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Toward Homogeneous Erythropoietin: Chemical Synthesis of the Ala[1]−Gly[28] Glycopeptide Domain by "Alanine" Ligation

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Abstract: The Ala[1]−Gly[28] glycopeptide fragment (**28**) of EPO was prepared by chemical synthesis as a single glycoform. Key steps in the synthesis include attachment of a complex dodecasaccharide (**7**) to a seven amino acid peptide via Lansbury aspartylation, native chemical ligation to join peptide **19** with the glycopeptide domain **18**, and a selective desulfurization at the ligation site to reveal the natural Ala[19]. This glycopeptide fragment (**28**) contains both the requisite N-linked dodecasaccharide and a C-terminal $^{\alpha}$thioester handle, the latter feature permitting direct coupling with a glycopeptide fragment bearing N-terminal Cys[29] without further functionalization.

Introduction

As a part of our continuing effort to bring chemical synthesis to the realm of "biologics", we are pursuing the preparation of homogeneous glycoproteins.[1,2] In contrast to biochemical methods that do not allow for homogeneous expression of glycoproteins, de novo chemical synthesis offers precise structural control for the preparation of homogeneous products while potentially deconvoluting key structure−function relationships of such complex structures. Central to our efforts to apply chemical synthesis to the preparation of "biologics" is our proposed total synthesis of erythropoietin.

Erythropoietin (EPO, **1**, Figure 1) is a glycoprotein hormone used to treat anemia associated with renal failure and cancer chemotherapy.[3] Extensive efforts have been made to study and understand the structure and function of EPO, including the role of glycosylation. Specifically, Higuchi et al. demonstrated that erythropoietin's (rhuEPO) three N-linked glycosyl groups were not required for in vitro activity but were required for in vivo activity, while the single O-linked glycosyl group did not bear any biological role.[4] Furthermore, the in vivo activity of EPO was shown to be directly related to the sialic acid content.[5] Interestingly, Kent and co-workers have synthesized a polymer-modified EPO that demonstrated superior in vivo activity compared to the glycosylated rhuEPO.[6] Despite the impressive biological and chemical studies that have been made to understand the structure and function of EPO, efforts to determine the exact biological role of defined EPO glycoforms have been hindered by difficulties associated with isolating significant quantities of homogeneous glycoforms.[7] In fact, this has not been accomplished by any biologically enabled means. As discussed in the preceding papers,[1] a potentially powerful solution to this problem is chemical synthesis, which would potentially enable access to homogeneous EPO glycoforms and related analogues.

Chemical synthesis of glycoproteins or glycopeptides bearing complex carbohydrates has only recently been realized. This is in sharp contrast to the plethora of nonglycosylated proteins that have been prepared, aided by the power of native chemical ligation[8,9] in polypeptide synthesis. Using native chemical ligation (NCL), Kajihara and Dawson reported the first synthesis of a complex glycoprotein, i.e., a single glycoform of monocyte chemotactic protein-3 containing human complex sialyloligosac-

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[‡] Columbia University.

(1) See preceding two papers: (a) Tan, Z.; Shang, S.; Halkina, T.; Yuan, Y.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2009**, *131*, 5424–5431. (b) Yuan, Y.; Chen, J.; Wan, Q.; Tan, Z.; Chen, G.; Kan, C.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2009**, *131*, 5432–5437.
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(3) For general reviews on EPO, see: (a) Sytkowski, A. J. *Erythropoietin*; Wiley-VCH Verlag GmbH and Co. KGaA: Weinheim, 2004. (b) Jelkmann, W. *Intern. Med.* **2004**, *43*, 649–659.
(4) Higuchi, M.; Masayoshi, O.; Kuboniwa, H.; Tomonoh, K.; Shimonaka, Y.; Ochi, N. *J. Biol. Chem.* **1992**, *267*, 7703–7709.
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(6) Kochendoerfer, G. G.; et al. *Science* **2003**, *299*, 884–887.
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(8) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776–779.

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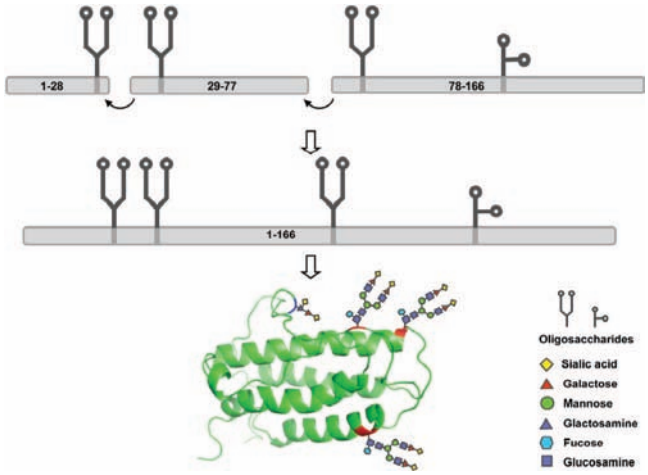


