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Strategies for the highly efficient synthesis of erythropoietin N-glycopeptide hydrazides

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A convergent synthesis for erythropoietin (EPO) 1-28 N-glycopeptide hydrazides was developed. In this approach, EPO 1-28 peptides were synthesized on the solid phase and converted to C-terminal hydrazides after cleavage from the resin. After selective deprotection of the Asp24 side chain, the desired glycosylamine was coupled by pseudoproline-assisted Lansbury aspartylation. Although the initial yields of the EPO 1-28 glycopeptides were satisfactory, they could be markedly improved by increasing the purity of the peptide using a reversed-phase high-performance liquid chromatography (RP-HPLC) purification of the protected peptide.

KEYWORDS
glycopeptide, HPLC, purification, solid-phase peptide synthesis

1 | INTRODUCTION

Erythropoietin (EPO) is a cytokine needed for the homeostasis of erythrocytes. Therapeutic EPO is expressed recombinantly in Chinese hamster ovary (CHO) cells and used mainly to treat anemic patients suffering from renal failure or cancer.[1] Owing to its high therapeutic relevance, human EPO is one of the best studied glycoproteins. The biological activity of EPO is modified by the sugar part[2]; however, the inherent microheterogeneity of glycoproteins at each glycosylation site precludes the availability of pure glycoforms for detailed structure–activity studies. The presence of three N-glycans is crucial for the biological activity of recombinant EPO, because non-glycosylated EPO expressed in *Escherichia coli* is suffering from low stability and a short serum half-life.[3–6] Thus, a number of solubility-enhancing mutations need to be introduced when expressing non-glycosylated EPO.[7] Additionally, the presence of a single glycan can significantly stabilize EPO against aggregation.[8]

Single pure glycoforms of glycoproteins are currently available only by synthetic methodology.[9] Despite its complexity (three N-glycans and one O-glycan), a number of EPO glycoforms and variants were successfully synthesized, providing the protein with natural or surrogate linkages for the glycans.[8,10–19] Synthetic approaches for EPO based on native chemical ligation require the synthesis of several glycopeptide building blocks with a length of up to 40 amino acids.

The convergent synthesis of N-glycopeptides following the aspartylation method developed by Lansbury[20] involves the coupling of a glycosylamine to an aspartyl side chain of a protected peptide. Activation of the aspartyl side chain carboxylate by coupling reagents can lead to an extensive formation of aspartimide and other byproducts. These side reactions can be significantly reduced by using a pseudoproline-assisted Lansbury aspartylation either in solution[21] or on the solid phase.[22] This methodology takes advantage of the Asn-X-Ser/Thr sequon for N-glycosylation. It was found that within this sequon, the incorporation of an n+2 Ser/Thr pseudoproline efficiently reduces the formation of aspartimide both during the synthesis of the

Abbreviations: Boc, tert-butyloxycarbonyl; Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; DCM, dichloromethane; DIC, N,N′-diisopropylcarbodiimide; DIPEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; Fmoc, fluorenylmethyloxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; HFIP, hexafluoroisopropanol; HOAt, 1-hydroxy-7-azabenzotriazole; MS, mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; *t*-Bu, tert-butyl; TFA, trifluoroacetic acid.

peptide and in the subsequent coupling to the glycan.[22] Even though pseudoprolines are widely used in peptide synthesis, a mechanistic explanation of how pseudoprolines efficiently reduce the formation of aspartimides remains to be established.

A frequently used ligation site for EPO is Cys29 whereby EPO 1-28 glycopeptide thioesters[23] are coupled with synthetic or recombinant[24] EPO 29-166 fragments. We are attempting the synthesis of small libraries of EPO with a defined N-glycosylation pattern. Towards this endeavor, EPO 1-28 glycopeptides were found to be versatile building blocks serving for the synthesis of EPO variants with one N-glycan (N24, see Figure 1). Herein, we present a systematic investigation of the large-scale convergent synthesis of EPO 1-28 glycopeptide hydrazides[25] suitable for native chemical ligation.

We found that the efficient synthesis of EPO 1-28 glycopeptides requires a high purity of both the glycosylamine and the selectively deprotected peptide. A key feature of this approach was the establishment of conditions allowing the prepurification of the protected EPO 1-28 hydrazide building blocks using nonaqueous reversed-phase high-performance liquid chromatography (RP-HPLC).[26]

2 | RESULTS AND DISCUSSION

As shown before,[22] EPO 1-28 glycopeptides can be synthesized on the solid phase in a straightforward manner. The aspartate of the N-glycosylation site 24 was protected with an allyl ester.[22] After mild cleavage from the resin, the 1-28 glycopeptides were converted to a thioester.[22,27] The glycopeptide thioester containing GlcNAc gave an overall yield of 37%. In contrast, the yields of the corresponding glycopeptide thioester containing a biantennary N-glycan nonasaccharide reached only 24% owing to retention of the glycopeptide on the solid phase.[22] This led to a redesigned strategy where the peptide carrying a C-terminal hydrazide serving as a latent thioester is synthesized first, and after Asp side chain deprotection, the desired glycan can be coupled in solution.[28] To avoid residual palladium species in the peptide after Pd-catalyzed deallylation[22,29] in solution Asp24 was protected with a phenylisopropyl ester.[30] Cysteine7 was protected as a mixed disulfide, reducing the risk of oxidation after global deprotection of the peptide.

