Glycoproteins

DOI: 10.1002/ange.200600538

Building Complex Glycopeptides: Development of a Cysteine-Free Native Chemical Ligation Protocol**

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The development of increasingly efficient and general methods for the merging of complex peptidic fragments remains a central objective in the field of polypeptide and glycopolypeptide synthesis. A number of traditional native chemical ligation (NCL) techniques have been applied to the problem of polypeptide assembly through convergent ligation.[1] Nearly all of these require the presence of an N-terminal cysteine residue to function as the acyl acceptor. Given the relative scarcity of cysteine residues in nature, a clear impetus arises for the realization of new NCL capabilities.

Our laboratory has a major interest in the development of methods for the preparation of homogeneous, fully synthetic polypeptides, and even proteins, that display multiple sites of glycosylation. A particularly relevant glycoprotein, which serves to coordinate and focus our efforts in this field, is the naturally occurring erythropoietin alpha (EPO; Figure 1).[2]

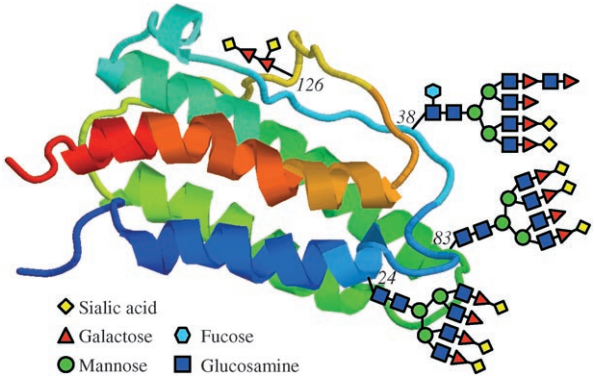

Figure 1. Structure of erythropoietin.

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[**] This work was supported by the National Institutes of Health (Grant CA28824 to S.J.D., and CA62948 to J.D.W.) and by New York State Department of Health, New York State Breast Cancer Research and Education Fund (to B.W.).