Figure 1. Retrosynthetic analysis and ribbon diagram of erythropoietin (EPO, **1**).

charide.[10] Erythropoietin glycopeptide fragments containing complex sialyloligosaccharides have been prepared by our group[1,2d] using either NCL or direct condensation methods.[2f,11] Recently, an asialo erythropoietin glycopeptide fragment was reported by Kajihara,[12] in which a serine ligation was effectively employed. Clearly, the chemical synthesis of complex glycoproteins and glycopeptides remains a significant and daunting challenge.

Our program directed to reaching EPO by total synthesis has already yielded valuable contributions to the rapidly growing field of (glyco)peptide ligation tools and methods. Several different C-terminal functional groups have been employed in our glycoprotein synthesis studies, including cyanophenolic esters[1b] and nitrophenolic esters.[2h] Our *o*-disulfide phenolic ester[2i] was developed to serve as a stable, latent thioester. These C-terminal groups have enabled orthogonal ligations such that *multiple* polypeptide and glycopeptide couplings can be accomplished from the N- to the C-terminus.[1b,2f] Our investigations into cysteine-free ligation methods have included the development of a thiol auxiliary that permits coupling of complex glycopeptide fragments,[2c,13] the discovery of a two-component isonitrile/carboxylic acid coupling method to construct amide bonds,[14] and alanine,[2g,15] valine,[16,17] and homocysteine[18,19] ligations, which utilize a mild and selective desulfurization method that is also compatible with complex carbohydrates.[20] The synthesis of homogeneous glycosylated EPO(1-28) (**2**, Figure 2), described below, serves to illustrate the value of "alanine" ligations.

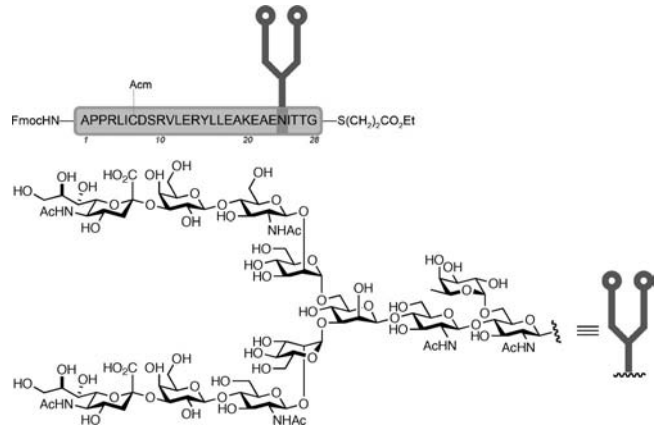


Figure 2. Structure of EPO(1-28) (**2**).

Results and Discussion

Our vision for the assembly of erythropoietin emphasizes maximum convergency. It projects the synthesis of three glycopeptide fragments that will subsequently be merged. Indeed the syntheses of EPO(78-166) and EPO(29-77) have been described earlier in this series.[1] In each case we were able to overcome potentially serious complications inherent in those domains. The EPO(1-28) segment is the shortest of the three peptide fragments, containing less than 20% of the EPO sequence. As the smallest fragment and (potentially) the last fragment in the synthesis of EPO (assuming a linear synthesis from the C- to the N-terminus), it may appear to be the simplest of the segments. However, the inherent challenge of the EPO(1-28) segment lies in the absence of any functional cysteine and glycine/proline residues upon which to base a retrosynthetic analysis. This peculiarity precluded the use of either NCL or direct condensation methods[21] for its assembly. This is critical because during our initial studies, and as reported in the

(9) For the first example of glycoprotein chemical synthesis using NCL, see: (a) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689. For recent examples of protein synthesis using NCL, see: (b) Torbeev, V. Y.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 1667–1670. (c) Durek, T.; Torbeev, V. Y.; Kent, S. B. H. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 4846–4851. For a review on NCL, see: (d) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923–960.

(10) Note that the sialyloligosaccharide was isolated from hen's egg yolk and does not contain any fucose unit. See: Yamamoto, N.; Tanabe, Y.; Okamoto, R.; Dawson, P. E.; Kajihara, Y. *J. Am. Chem. Soc.* **2008**, *130*, 501–510.