The EPO 1-28 peptide was assembled on Fmoc-Gly-trityl-ChemMatrix resin (**1**)[31] incorporating two pseudoproline dipeptides (see Figure 2). After mild cleavage from the resin (**2**), the protected peptidyl acid **3** was converted to the Boc-hydrazide **4** using DIC/Cl-HOBt and *tert*-butyl carbazate in analogy to the in situ thioesterification method developed by Flemer.[27] Peptide **4** was purified by flash chromatography. A brief treatment of **4** with 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) cleaved the phenylisopropyl ester of Asp24 selectively, and the aspartyl peptide **5** was precipitated with diethylether. Gratifyingly, the course of the deprotection could be followed by RP-LC–MS of the protected peptides **4** and **5**, revealing some loss of the acetonides (M-40) of the two pseudoprolines. The pseudoproline-assisted Lansbury aspartylation

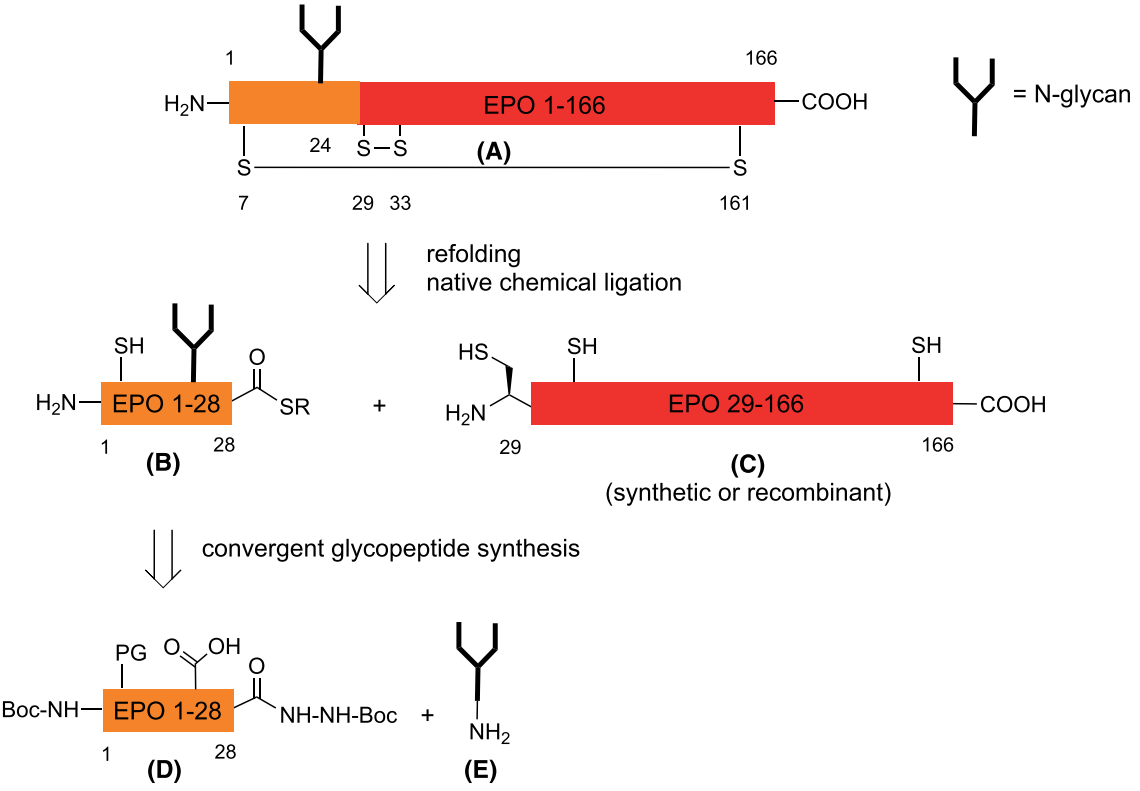

FIGURE 1 Retrosynthesis of erythropoietin (EPO) glycoforms **A** with a single N-glycan at Asn24. The building blocks **B** and **C** can be joined by a native chemical ligation at Cys29. The glycopeptide thioester **B** is accessible via a convergent glycopeptide synthesis from the glycosylamine **E** and the hydrazide **D** with a free Asp side chain at the glycosylation site 24