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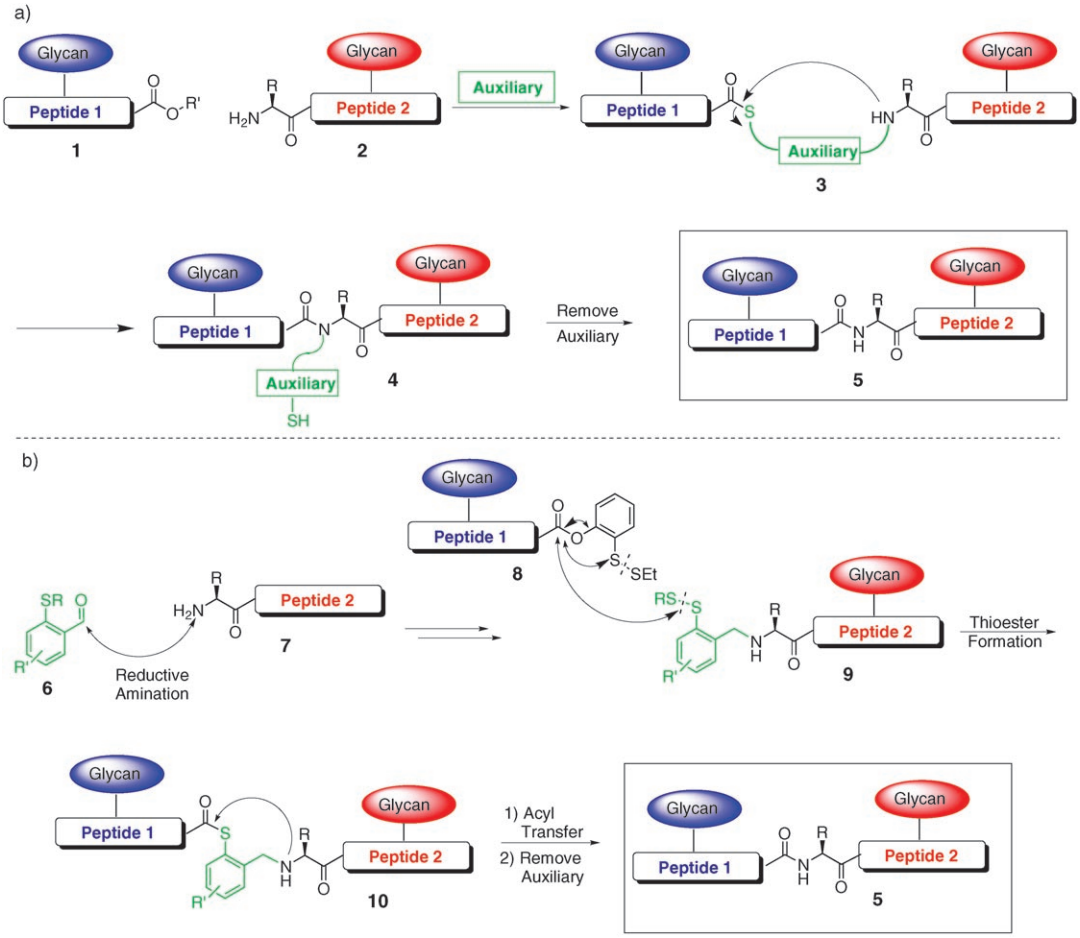
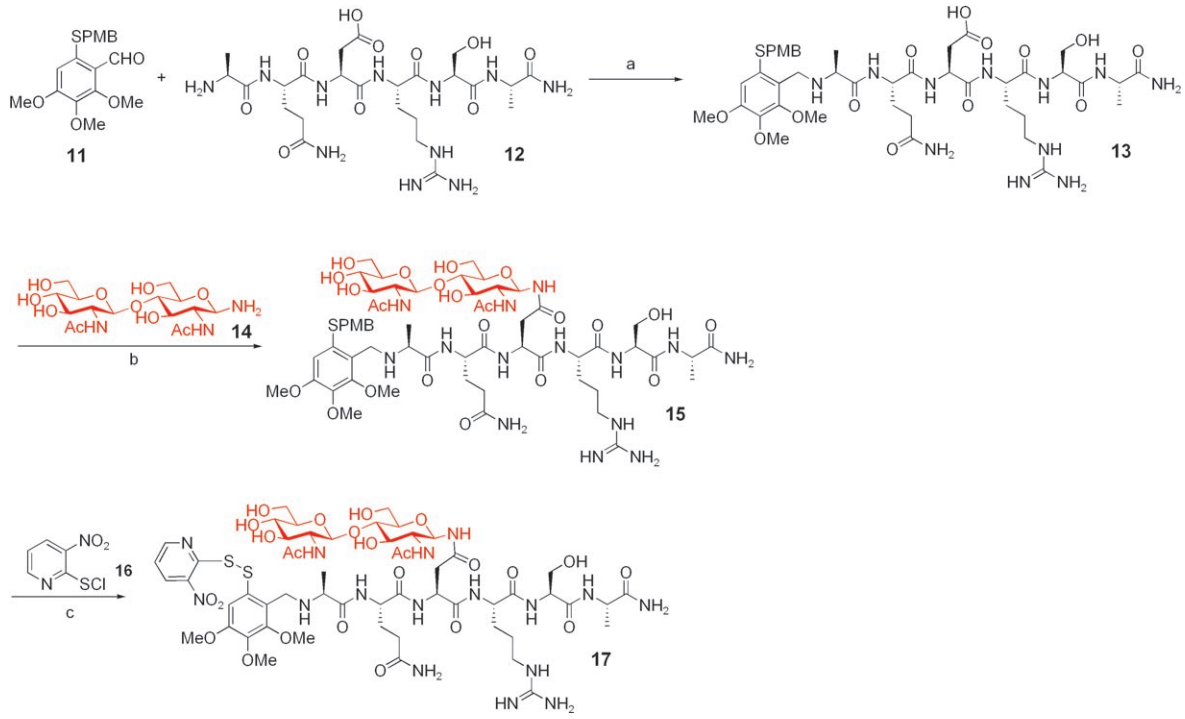
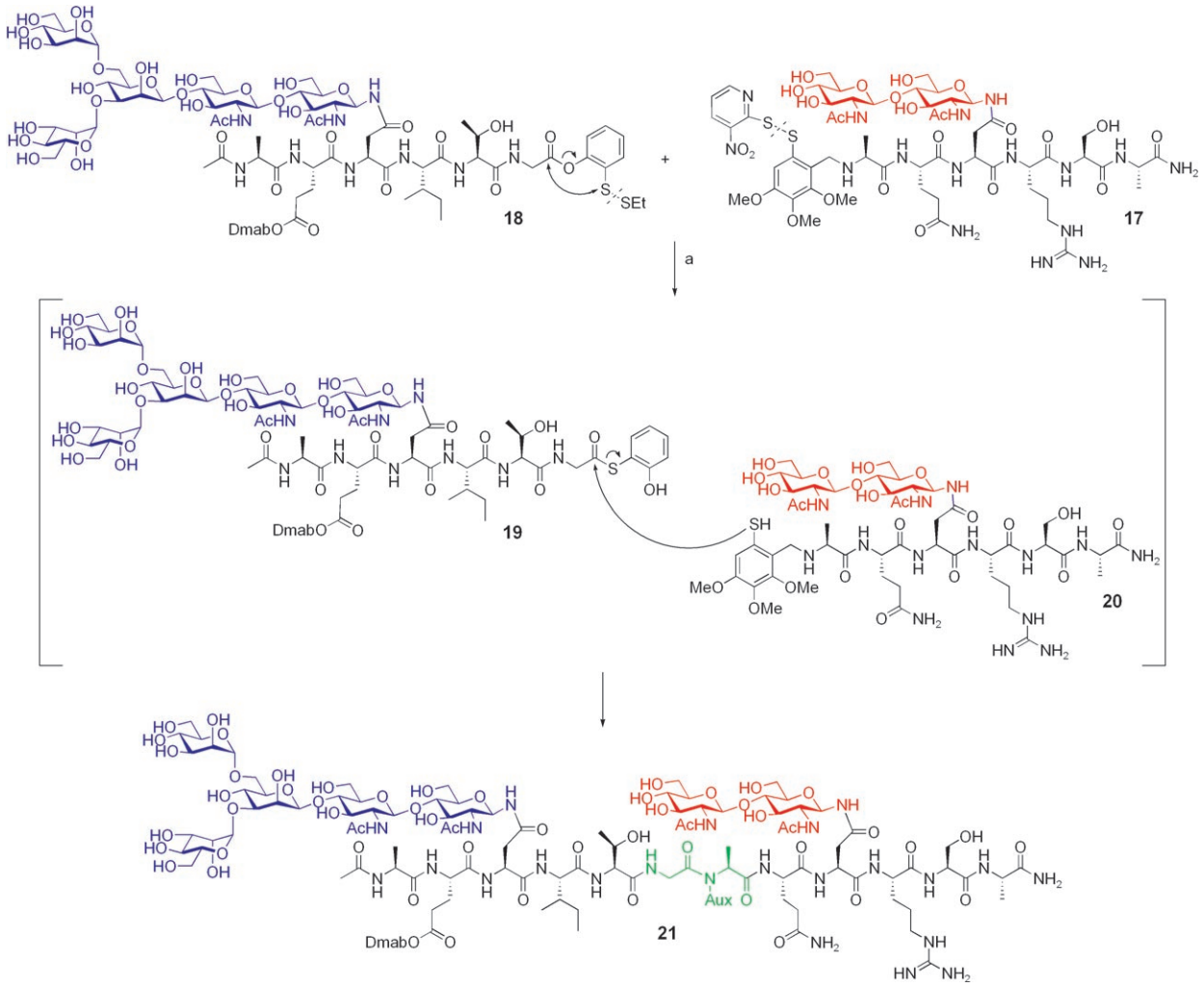