(11) For other direct condensation methods, see: (a) Blake, J. *Int. J. Pept. Protein Res.* **1981**, *17*, 273–274. (b) Aimoto, S.; Mizoguchi, N.; Hojo, H.; Yoshimura, S. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 524–531. (c) Aimoto, S. *Biopolymers* **1999**, *51*, 247–265. (d) Payne, R. J.; Ficht, S.; Greenberg, W. A.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2008**, *47*, 4411–4415.

(12) Okamoto, R.; Kajihara, Y. *Angew. Chem., Int. Ed.* **2008**, *47*, 5402–5406.

(13) For other examples of auxiliary-based cysteine-free ligations, see: (a) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891–5896. (b) Botti, P.; Carrasco, M. R.; Kent, S. B. H. *Tetrahedron Lett.* **2001**, *42*, 1831–1833. (c) Marinzi, C.; Bark, S. J.; Offer, J.; Dawson, P. E. *Bioorg. Med. Chem.* **2001**, *9*, 2323–2328. (d) Low, D. W.; Hill, M. G.; Carrasco, M. R.; Kent, S. B. H.; Botti, P. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6554–6559. (e) Offer, J.; Boddy, C. N. C.; Dawson, P. E. *J. Am. Chem. Soc.* **2002**, *124*, 4642–4646. (f) Macmillan, D.; Anderson, D. W. *Org. Lett.* **2004**, *6*, 4659–4662. (g) Payne, R. J.; Ficht, S.; Tang, S.; Brik, A.; Yang, Y.-Y.; Case, D. A.; Wong, C.-H. *J. Am. Chem. Soc.* **2007**, *129*, 13527–13536, and references therein.

(14) Li, X.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2008**, *130*, 5446–5448.

(15) For other studies on alanine ligations, see: (a) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526–533, and references therein. (b) Pentelute, B.; Kent, S. B. H. *Org. Lett.* **2007**, *9*, 687–690.

(16) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 8521–8524.

(17) For other studies on valine ligations, see: Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem., Int. Ed.* **2008**, *47*, 6807–6810.

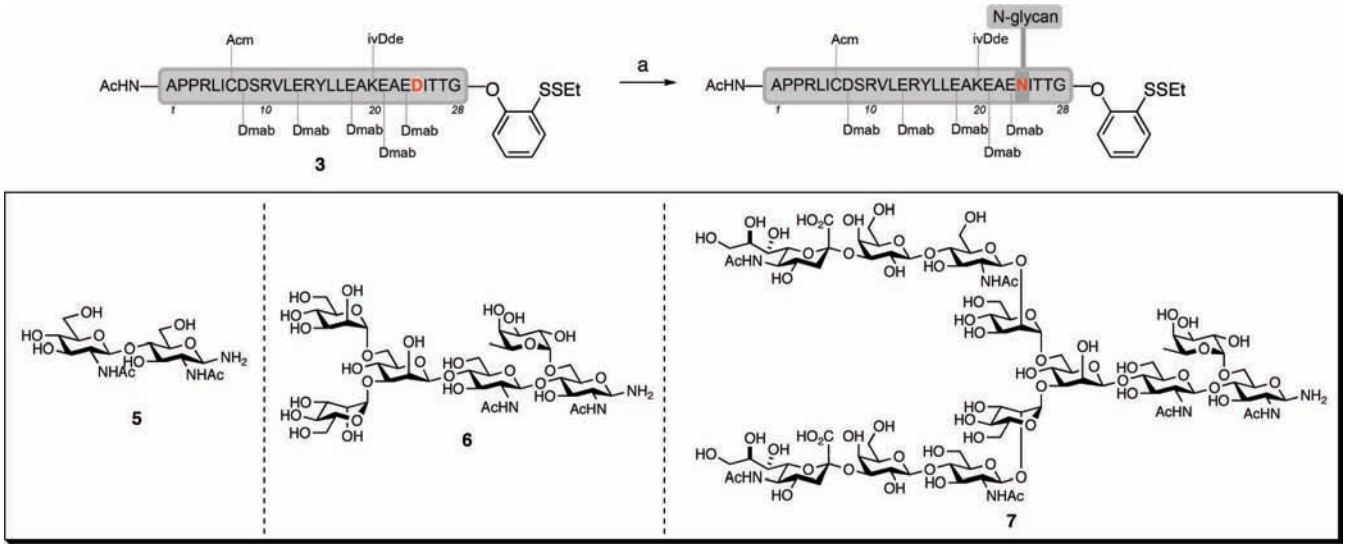
(18) Li, X.; Danishefsky, S. J. Unpublished results.

(19) For other studies on homocysteine ligations, see: Tam, J. P.; Yu, Q. *Biopolymers* **1998**, *46*, 5402–5406.

(20) In addition to alanine, valine, and homocysteine ligations, a phenylalanine ligation has also been reported. See: (a) Botti, P.; Tchertchian, S. WO 133962, 2006. (b) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064–10065.