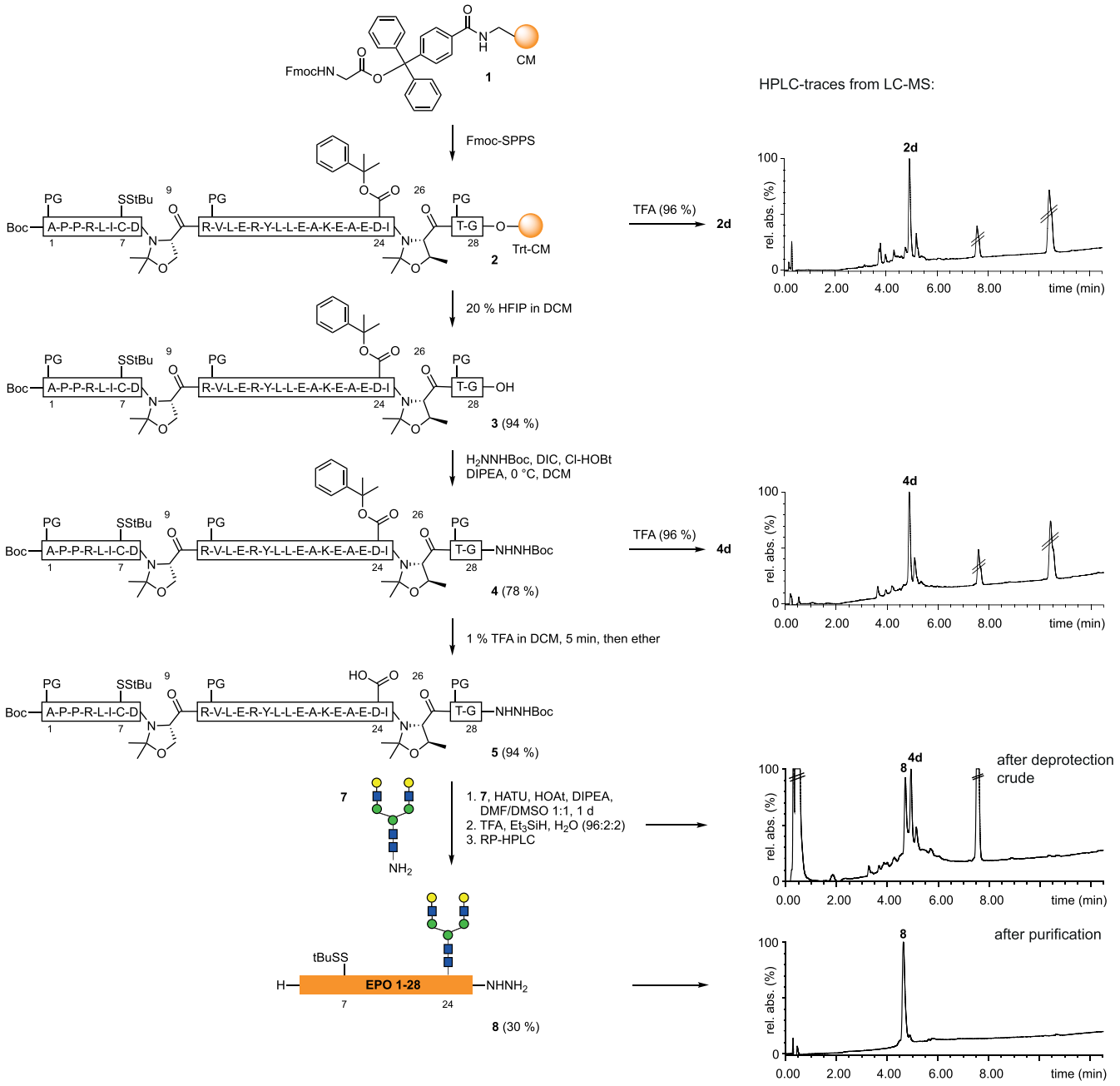

FIGURE 2 Synthesis of erythropoietin (EPO) 1-28 glycopeptide hydrazide **8**. The 1-28 peptide was assembled by Fmoc-solid-phase synthesis on a trityl-ChemMatrix resin, cleaved, and converted to the hydrazide **4**. Selective cleavage of the phenylisopropyl ester rendered the aspartyl peptide **5**, which was coupled with the complex-type glycosyl amine **7**. After global deprotection, the glycopeptide hydrazide **8** was obtained. On the right side, the high-performance liquid chromatography (HPLC) traces from the HPLC–MS analysis of the peptides are shown