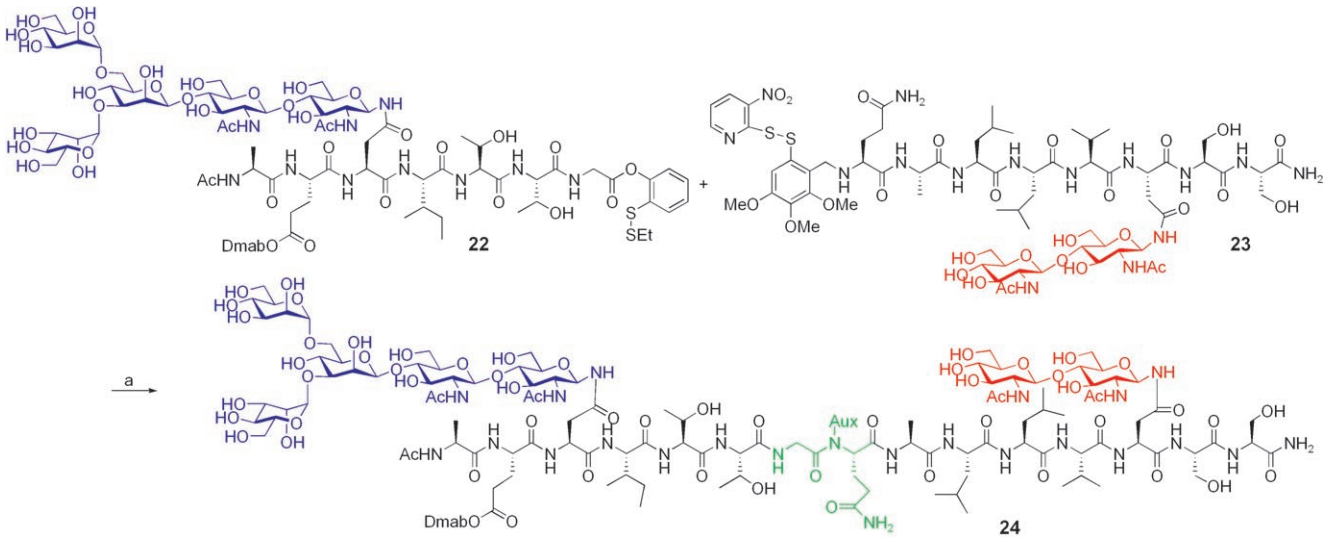
Scheme 1. Cysteine-free ligation strategy. See text for details.



Scheme 2. Synthesis of compound **17**. Reaction conditions, a) $NaCNBH_3$, MeOH/DMF, 60%; b) **14**, HATU, iPr_2NEt, DMSO, 62%; c) **16**, TFE/CH_2Cl_2, 70%. Ac = acetyl, DMF = *N*,*N*-dimethylformamide, DMSO = dimethyl sulfoxide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, PMB = *para*-methoxybenzyl, TFE = 2,2,2-trifluoroethanol.

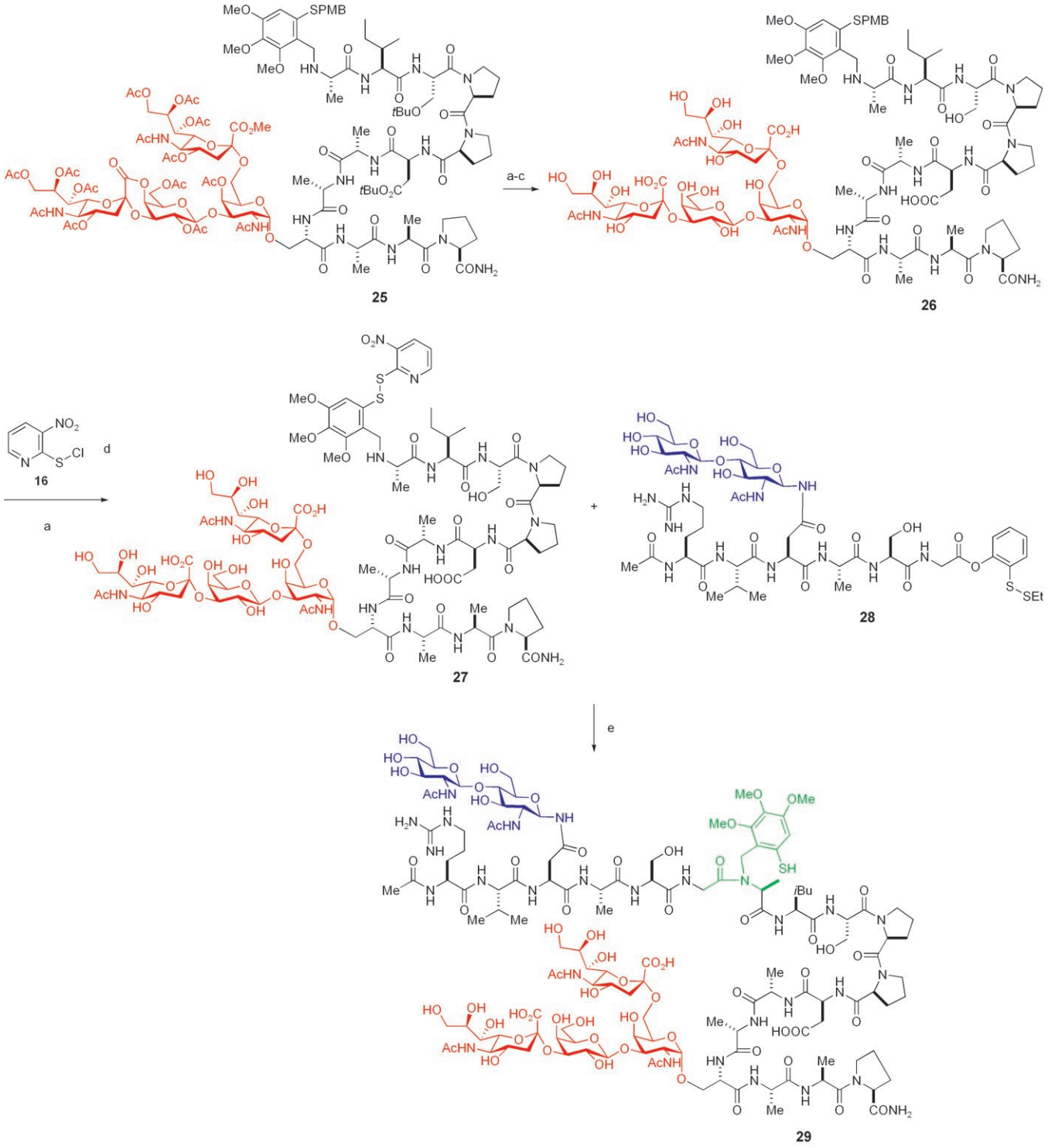


Scheme 3. Synthesis of **21**. Reaction conditions, a) TCEP, PBS (pH 8.0), 37%. Aux = auxiliary, Dmab = 4-{N-[1-(4,4-dimethyl-2,6-dioxocyclohexyl-idene)-3-methylbutyl]-amino}benzyl, PBS = phosphate-buffered saline buffer solution, TCEP = tricarboxyethylphosphine.



Scheme 4. Ligation of **22** and **23**. Reaction conditions, a) TCEP, DMF, Na_2HPO_4, 32 °C, 54%.