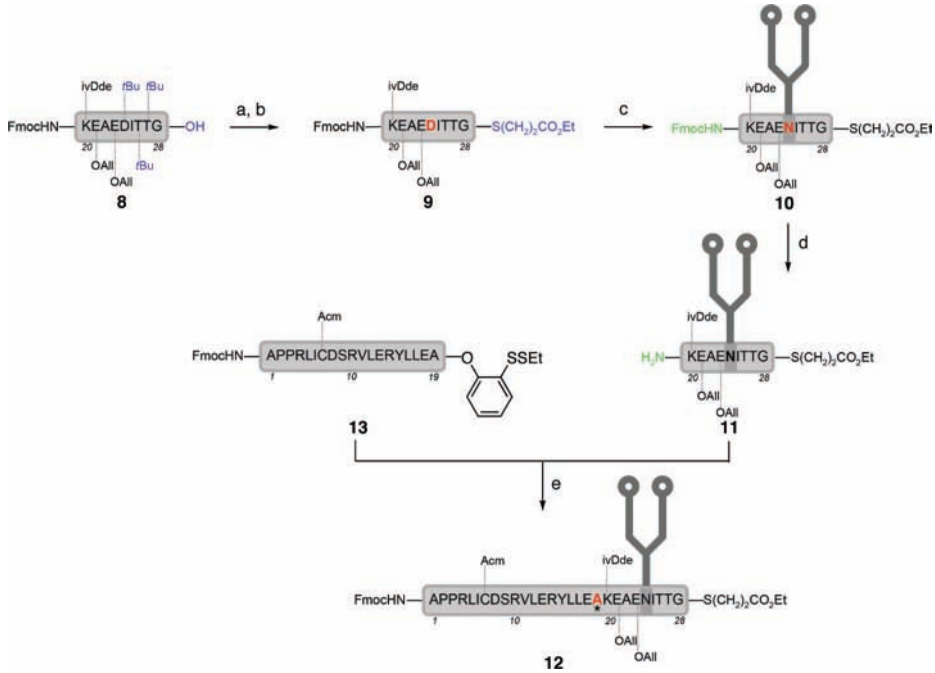
(21) Following completion of our studies, C.-H. Wong and co-workers published a direct aminolysis method that could, in theory, be applied to the synthesis of our glycopeptide fragment. See ref 11d.

Scheme 1. Initial Lansbury Aspartylation Studies[a]



[a] Reagents and conditions: (a) HATU, *i*Pr$_2$NEt, DMSO, with glycan **5**, 70%; with glycan **6**, 30%; with glycan **7**, 0%.

Scheme 2. Synthesis of **12** via TCEP-Assisted Phenolic Ester-Directed Amide Coupling[a]



[a] Reagents and conditions: (a) ethyl thiopropionate, EDCI, HOBt, DMF, 91%; (b) 88% TFA/CH_2Cl_2 (1:2), 5% H_2O, 5% phenol, 2% *i*Pr$_3$SiH, 23%; (c) HATU, *i*Pr$_2$NEt, DMSO, glycan **7**; (d) piperidine, 35% over 2 steps; (e) **13**, HCl·P($CH_2CH_2CO_2H$)$_3$, HOOBt, 2,6-di-*t*Bu-DMAP, DMSO.

preceding paper,[1a] we found that the direct attachment of dodecasaccharide **7**[22] to Asp[24] of the EPO(1-28) peptide sequence **3**[23] via Lansbury aspartylation[24] was unsuccessful, providing only aspartimide byproduct. In contrast, the joining of disaccharide **5** and hexasaccharide **6** to the 28-residue peptide **3** by Lansbury aspartylation proceeded in 70% and 30% yields, respectively (Scheme 1). Such unacceptably poor reactivity must surely be the consequence of the increased steric bulk presented by dodecasaccharide **7**, rendering aspartimide formation not only kinetically competitive (as with **6**), but dominant.

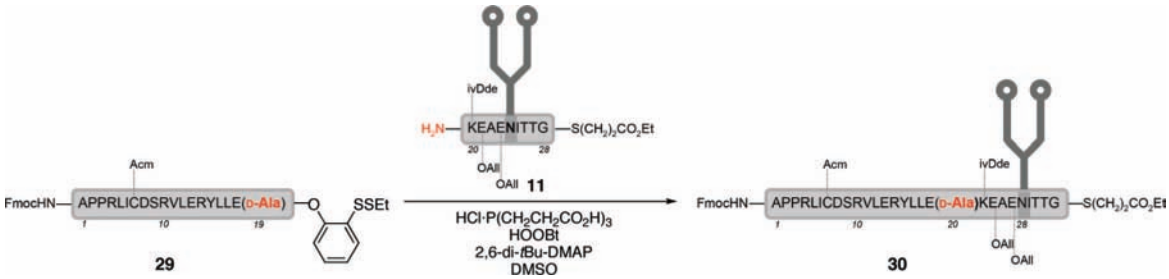
Recognizing the need to employ a smaller peptide to permit functional aspartylations of larger glycosylamines, we revised our plan for the synthesis of the EPO(1-28) fragment to include a strategic dipeptide scission between Ala[19] and Lys[20]. We first favored this disconnection with the goal of applying our TCEP-assisted phenolic ester-directed amide coupling method[2f] to