was carried out by activating peptide **5** (1.5 eq.) with HATU/HOAt and subsequent addition of freshly prepared biantennary glycosylamine **7** (1.0 eq.).[22] The amine **7** was obtained by reduction of the corresponding glycosyl azide using propane dithiol[32] in the presence of *N*,*N*-diisopropylethylamine (DIPEA), which promotes the reduction and prevents anomerization of the glycosyl amine.[33] The glycopeptide hydrazide was deprotected after 16 h and purified by HPLC, giving **8** in a yield of 30%. The yield for the coupling in solution barely exceeded the overall yield obtained after the aspartylation of EPO 1-28 on the solid phase (24% for the biantennary N-glycan nonasaccharide *vide supra*). We suspected that impurities were responsible for the moderate yield of glycopeptide **8**. Because glycosylamine **7** was prepared from the corresponding glycosyl azide **6**, which was purified by HPLC,[28] we focused on increasing the purity of the selectively deprotected peptide **5**.

However, the solubility of the selectively deprotected aspartyl peptide **5** in MeOH was low, whereas the fully protected precursor **4** showed satisfactory solubility. In CH_3CN, both peptides **4** and **5** were poorly soluble. Thus, preparative purification conditions for **4** were initially investigated on a C8 column using MeOH/water as

an eluent. With the use of an isocratic elution (95% MeOH/water), peptide **4** could be separated from a number of overlapping peaks with shorter retention time.

Owing to the broad shape of most of the peaks and low reproducibility, a nonaqueous[26] mixture of organic solvents (70% CH_3CN/MeOH isocratic) was tested for the purification, which separated **4** reliably and additionally provided 11 minor fractions (Figure 3C).

Because the masses of the protected peptides in the minor fractions could not be determined by LC–MS directly, the fractions were separately deprotected and then analyzed. HPLC–MS revealed that all the peaks eluting prior to peptide **4** corresponded to truncated peptides. Only the two fractions with longer retention times gave a higher mass than the target peptide **4d** (Data S10b). The peptide **4** purified by HPLC resulted in a significantly increased purity of **4d** where only traces of the previously observed side products were visible (Figure 3E). Despite a good separation of the side products on a 10-mg scale using a 2 × 25-cm C8 column, the purification of amounts over 15 mg decreased the resolution significantly. Thus, another stationary phase was tested. Gratifyingly, a polystyrene-based HPLC column using a nonaqueous gradient system (0–40% CH_3CN/MeOH) also provided **4** in similar purity as before (Figure 3E). In a single run, over 30 mg of **4** could be purified over a 2.5 × 30 cm Nucleogel column, removing many side products and contaminants mainly with shorter retention times.

Subsequently, the phenylisopropyl ester of peptide **4** purified by HPLC was selectively removed by 1% TFA/DCM, and the resulting peptide **5** was aspartylated using glycosyl amine **7**. To our delight, the final yield of glycopeptide **8** was raised to 60%, thus virtually doubling the yield in this step. It can be assumed that the numerous side products previously present in crude **5** (no HPLC) were also converted to glycopeptides and other products, which needed to be removed in the final HPLC step, thus lowering the coupling yield considerably.

We next attempted to also increase the purity of **5** by HPLC because the selective deprotection of **4** under acidic conditions might give rise to unwanted side products. The cleavage of the Asp24 PhiPr group of **4** under different conditions was monitored by HPLC–MS. The use of 1% TFA/DCM led to rapid deprotection and also to the appearance of additional products after prolonged incubation (more than 5 min). This deprotection was dependent on