This multiply glycosylated protein has found widespread therapeutic application in the treatment of anemia. Despite the clear-cut clinical importance of this compound, attempts to rigorously evaluate the role of glycosylation on the activity and stability of erythropoietin have thus far been complicated by the daunting difficulties associated with isolating significant quantities of homogeneous EPO.[3] On the basis of our long-term involvement in the arena of carbohydrate and glycopeptide total synthesis,[4] we reasoned that if the considerable powers of chemical synthesis were brought to bear on this problem in a principled and focused fashion, it might be possible to gain access to fully synthetic, homogeneous erythropoietins. Needless to say, such a capability would likely enable access to a range of EPO analogues for structure–activity-relationship investigations. Broadly speaking, an undertaking of this magnitude could well lead to the



Scheme 5. Ligation of O-linked glycodomains. Reaction conditions, a) TFA, PhOH, H_2O, TESH; b) 0.1 N NaOH, MeOH; c) H_2NNH_2, MeOH, 61% over 3 steps; d) **16**, TFE, 67%; e) TCEP, DMF, Na_2HPO_4, 65%. TFA = trifluoroacetic acid, TESH = triethylsilane.

development of generally useful strategies and protocols of utility to the entire field of glycoprotein synthesis.

Erythropoietin is a 166-residue protein possessing four sites of glycosylation. Three of these are N linked to asparagine residues and one is O linked to a serine residue. In considering a strategy for the de novo synthesis of erythropoietin, we took note of the paucity of cysteine residues on the molecule. A maximally convergent route to erythropoietin would involve the preparation of the four different glycopeptide fragments, which would subsequently be joined through some form of ligation to furnish the fully glycosylated protein backbone. Thus, in light of the fact that the four cysteine residues of erythropoietin do not segregate into nearly equal-sized carbohydrate-bearing domains, we were led to consider glycopeptide ligation methods that do not require cysteine-based acyl acceptors. The development of a repertoire of ligation methods could well be critical to the success of our EPO-directed venture.

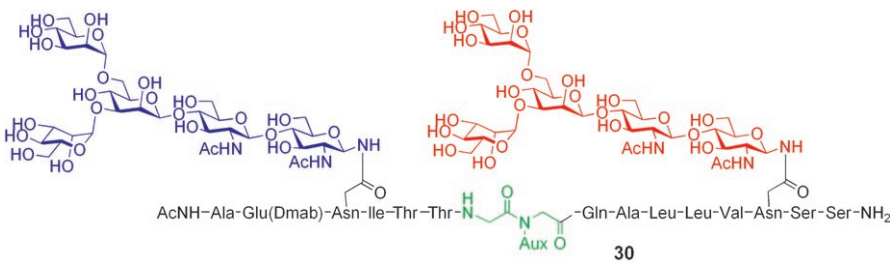
Scheme 1a shows, in the most general terms, a scenario wherein two differentially glycosylated peptide fragments (**1** and **2**) would be temporarily engaged through an auxiliary linker[5] such that the C-terminal coupling fragment would be activated as a thioester (**3**). Having been coaxed into proximity, the N-terminal coupling partner would attack the thioester, thus forming the key amide bond (**4**). Removal of the auxiliary would provide the doubly glycosylated peptide **5**. In this way, the limitation of a cysteine acyl acceptor situated at the N-terminus would have been removed.

One conceivable manifestation of this general strategy would commence with the covalent appendage of a sulfur-displaying auxiliary of the type **6** to the N terminus of an appropriate peptide fragment (**7**) through reductive amination.[6] In this early feasibility demonstration, we benefited from a recently disclosed clever idea of Dawson et al. for a non-cysteine-based ligation, albeit by a different organizing step.[7] The resultant intermediate would be advanced to the glycopeptide **9**. At this point, we envisioned joining the two fully functionalized glycopeptide fragments under conditions analogous to those that we had previously developed in the context of a cysteine-based NCL method.[5a] Thus, the coupling partner, **8**, would be equipped with a C-terminal phenolic ester, as shown in Scheme 1b. Under disulfide reducing conditions, the C-terminal coupling partner, **8**, would be activated to form a thioester with the auxiliary sulfur functionality of the N-terminal fragment, thus providing an intermediate of the type **10**. Amide-bond formation followed by auxiliary removal would provide the bifunctional glycopeptide **5**.

In the end, we elected to equip the sulfur atom of the auxiliary with a PMB protecting group.[8] We anticipated that deprotection of this group could be accomplished under mild conditions that are compatible with survival of glycopeptide functionality (see Scheme 2). Thus, aldehyde **11** was prepared through slight modification of a known procedure.[7a] Reductive amination with hexapeptide **12** provided **13** in 60% yield. This ability to connect a substantial N-terminal domain to the auxiliary by reductive amination is an important feature of this methodology. Next, the disaccharide **14** was appended to the peptide through reducing-end amination and aspartylation.[9] At this stage, we sought to convert the S–PMB group into an aromatic disulfide under mild conditions that would leave the sensitive glycopeptide functionality intact. We were pleased to find that, following treatment of **15** with sufenyl chloride **16** in TFE/DCM, compound **17** was obtained.[10] Notably, this transformation represents an appealing alternative to previously described conditions that typically require exposure to harsh reagents such as anhydrous HF or $Hg(OAc)_2$ and TFA.[6a,7a]

With the auxiliary-bearing glycopeptide fragment **17** in hand, we were now prepared to investigate the viability of our cysteine-free coupling strategy. In this context, we first examined a Gly–Ala ligation. As expected, upon exposure to TCEP in PBS buffer solution (pH 8.0), glycopeptides **17** and **18** each underwent reductive disulfide cleavage (see Scheme 3). Presumably, the C-terminal glycopeptide then suffered an O→S acyl transfer of the type previously described to generate the activated thioester (**19**).[11] As anticipated, **19** and **20** were temporarily joined through a thioester exchange reaction. Following intramolecular acyl transfer, the fully functionalized glycopeptide **21** was isolated in 37% yield.[12]