(22) Wu, B.; Hua, Z.; Warren, J. D.; Ranganathan, K.; Wan, Q.; Chen, G.; Tan, Z.; Chen, J.; Endo, A.; Danishefsky, S. J. *Tetrahedron Lett.* **2006**, *47*, 5577–5579.

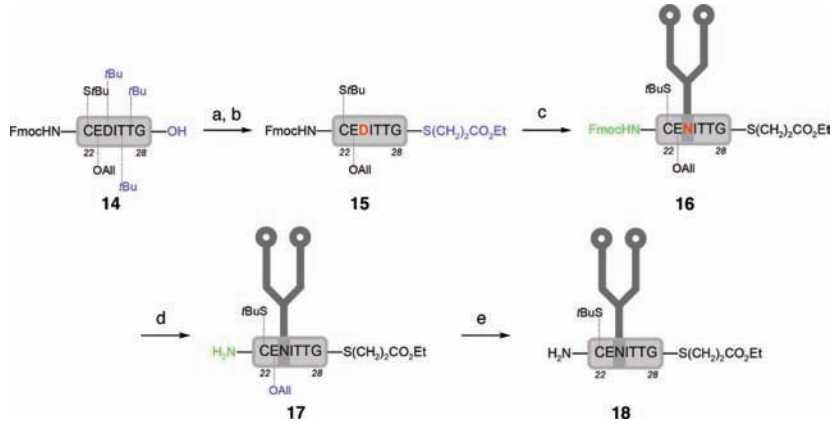
(23) The synthesis of the EPO(1-28) peptide via solid-phase peptide synthesis (SPPS) required the use of a commercially available pseudoproline-protected dipeptide for residues Asp[8] and Ser[9] (Fmoc−Asp(OtBu)−Ser($\Psi^{Me,Me}$pro)−OH) to avoid unwanted aspartimide formation.

(24) (a) Anisfeld, S. T.; Lansbury, P. T., Jr. *J. Org. Chem.* **1990**, *55*, 5560–5562. (b) Cohen-Anisfeld, S. T.; Lansbury, P. T., Jr. *J. Am. Chem. Soc.* **1993**, *115*, 10531–10537.

Scheme 3. Synthesis of D-Ala[19] Diastereomer of EPO(1-28)

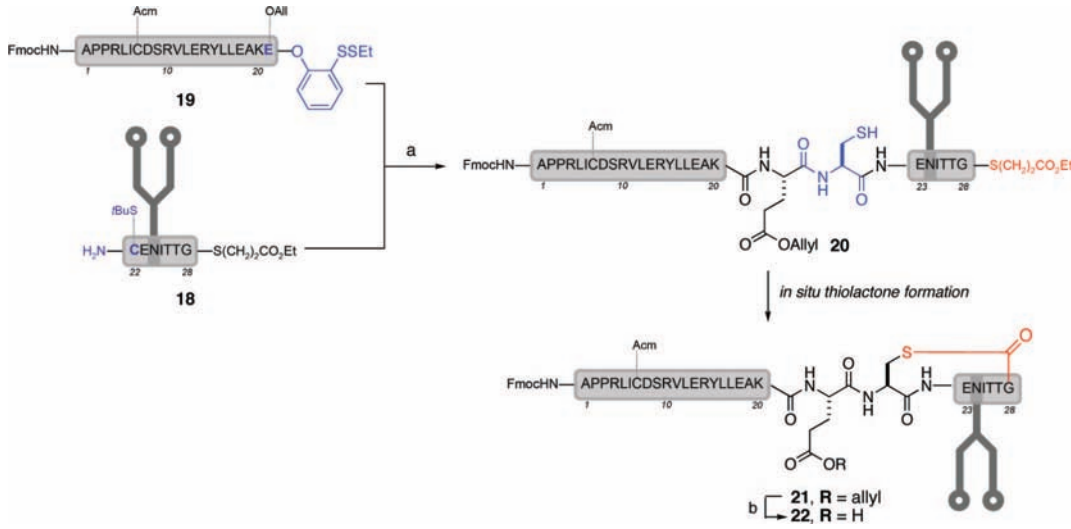


Scheme 4. Synthesis of Glycopeptide **18**[a]



[a] Reagents and conditions: (a) ethyl thiopropionate, EDCI, HOBt, DMF; (b) 88% TFA, 5% H_2O, 5% phenol, 2% iPr_3SiH, 45% over two steps; (c) HATU, iPr_2NEt, DMSO, glycan **7**; (d) piperidine, 65% over two steps; (e) $Pd(PPh_3)_4$, $PhSiH_3$, DMSO, 90%.