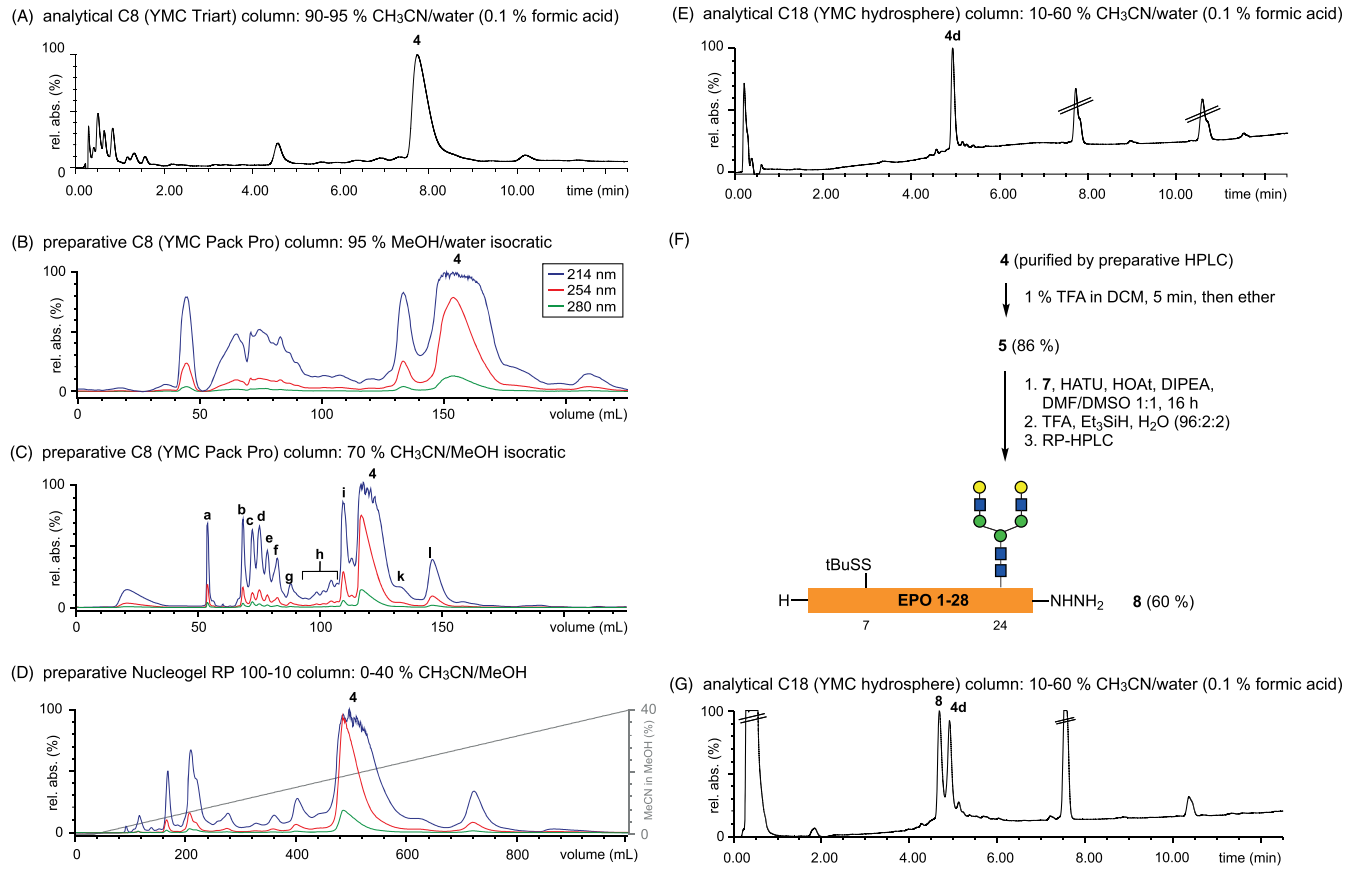

FIGURE 3 High-performance liquid chromatography (HPLC) conditions for the purification of protected hydrazide **4**: A, standard LC–MS conditions (C8, CH_3CN/H_2O gradient); B, preparative HPLC (C8, MeOH/H_2O isocratic); C, preparative HPLC (C8, CH_3CN/MeOH isocratic); D, preparative HPLC (polystyrene, CH_3CN/MeOH gradient); E, UPLC–MS of peptide **4** (purified by HPLC) after deprotection; F, synthesis of glycopeptide hydrazide **8** starting from **4** purified by HPLC; the yield is calculated based on the glycosylamine **7** (1 eq.), because peptide **5** was used in excess (1.5 eq.) G, LC–MS of crude **8** after the pseudoproline-assisted Lansbury aspartylation starting from **4** purified by HPLC; the excess of peptide **5** (1.5 eq.) increases the peak of deprotected peptide **4d** in the chromatogram

the concentration of peptide **4** with significantly less side products in a 3 mM solution of **4** compared with 1 mM of **4**. In contrast, we found a clean and concentration-independent deprotection of **4** using neat hexafluoroisopropanol (HFIP) over 6 h without significant amounts of side products even after 48 h of incubation.[34] The high selectivity during the deprotection can be attributed to the low acidity of HFIP (pKa 9.3),[35] providing milder reaction conditions than 1% TFA/DCM.

For the purification of **5** by nonaqueous HPLC, the poor solubility of **5** in neat MeOH was improved by dissolving **5** in a larger volume of MeOH containing DIPEA. However, the use of DIPEA in the loading solution resulted in a strong tailing of the major peaks eluting from the polystyrene-based Nucleogel column (0–21.5% CH_3CN/MeOH). Finally, a concentrated solution of **5** in DMF was applied in the loading step resulting in symmetric and sharp peaks. The gradient profile of the chromatography was adjusted accordingly by adding a conditioning step with 0% acetonitrile over 20 min after the loading. This procedure allowed the purification of **5** in amounts of up to 50 mg per run. An analytical deprotection of **5** showed a further reduction of side products in LC–MS (Figure 4D).