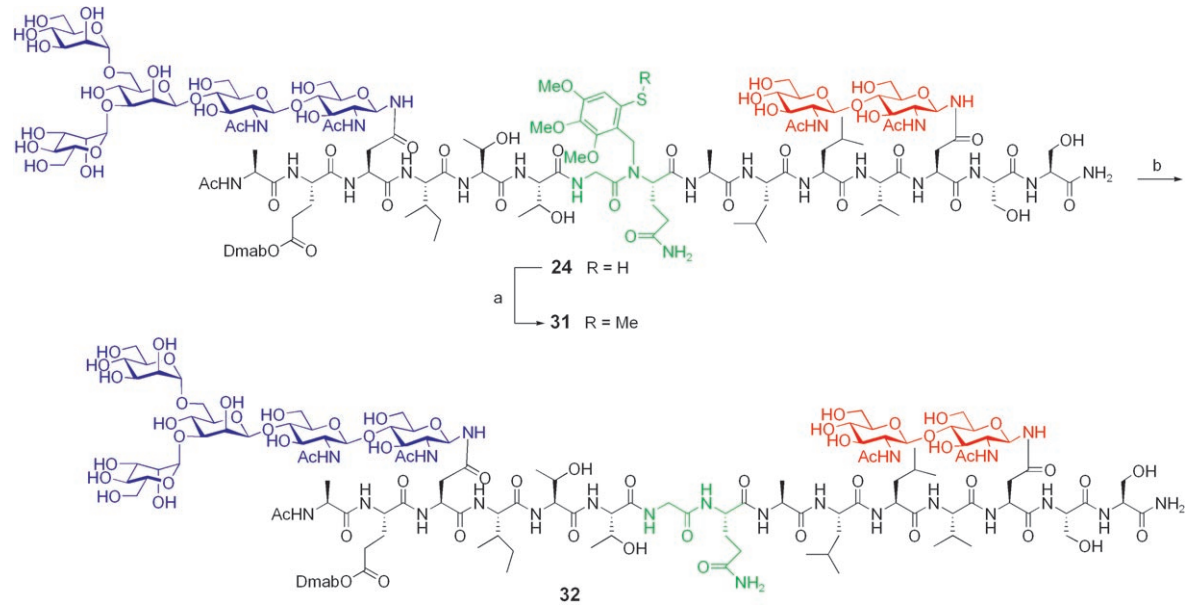
Encouraged by this early success, we next sought to explore the generality of the method by attempting ligation at a more challenging Gly–Gln center. Thus, glycopeptides **22** and **23** (Scheme 4) were prepared and subjected to ligation conditions (TCEP in PBS buffer solution; pH 8.0). Unfortunately, the yield of **24** was found to be quite low, and a significant quantity of carboxylic acid arising from the hydrolysis of **22** was observed. We postulated that, although in the previous instance (**17** + **18** → **21**) the rate-determining step of the sequence had been the joining of the two fragments through transthioesterification, in the case at hand, the increased steric hindrance around the reacting center had caused the intramolecular acyl transfer to become rate limiting. Consequently, hydrolysis of the tethered intermediate had the opportunity to intervene as a competitive side reaction. Although we were unable to improve upon the product distribution by adjusting the pH of the system, we did find that, by introducing DMF as a cosolvent with a small amount of Na_2HPO_4, we were able to isolate the ligation product **24** in a more-acceptable 54% yield.



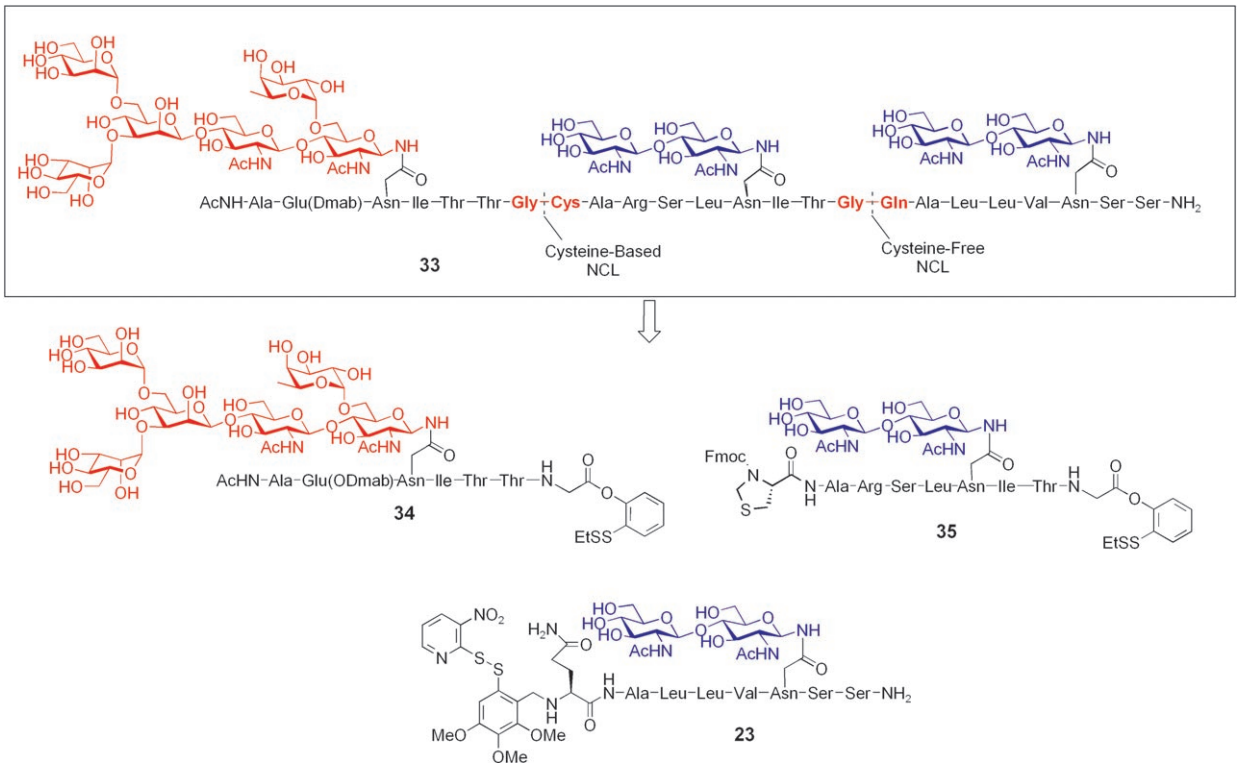
Scheme 6. Bifunctional glycopeptide.

Having demonstrated the capacity of our methods to successfully ligate two N-linked glycopeptide domains, we next sought to investigate its compatibility with O-linked glycodomains. Thus, intermediate **25** was advanced to **26** through a three-step sequence, as shown in Scheme 5.[13] The latter was converted into the N-terminal coupling partner, **27**, according to previously developed reaction conditions. We were pleased to find that, upon exposure to TCEP and DMF with Na_2HPO_4, **27** and **28** readily underwent cysteine-free native chemical ligation to provide glycopeptide **29**, which possesses both N- and O-linked carbohydrate domains. Notably, no carbohydrate decomposition products were observed.