Scheme 5. Native Chemical Ligation between **18** and **19**[a]



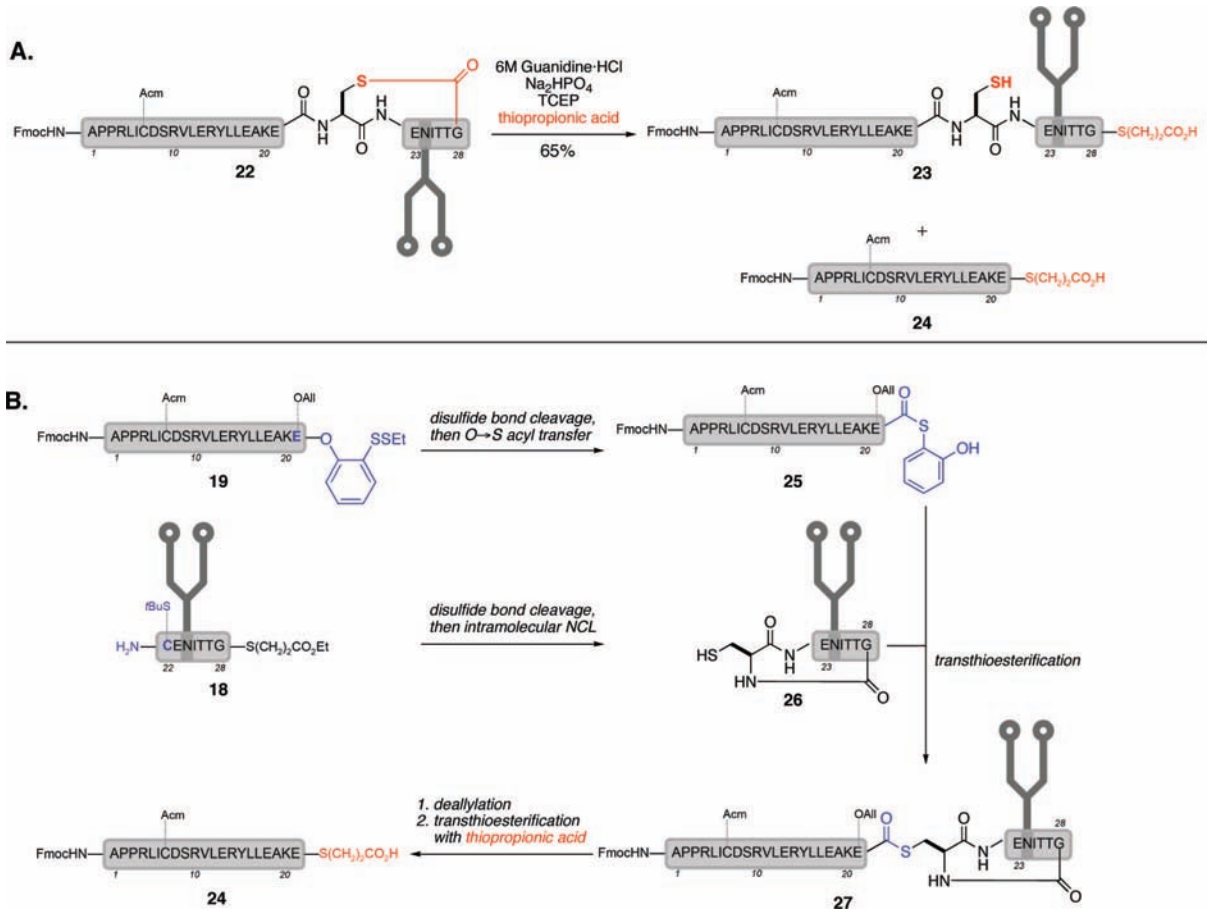
[a] Reagents and conditions: (a) 6M Guanidine·HCl, Na_2HPO_4, TCEP·HCl, TCEP, thiopropionic acid, 20%; (b) $Pd(PPh_3)_4$, $PhSiH_3$, DMSO, 90%.

assemble the fragment, despite concerns about the potential for epimerization at Ala[19]. Under this direction, the protected Lys[20]−Gly[28] sequence (**9**) was prepared and effectively joined with dodecasaccharide **7** by Lansbury aspartylation to provide, after Fmoc removal, our target fragment **11** (35%) (Scheme 2). Efforts to ligate the FmocHN−Ala[1]−Ala[19]−CO_2Ph(*o*-SSEt) fragment (**13**) with glycopeptide **11** using our direct condensation method proceeded successfully to give **12**, but the stereointegrity of the alanine ligation site (see asterisk) could not be verified.