Peptide **5** purified by HPLC was subsequently tested in a Lansbury aspartylation with the biantennary glycosylamine **7** (Figure 4). After coupling, deprotection and purification the isolated yield was further improved (71%), indicating that impurities contained in the selectively deprotected peptide **5** that are removable by HPLC are the cause of low yields in pseudoproline-assisted Lansbury aspartylations. Because the N-glycans of EPO are mainly of the tetraantennary complex type, a synthetic tetraantennary glycosylamine **10**[36] (1 eq.) was coupled to purified **5** (1.3 eq.). The reaction was carried out for 16 h

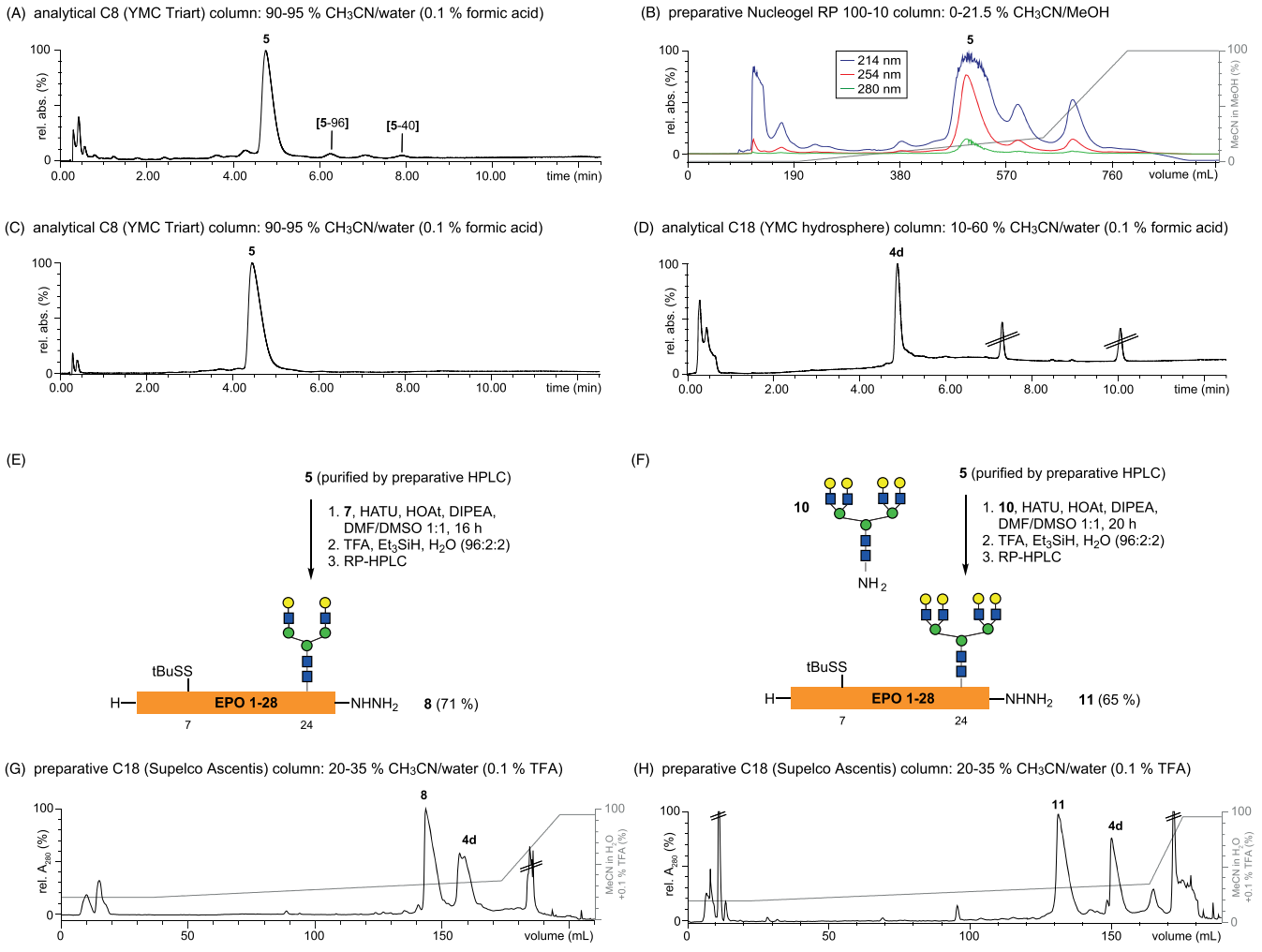

FIGURE 4 High-performance liquid chromatography (HPLC) conditions for the purification of protected hydrazide **5** and aspartylation of the purified peptide **5**: A, peptide **5** obtained by deprotection of **4** in neat hexafluoroisopropanol (HFIP) monitored by LC–MS (C8, CH_3CN/H_2O gradient); B, preparative HPLC of **5** (Nucleogel, CH_3CN/MeOH gradient); C, analysis of **5** (purified by HPLC) by LC–MS: (C8, CH_3CN/H_2O gradient); D, deprotection of **5** (purified by HPLC) gave **4d**, LC–MS: (C8, CH_3CN/H_2O gradient); E, synthesis of biantennary glycopeptide hydrazide **8** using peptide **5** purified by HPLC; F, synthesis of tetraantennary glycopeptide hydrazide **11** using peptide **5** purified by HPLC; G, preparative HPLC of crude glyopeptide hydrazide **8** after deprotection; H, preparative HPLC of crude glyopeptide hydrazide **11** after deprotection

