic protein was purified by HPLC. The analytical HPLC chromatogram of the synthetic [Lys[24,38,83]]EPO is shown in Figure 6A; the ESI-MS spectrum was obtained by direct infusion,[27] giving a mass of (18277.9 ± 0.5) Da (Figure 6B), which is in excellent agreement with the calculated mass of 18278.1 Da. (average isotopes). The loss of four Daltons ((4.7 ± 1) Da) in mass was consistent with the formation of two intramolecular disulfide bonds after folding. The circular dichroism (CD) spectrum of synthetic [Lys[24,38,83]]EPO was measured in phosphate buffer (10 mM, pH 6.2) at a concentration of 5.9 μM. The spectrum is in good agreement with the reported highly helical secondary structure of this protein molecule (Figure 6C). The CD spectrum, with the characteristic shape of a minimum around 208 and a shoulder around 222 nm, is essentially identical to that reported for recombinant [Lys[24,38,83]]EPO[17] (see the Supporting Information for comparison of CD spectra) and is closely similar to the CD spectra reported for recombinant EPO[28] and polymer-modified EPO analogues.[12a]

The in vitro biological activity of synthetic [Lys[24,38,83]]EPO was evaluated in a cell proliferation assay.[29] TF-1 cells (human erythroblast) were incubated with different concentrations of synthetic [Lys[24,38,83]]EPO and native (recombinant) EPO for 72 h before the uptake of [^{3}H]thymidine was quantified.[29,30] Lysine-mutated human EPO had an EC_{50} value of 0.02 μg mL^{-1} (Figure 6D), which indicates that it is fully active for EPO receptor binding.

In summary, we have developed and prototyped a fully convergent chemical synthesis of a nonglycosylated human EPO. A key feature of the successful synthesis was native chemical ligation at Xaa-Ala sites enabled by the use of metal-free desulfurization (dethiylation).[22] The combination of characterizations using HPLC, mass spectrometry, CD spectroscopy, and cell proliferation assay showed that the synthetic [Lys[24,38,83]] human EPO has a well-defined covalent structure, was folded correctly, and was biologically active. This convergent chemical synthesis of EPO complements other synthetic and semi-synthetic approaches to the EPO glycoprotein and will provide a flexible approach to the preparation of EPO analogues with any desired modification on the polypeptide backbone. Our convergent synthetic route also provides a versatile platform for the preparation of neoglycan analogues of EPO to explore the effects of carbohydrate structure on biological function.

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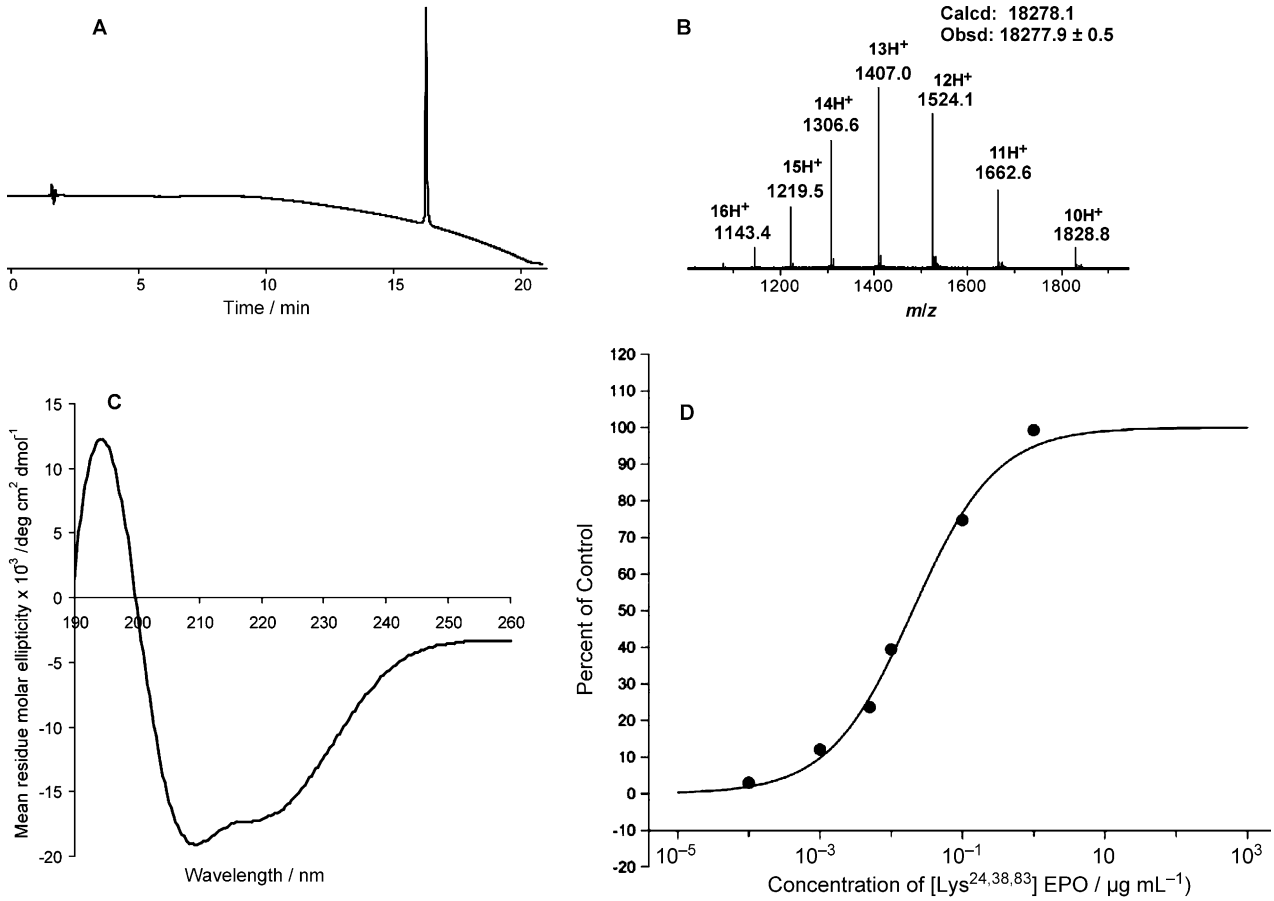

Figure 6. Characterization of folded, lysine-mutated human EPO (synthetic [$Lys^{24,38,83}$]EPO). A) Analytical HPLC trace ($\lambda = 214$ nm). The chromatographic separation was performed on an analytical C3 column (Agilent ZORBAX 300SB, 4.6×150 mm) using a linear gradient of 5–65% buffer B in buffer A over 15 min (buffer A = 0.1% trifluoroacetic acid (TFA) in H_2O; buffer B = 0.08% TFA in acetonitrile). B) ESI-MS spectrum of [$Lys^{24,38,83}$]EPO obtained by direct infusion. C) CD spectrum of lysine-mutated human EPO (5.9 µM) in phosphate buffer (10 mM, pH 6.2). D) Cell proliferation assay.[29] The growth responses of TF-1 cells (Human erythroblast) to the addition of synthetic [$Lys^{24,38,83}$]EPO and recombinant EPO (data not shown) were measured after 72 h incubation. Synthetic [$Lys^{24,38,83}$]EPO had a half maximal effective concentration (EC_{50}) value of 0.02 µg mL^{-1}.

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