We next validated our methodology in the context of more-complex glycan fragments, including those containing characteristic non-reducing and sialic acid moieties. The



Scheme 7. Synthesis of **32**. Reaction conditions, a) methyl *p*-nitrobenzene sulfonate; b) 95% TFA.

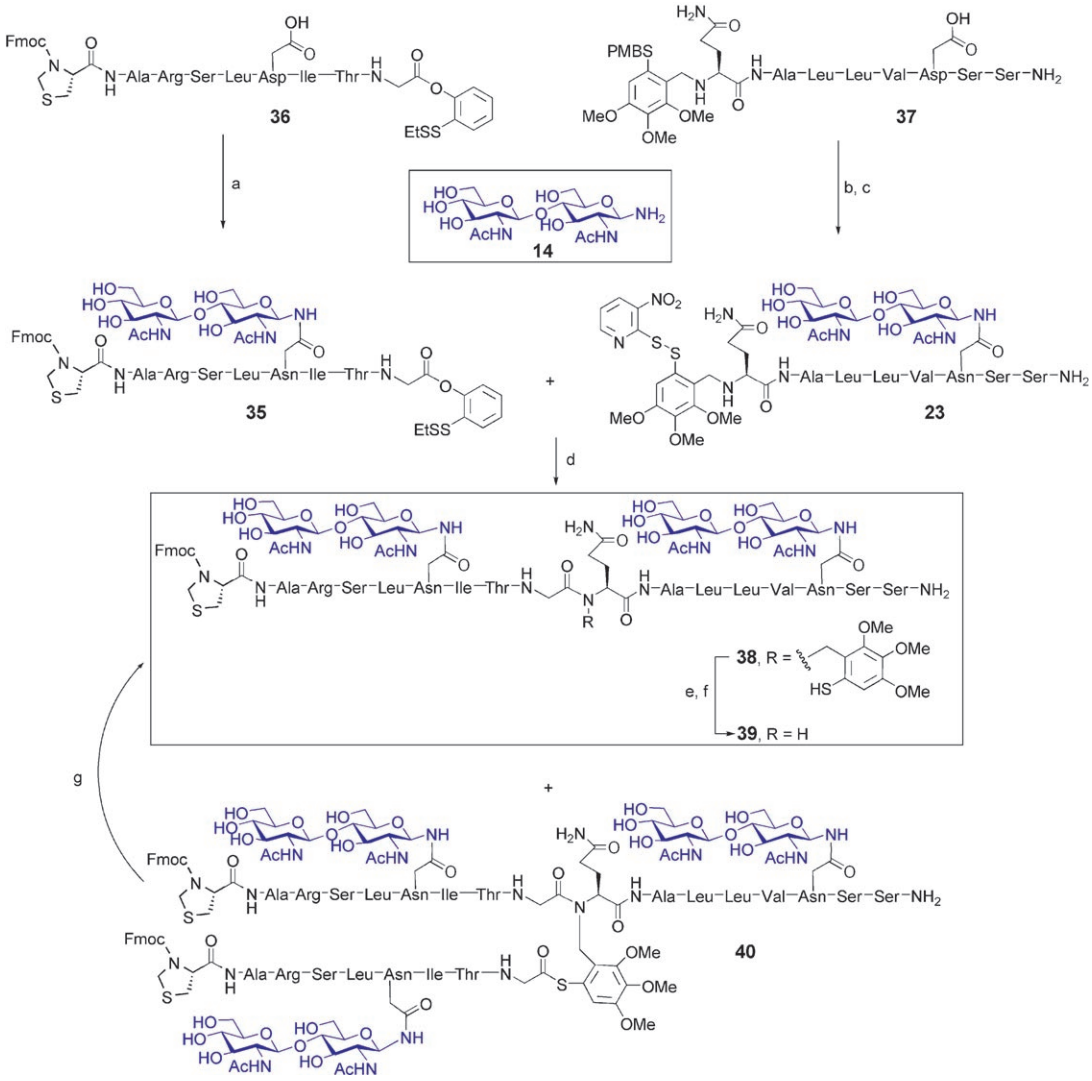


Scheme 8. Trifunctional glycopeptide.

extent of sialidation is apparently a determinant of EPO stability.[14] Thus, the coupling of two glycopeptide fragments, each displaying an N-linked core pentasaccharide, was found to proceed smoothly to provide the bifunctional glycopeptide **30** (Scheme 6).

The final phase of this investigation would be the development of appropriately mild conditions for the cleavage of the thiol auxiliary. In this context, TFA with a scavenger has been used in similar types of systems. However, in our hands, the treatment of the ligation product with 95% TFA with a triisopropyl silane (TIPS) scavenger resulted in a mixture of the desired native glycopeptide along with another compound of the same molecular weight as the starting glycopeptide. The latter was tentatively assigned to be the thioester intermediate, arising from acid-mediated intramolecular N→S acyl transfer.[15] Presumably, the otherwise endothermic step is driven by irreversible protonation of the benzylic amine. In light of this finding, a two-step sequence (Scheme 7) was devised for the removal of the auxiliary. First, intermediate **24** was treated with methyl *p*-nitrobenzene sulfonate.[16] This step accomplished selective methylation of the sulfur on the aromatic ring to provide intermediate **31**. The latter was not purified, rather, it was exposed to the action of 95% TFA, thereby providing the native glycopeptide **32**, free of any observable thioester by-product.

Having successfully field tested our novel cysteine-free ligation protocol in the context of a convergent bis-domainal glycopeptide synthesis, we next turned to the challenge of synthesizing longer peptide chains that contain more than two sites of glycosylation. Indeed, to consider the total synthesis of a complex glycoprotein, such as EPO, it would be critical to be able to couple, in a reiterative fashion, multiple glycopeptide fragments. Along these lines, we recently disclosed a method to generate differentially glycosylated trifunctional glycopeptides based on a cysteine-dependent native chemical ligation protocol.[17] As a demonstration of the applicability of our new non-cysteine NCL technology to complex targets, we