and was subsequently deprotected with a TFA cocktail. After workup and purification, nearly 5 mg of the EPO 1-28 glycopeptide **11** containing a galactosylated tetraantennary N-glycan was isolated, corresponding to a yield of 65%.

3 | CONCLUSION

In summary, the yields of EPO 1-28 glycopeptide hydrazides by convergent synthesis were found to depend strongly on the purity of the selectively deprotected aspartyl peptide. Conditions were established to monitor the synthesis and also purify the peptide hydrazides by nonstandard conditions (nonaqueous reversed-phase chromatography). With the protected peptides purified by HPLC, the pseudoproline-assisted Lansbury aspartylations proceeded in high yields and thus permitted the rapid and efficient derivatization of a single peptide with the desired glycosyl amines. Because the solubility of protected peptides is affected by the sequence and the protecting groups, purification schemes need to be established for each peptide individually.

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REFERENCES

1. Jelkmann W. Physiology and pharmacology of erythropoietin. *Transfus Med Hemother*. 2013;40(5):302-309.
2. Takeuchi M, Inoue N, Strickland TW, et al. Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proc Natl Acad Sci U S A*. 1989;86(20):7819-7822.
3. Takeuchi M, Takasaki S, Shimada M, Kobata A. Role of sugar chains in the in vitro biological activity of human erythropoietin produced in recombinant Chinese hamster ovary cells. *J Biol Chem*. 1990;265(21):12127-12130.
4. Narhi LO, Arakawa T, Aoki KH, et al. The effect of carbohydrate on the structure and stability of erythropoietin. *J Biol Chem*. 1991;266(34):23022-23026.
5. Wasley LC, Timony G, Murtha P, et al. The importance of N- and O-linked oligosaccharides for the biosynthesis and in vitro and in vivo biologic activities of erythropoietin. *Blood*. 1991;77(12):2624-2632.
6. Delorme E, Lorenzini T, Giffin J, et al. Role of glycosylation on the secretion and biological activity of erythropoietin. *Biochemistry*. 1992;31(41):9871-9876.
7. Narhi LO, Arakawa T, Aoki K, et al. Asn to Lys mutations at three sites which are N-glycosylated in the mammalian protein decrease the aggregation of *Escherichia coli*-derived erythropoietin. *Protein Eng*. 2001;14(2):135-140.
8. Streichert K, Seitz C, Hoffmann E, et al. Synthesis of erythropoietins site-specifically conjugated with complex-type N-glycans. *Chembiochem*. 2019;20(15):1914-1918.
9. Unverzagt C, Kajihara Y. Chemical assembly of N-glycoproteins: a refined toolbox to address a ubiquitous posttranslational modification. *Chem Soc Rev*. 2013;42(10):4408-4420.
10. Kochendoerfer GG, Chen SY, Mao F, et al. Design and chemical synthesis of a homogeneous polymer-modified erythropoiesis protein. *Science*. 2003;299(5608):884-887.
11. Macmillan D, Bill RM, Sage KA, Fern D, Flitsch SL. Selective in vitro glycosylation of recombinant proteins: semi-synthesis of novel homogeneous glycoforms of human erythropoietin. *Chem Biol*. 2001;8(2):133-145.
12. Hirano K, Macmillan D, Tezuka K, Tsuji T, Kajihara Y. Design and synthesis of a homogeneous erythropoietin analogue with two human complex-type sialyloligosaccharides: combined use of chemical and bacterial protein expression methods. *Angew Chem Int Ed*. 2009;48(50):9557-9560.
13. Murakami M, Okamoto R, Izumi M, Kajihara Y. Chemical synthesis of an erythropoietin glycoform containing a complex-type disialyloligosaccharide. *Angew Chem Int Ed*. 2012;51(15):3567-3572.
14. Liu S, Pentelute BL, Kent SB. Convergent chemical synthesis of [lysine(24, 38, 83)] human erythropoietin. *Angew Chem Int Ed*. 2012;51(4):993-999.
15. Wang P, Dong S, Brailsford JA, et al. At last: erythropoietin as a single glycoform. *Angew Chem Int Ed*. 2012;51(46):11576-11584.
16. Adams AL, Cowper B, Morgan RE, Premdjee B, Caddick S, Macmillan D. Cysteine promoted C-terminal hydrazinolysis of native peptides and proteins. *Angew Chem Int Ed*. 2013;52(49):13062-13066.
17. Wang P, Dong S, Shieh JH, et al. Erythropoietin derived by chemical synthesis. *Science*. 2013;342(6164):1357-1360.
18. Murakami M, Kiuchi T, Nishihara M, et al. Chemical synthesis of erythropoietin glycoforms for insights into the relationship between glycosylation pattern and bioactivity. *Sci Adv*. 2016;2(1):e1500678.
19. Yang Q, An Y, Zhu S, et al. Glycan remodeling of human erythropoietin (EPO) through combined mammalian cell engineering and chemoenzymatic transglycosylation. *ACS Chem Biol*. 2017;12(6):1665-1673.
20. Cohen-Anisfeld ST, Lansbury PT. A practical, convergent method for glycopeptide synthesis. *J Am Chem Soc*. 1993;115(23):10531-10537.
21. Wang P, Aussedat B, Vohra Y, Danishefsky SJ. An advance in the chemical synthesis of homogeneous N-linked glycopolypeptides by convergent aspartylation. *Angew Chem Int Ed*. 2012;51(46):11571-11575.
22. Ullmann V, Rädisch M, Boos I, et al. Convergent solid-phase synthesis of N-glycopeptides facilitated by pseudoprolines at consensus-sequence Ser/Thr residues. *Angew Chem Int Ed*. 2012;51(46):11566-11570.
23. Kan C, Trzupek JD, Wu B, et al. Toward homogeneous erythropoietin: chemical synthesis of the Ala1-Gly28 glycopeptide domain by "alanine" ligation. *J Am Chem Soc*. 2009;131(15):5438-5443.
24. Macmillan D, Arham L. Cyanogen bromide cleavage generates fragments suitable for expressed protein and glycoprotein ligation. *J Am Chem Soc*. 2004;126(31):9530-9531.
25. Fang GM, Li YM, Shen F, et al. Protein chemical synthesis by ligation of peptide hydrazides. *Angew Chem Int Ed*. 2011;50(33):7645-7649.
26. Parris NA. Non-aqueous reversed-phase liquid chromatography. A neglected approach to the analysis of low polarity samples. *J Chromatogr*. 1978;157(1):161-170.
27. Flemer S Jr. Efficient method of circumventing insolubility problems with fully protected peptide carboxylates via in situ direct thioesterification reactions. *J Pept Sci*. 2009;15(11):693-696.
28. Graf CGF, Schulz C, Schmälzlein M, et al. Synthetic glycoforms reveal carbohydrate-dependent bioactivity of human saposin D. *Angew Chem Int Ed*. 2017;56(19):5252-5257.
29. Vetter D, Tumelty D, Singh SK, Gallop MA. A versatile solid-phase synthesis of N-linked glycopeptides. *Angew Chem Int Ed Engl*. 1995;34(1):60-63.
30. Fernandez-Tejada A, Vadola PA, Danishefsky SJ. Chemical synthesis of the beta-subunit of human luteinizing (hLH) and chorionic