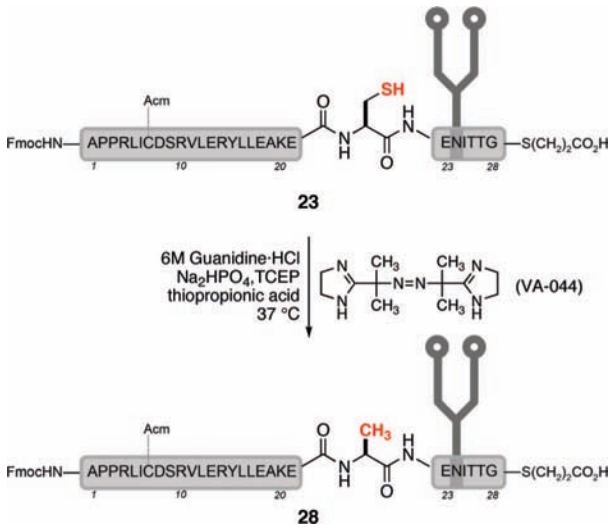
One way to determine whether the condensation product (**12**) suffered from epimerization during the key peptide coupling is to compare the retention times of **12** and the corresponding D-Ala[19] diastereomer. Of course, we recognized the possibility that the two diastereomers might coelute. Following the same sequence of steps as shown in Scheme 2, and using the D-Ala[19] peptide **29**, the diastereomer **30** was prepared (Scheme 3). Coinjection of the two glycopeptides yielded a single peak, offering no resolution. Increasing the concentration of the D-Ala[19] diastereomer did not change the peak shape. Despite these efforts, the stereointegrity of the alanine ligation site remained uncertain.[25]

Given the knowledge that the attachment of dodecasaccharide **7** to a shorter sequence was successful, we turned to an

Scheme 6. (A) Thiolactone Opening. (B) Possible Origin of Observed Byproduct from Thiolactone Opening



Scheme 7. Key Desulfurization Step



alternative approach to address the issue of stereointegrity. As stated earlier, the absence of any useful cysteine and glycine/proline residues precluded the use of either NCL or direct condensation methods. However, it was noted that several alanine residues are present in close proximity to Asp^{24}, the site for glycosyl attachment. An established extension of NCL has been the conversion of cysteine to alanine via desulfurization,[15] which enables ligation at alanine sites with the benefits of NCL (e.g., avoidance of epimerization, chemoselectivity). Recently, our laboratory had developed a desulfurization method that is compatible with oligosaccharides and is both extremely mild and selective.[2g] We anticipated that the versatility of this method would readily accommodate the different functional groups present within EPO(1-28), thus enabling the use of NCL to address our earlier problems in the synthesis of the EPO(1-28) fragment.

The EPO(1-28) fragment contains two alanine residues (Ala^{19} and Ala^{22}) in close proximity to the N-glycan, either of which could serve as the ligation site. We elected to implement a ligation between Glu^{21} and Ala^{22} to obtain a shorter (glyco)-peptide segment that would be more suitable for the essential Lansbury aspartylation as it is (1) smaller in size and (2) free of Lys^{20} and Glu^{21}, the side chains of which would necessarily be protected during the aspartylation reaction. This disconnection also yields the longer 21-amino acid peptide terminating at Glu^{21}. It should be noted that Botti has demonstrated that NCL at C-terminal glutamates and aspartates requires the side chains to be protected to avoid formation of the unnatural γ-amide bond during ligation.[26] While inconvenient, a glutamate protecting group would be necessary regardless of which disconnection was selected.

The requisite glycopeptide segment **18** was prepared, as shown in Scheme 4. Starting from peptide **14** (95% yield via

(25) Alternatively, trypsin could be used to provide the short peptide fragment YLLEAK, which should be readily resolved if Ala^{19} has epimerized. While we cannot show that Ala^{19} has not epimerized, model studies with the corresponding disaccharide substrates indicate formation of a separable mixture of diastereomers in a 4:1 ratio.