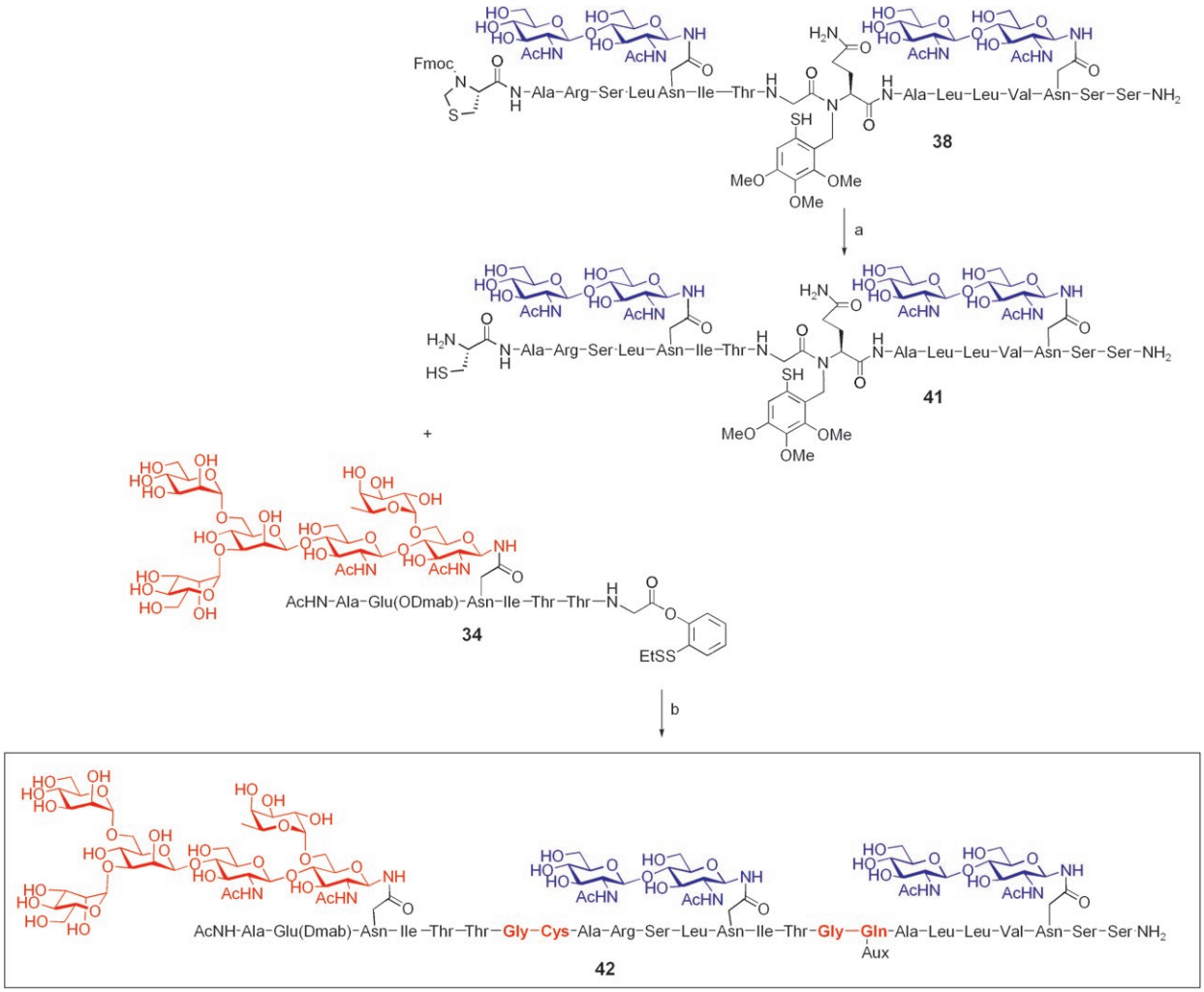
Scheme 9. Synthesis of **40**. Reaction conditions, a) **14**, HATU, iPr$_2$NEt, DMSO, 72%; b) **14**, HATU, iPr$_2$NEt, DMSO, 83%; c) TFE, DCM, **16**, 69%; d) TCEP, DMF, Na_2HPO_4, 58% (**38**) + 11% (**40**); e) methyl *p*-nitrobenzene sulfonate; f) 95% TFA; g) PhSH or MesNa, PBS. DCM = dichloromethane, Fmoc = 9-Fluorenylmethoxycarbonyl, MesNa = 2-mercaptoethane sulfonic acid, sodium salt.

next sought to apply a combination of our cysteine-dependent and cysteine-free NCL protocols to the preparation of the multiply glycosylated peptide, **33**. Under our synthetic plan, we would first prepare each of the three glycopeptide fragments (**34**, **35**, and **23**) according to glycal assembly and glycopeptide synthesis protocols that have been validated and optimized over the course of many years in our laboratory (Scheme 8).[4] Fragments **23** and **35** would then be joined according to our newly developed cysteine-free ligation method to form the Gly–Gln junction. Next, following deprotection of the N-terminal cysteine residue, the bifunctional peptide would be merged with glycopeptide **34** through cysteine-based ligation to afford the fully functionalized target compound.

Thus, polypeptides **36** and **37** were prepared for the cysteine-free ligation event. It is noted that the termini have been suitably equipped in anticipation of the reiterative sequence. Thus, peptide **37** bears the requisite N-terminal auxiliary for the cysteine-free coupling, whereas fragment **36**, which will serve as the middle glycopeptide component, incorporates the C-terminal phenolic ester for the first cysteine-free ligation as well as a 1,3-thiazolidine-4-carboxo (Thz)-protected N-terminal cysteine residue, which is unmasked prior to the second, cysteine-based ligation event.[18]

Each peptide fragment was subjected to glycosylation with disaccharide **14**, and, following conversion of the N-terminal auxiliary S–PMB group to the requisite disulfide, glycopeptides **35** and **23** were in hand. As hoped, the coupling of the two fragments proceeded readily in the presence of TCEP to afford the ligated product **38** in 58% yield, along with 11% of the thioester **40**. The latter could be converted into **38** upon treatment with thiophenol or MesNa.[19] At this stage, the thiol auxiliary could be removed according to the two-step sequence shown in Scheme 9 (**38**→**39**); alternatively, the auxiliary could also be maintained in the subsequent ligation event without causing detriment.