gonadotropin (hCG) glycoprotein hormones. *J Am Chem Soc*. 2014; 136(23):8450-8458.
31. Heinlein C, Varon Silva D, Tröster A, Schmidt J, Gross A, Unverzagt C. Fragment condensation of C-terminal pseudoproline peptides without racemization on the solid phase. *Angew Chem Int Ed*. 2011;50(28): 6406-6410.
32. Bayley H, Standring DN, Knowles JR. Propane-1,3-dithiol: a selective reagent for the efficient reduction of alkyl and aryl azides to amines. *Tetrahedron Lett*. 1978;39:3633-3634.
33. Ogawa T, Nakabayashi S, Shibata S. Synthetic studies on cell surface glycans. Part XVIII. Synthetic studies on nephritogenic glycosides. Synthesis of *N*-(β-L-aspartyl)-α-D-glucopyranosylamine. *Agric Biol Chem*. 1983;47(2):281-285.
34. Moretto A, Crisma M, Formaggio F, et al. Slow *tert*-butyl ester acidolysis and peptide 3(10)-helix to alpha-helix transition in HFIP solution. *Biopolymers*. 2007;88(2):233-238.
35. Filler R, Schure RM. Highly acidic perhalogenated alcohols. A new synthesis of perfluoro-tert-butyl alcohol. *J Org Chem*. 1967;32(4): 1217-1219.
36. Canales A, Boos I, Perkams L, et al. Breaking the limits in analyzing carbohydrate recognition by NMR spectroscopy: resolving branch-selective interaction of a tetra-antennary N-glycan with lectins. *Angew Chem Int Ed*. 2017;56(47):14987-14991.

SUPPORTING INFORMATION

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