(26) Villain, M.; Gaertner, H.; Botti, P. *Eur. J. Org. Chem.* **2003**, 3267–3272.

solid phase peptide synthesis), condensation with ethyl thiopropionate followed by removal of the *t*-butyl groups provided thioester **15**. Attachment of dodecasaccharide **7** to peptide **15** via Lansbury aspartylation followed by in situ Fmoc cleavage proceeded in moderate yield to afford glycopeptide **17** in 65% yield over the two-step sequence. Finally, deallylation with $Pd(PPh_3)_4$ and $PhSiH_3$ afforded the NCL partner **18** in 90% yield.

Under NCL conditions, glycopeptide **18** was coupled with the longer peptide segment **19**[27] to yield the desired ligation product **20**, as well as the corresponding thiolactone **21** (Scheme 5). Allowing the reaction to stir longer resulted in what was thought to be exclusive formation of the thiolactone (vide infra), which was isolated in 20% yield. Following ligation, the allyl protecting group on Glu[21] was removed to avoid complications during the critical desulfurization.

Before desulfurization could be attempted, it was necessary to free the cysteine side chain from the thiolactone. Treatment of **22** with thiopropionic acid effectively opened the thiolactone to afford the acyclic **23** (Scheme 6A); however, a byproduct (**24**), identified as EPO(1-21)$COS(CH_2)_2CO_2H$, was also formed during the reaction. There are several possible explanations for the formation of this product. One possibility is that during the NCL reaction between **19** and **18**, a competitive intramolecular NCL within glycopeptide **18** had led to lactam **26**,[28] which subsequently underwent a transthioesterification with **19** to afford thioester **27** (Scheme 6B). Following removal of the allyl side chain group, the $^{\alpha}$thioester bond was readily cleaved in the presence of thiopropionic acid to give the observed byproduct. Alternatively, it is possible, though less likely, that under the mildly acidic conditions used to open the thiolactone, the Glu[21]−Cys[22] amide bond underwent an N → S acyl tranfer to generate the corresponding $^{\alpha}$thioester, which was subsequently cleaved to give the observed $^{\alpha}$thiopropionate ester.

Despite the loss of material, we were ready to test glycopeptide **23** in the key desulfurization reaction (Scheme 7). Treatment of **23** with VA-044 (a water-soluble radical initiator), TCEP, and thiopropionic acid in buffered conditions at 37 °C cleanly afforded the reduced product **28** (67% yield). The use of thiopropionic acid as the radical propagator also served to open any thiolactone that formed during the reaction. The final product, **28**, features both the biantennary glycan and a C-terminal $^{\alpha}$thioester, two critical features necessary for the convergent preparation of synthetic homogeneous EPO.

Conclusion

As described above, we have prepared the Ala[1]−Gly[28] glycopeptide fragment (**28**) of EPO by chemical synthesis. Key steps in the synthesis included attachment of a complex dodecasaccharide (**7**) to a seven amino acid peptide via Lansbury aspartylation, native chemical ligation to join peptide **19** with the glycopeptide domain **18**, and a selective desulfurization at the ligation site to expose the natural Ala[19]. This fragment presents both the requisite N-linked dodecasaccharide and a C-terminal $^{\alpha}$thioester handle, the latter feature permitting direct coupling with a glycopeptide fragment bearing N-terminal Cys[29] without further functionalization.

In summary the preparation of this Ala[1]−Gly[28], in the context of the accompanying reports, describing the syntheses of EPO(29-77), featuring the N-linked glycan, and EPO(78-166), presenting both the N-linked glycan and the O-linked glycophorin, suggests that the realization of our ultimate goal, i.e., biologically active homogeneous synthetic erythropoietin, is within reach. The assembly of these three fragments, corresponding to a full length homogeneous erythropoietin, represents the closest and most convergent approach to the chemical synthesis of a single EPO glycoform. The primary drawback in our synthetic efforts is the limited availability of the complex dodecasaccharide **7**. We note that much of the difficulty arises from the lack of commercial availability of the key building blocks. This situation could well change as the role of oligosaccharide chemistry in the synthesis of biologics becomes better understood.

We also think that the new amide bond forming chemistry delineated in this project, and summarized in the background section, could well allow for the combination of these fragments, recognizing that, with all complex target oriented total synthesis, intervention of the unexpected is predictable. That being said, we are hopeful that the convergent nature of our synthesis and its flexibility will enable its adaptation to reach our goals.

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Supporting Information Available: Experimental procedures, spectroscopic and analytical data for all new compounds, and complete ref 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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(27) Peptide **19** was prepared in two steps from **33**, see the Supporting Information for synthesis.

(28) Other examples of competitive intramolecular NCL have been observed. See: (a) Bang, D.; Pentelute, B. L.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2006**, *45*, 3985–3988. (b) Torbeev, V. Y.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 1667–1670.