As we had previously demonstrated, the cysteine residue was readily unmasked through exposure of **38** to 10% morpholine in DMF (to remove the Fmoc group) followed by treatment with an aqueous solution of $MeONH_2 \cdot HCl$ (Scheme 10). Native chemical ligation between **41** and **34** was



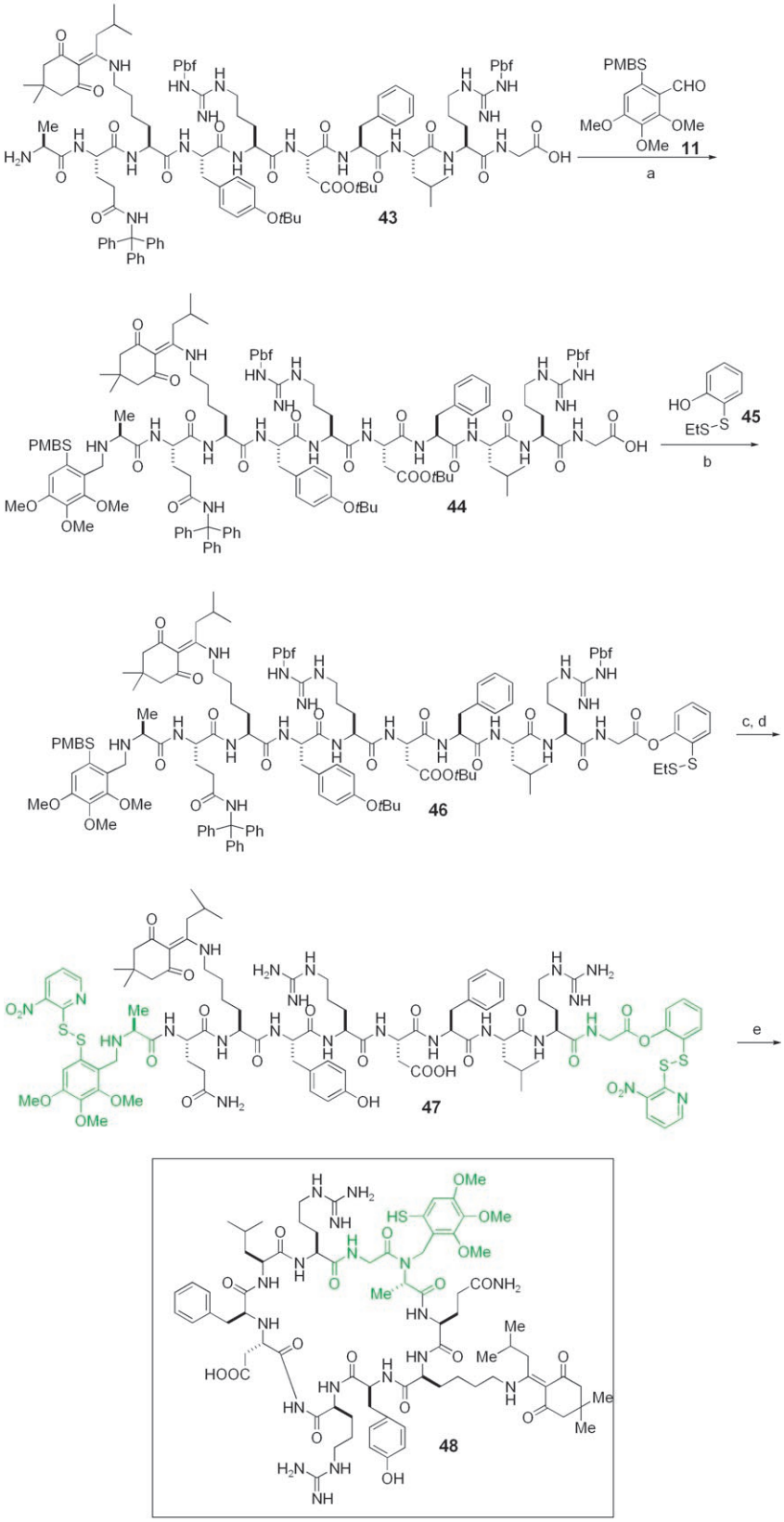
Scheme 10. Synthesis of **42**. Reaction conditions, a) (i) 10% morpholine in DMF; (ii) 0.4 M $MeONH_2 \cdot HCl$, 60%; b) MesNa, TCEP, PBS (pH 8.0), 57%.

carried out in the presence of MesNa and TCEP and afforded the multifunctional glycopeptide **42** in 57% yield.

Finally, the viability of our newly developed cysteine-free ligation protocol was demonstrated in another area of peptide chemistry of great interest to those at the forefront of chemistry and glycobiology, that is, the synthesis of cyclic peptides.[20] Cyclic peptides often possess enhanced biological specificity, activity, and metabolic stability in comparison to their linear counterparts. This is as a consequence of their constrained conformations and their enhanced levels of resistance to protease digestion. Although traditional strategies for cyclic-peptide formation are restricted to macrolactam or disulfide formation, Tam and co-workers[21] have disclosed that cyclic peptides can be accessed through native chemical ligation.[22] Recently, our research group reported on a newly modified protocol for native chemical ligation that allows for formation of cyclic peptides possessing a cysteine residue.[23] However, given the scarcity of cysteine residues in nature, the applicability of standard cysteine-based ligation methods may be somewhat limited. Clearly, the development of a broadly useful, cysteine-independent ligation protocol could well have profound ramifications for the field of cyclic-peptide synthesis.

The linear polypeptide **43** was prepared through solid-phase peptide synthesis. Reductive amination with aldehyde **11** served to introduce the N-terminal auxiliary (**44**). The C terminus was functionalized through HATU-mediated esterification with phenol **45**, providing **46**. Following protecting-group removal and exposure to 3-nitro-2-pyridinesulfenyl chloride, the requisite disulfide cyclization precursor was in hand. Thus, the linear peptide bis-disulfide (**47**) was treated with TCEP and Na_2HPO_4 in DMF to provide the desired cyclized peptide (**48**) in good yield. Importantly, no dimers or oligomers were observed in liquid chromatograpy–MS analysis (see Scheme 11).

Obviously, in undertaking a target of the complexity of erythropoietin, opportunities for complications and even failure are always inherent. However, we feel that in principle the basis of a realistic total synthesis of homogeneous erythropoietin has been set forth above. Always



Scheme 11. a) **11**, $NaCNBH_3$, MeOH, DMF, 66%; b) **45**, HATU, DIPEA, DMF; c) TFA, PhOH, TESH, H_2O, 57% for 2 steps; d) **16**, TFE, 60%; e) TCEP, Na_2HPO_4, DMF, 78%. DIPEA = *N*,*N*-diisopropylethylamine, Pbf = 2,2,4,6,7-pentymethyldihydrobenzofuran-5-sulfonyl.

mindful of the risks, we remain busily engaged in pursuing this goal.

Received: February 9, 2006
Revised: April 5, 2006
Published online: May 19, 2006

Keywords: cysteine-free ligation · erythropoietin · glycoproteins · native chemical ligation · peptides

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