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Promising agents at the interface of biology and oncology derived through chemical synthesis*

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Abstract: This account traces the development of our synthetic glycopeptide- and glycoprotein-based research program over the past decade. We recount the syntheses of a number of biologically relevant, natural product-inspired glycopeptide constructs, including those associated with prostate specific antigen (PSA) and with the gp120 surface envelope protein of HIV. We also describe our progress toward the synthesis of the multiply glycosylated protein, erythropoietin (EPO). Particular emphasis is placed on the development of enabling methodologies which allow for the ligation of complex glycopeptide fragments, thus rendering it possible to access, through purely synthetic means, homogeneous, multidomainal glycopeptide and glycoprotein constructs.

Keywords: carbohydrates; glycopeptides; glycoproteins; synthesis; erythropoietin; ligation.

INTRODUCTION

In the parlance of pharma seeking to define its mission, pharmaceutical agents are often divided into two categories. Certainly, a productive route for over a century has been what are now known as cell-permeable small molecules. More often than not, these "small molecules" are prepared by chemical synthesis following leads developed via sample collections from the pharma discovery laboratory or from small-molecule natural product (SMNP) collections [1]. Often, the actual optimization of phenomenological function precedes clear definition of the existence of a molecular target, not to speak of the precise structure of the target. Clearly, the trend in the pharma discovery style is in the direction of focusing on the prior definition of targets. The small molecule is graded on the basis of how it performs relative to the molecular target rather than to some gross functional phenomenon. While this line of progression is in keeping with the maturing of pharmacology into a molecular science which craves increasingly precise discovery venues, this otherwise admirable direction is not without potential costs. In this age of focus on target-constrained rationality, one wonders whether, if penicillin had first been encountered in 2007, it would have survived the barrage of skepticism it would surely have encountered from pharma sophisticates. A real challenge to increasingly rational discovery trajectories is that of maintaining an openness and receptivity to the joy of spontaneous discovery even from seemingly amateurish programs.

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A more recent and powerful innovation is embodied in the term "biologics" or "biologicals". Here, the intent is that one takes advantage of some large natural product or naturally derived product, usually a protein, an oligonucleotide, or an oligosaccharide. In the field of biologics, the molecular target is generally very well established before the proposed agent is optimized and developed extensively. Happily, previous biases against biologicals have been overcome, and, indeed, in recent times, several biologicals have reached blockbuster status, particularly in oncology [2].

Another dichotomy that has grown up and been nurtured relates to the origins of these broad classes of pharmaceutical agents. The general notion is that the small molecule is derived from chemistry (starting with a strong aromatic or heterocyclic bias) which was directed through synthesis toward a particular phenomenon or target. Conversely, the biologics have been seen to be the province of biology with its wonderful powers of amplification. The unspoken, but still powerful, assumption was that chemistry had little to offer to the universe of biologicals.

About eight years ago, we began to develop considerable curiosity about glycosylated polypeptides. In particular, we were interested in learning whether or not there were any advantages to having access to homogeneous versions of such systems. In that way, one could study the consequences of glycosylation on molecular structure and even perhaps biological function of "biologics". Indeed, at that time, we began to gain confidence that, with appropriate diligence and application, fully synthetic routes to homogeneous glycopolypeptides and perhaps even glycoproteins, while not yet feasible, could eventually become possible. Accordingly, we undertook a program directed at the total synthesis of homogeneous glycosylated polypeptides and glycoproteins.

One of the interesting features in our contemplations of such a course was the recognition that what we were proposing to ourselves was really something intermediate between "biologicals" and "small molecules". We were contemplating structures of a size, scope, and structural character that might well be candidates for the term "biologicals". On the other hand, we would be exploring synthetic routes which would, in principle, allow one to make ambitious mutations from the "wild type" based on design considerations without recourse to biological means. In other words, purely chemical means would be marshaled to assemble biologicals. This was a rather new idea for its time, and several years of concentrated application were required to achieve progress. Happily, as will be seen below, very substantial advances on the chemical front have been realized and a new era of fully synthetic homogeneous biologicals, whose structure can be edited and hopefully optimized in the tradition of small molecules, will become a reality.

We were urged to review our findings in this field, and this invitation led to the submission herein. In particular, we have chosen to emphasize the newly emerging synthetic logic and enabling synthetic methodologies which are coming to full fruition, thereby underscoring the concept of the fully synthetic biological. We have elected to focus on natural product-inspired constructs which involve suitably varied high-mannose oligosaccharides. These are, in turn, appended to polypeptides with very high positional specificity to create biologics of important consequence. The progress has been gratifying, as is shown in this report.

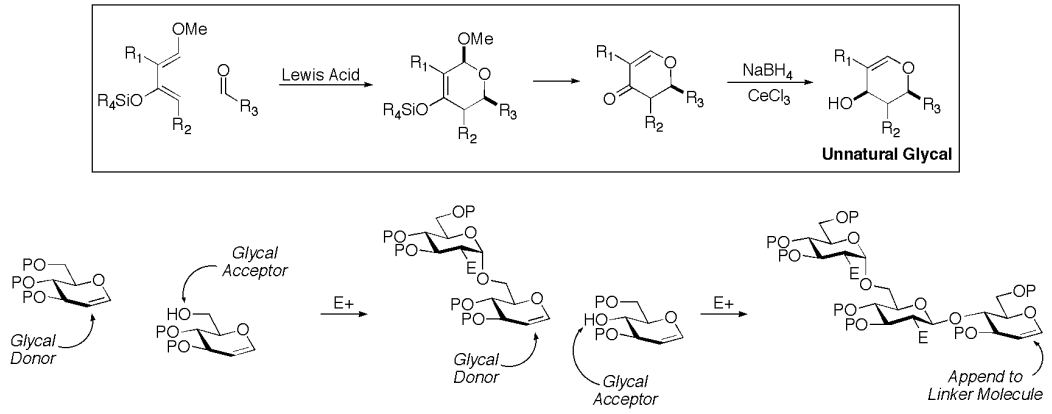
GLYCOPEPTIDE SYNTHESIS: GLYCAL ASSEMBLY

The post-translational glycosylation of proteins is a complex biological process, requiring the development and maintenance of elaborate systems of cellular machinery. Glycosylated proteins are observed widely throughout nature, and, given the elaborate systems required to synthesize such biomacromolecules in living cells, it seems likely that the carbohydrate sectors serve an important biological function. Indeed, protein glycosylation is seen to play a critical role in a range of cellular functions, including the mediation of protein folding [3], protection against proteolysis [4], promotion of cellular differentiation [5], and facilitation of intercellular communications [6].

A central challenge to this field of research is the isolation of useful quantities of structurally homogeneous glycoproteins. Indeed, many glycosylated proteins are heterogeneous in nature, consist-

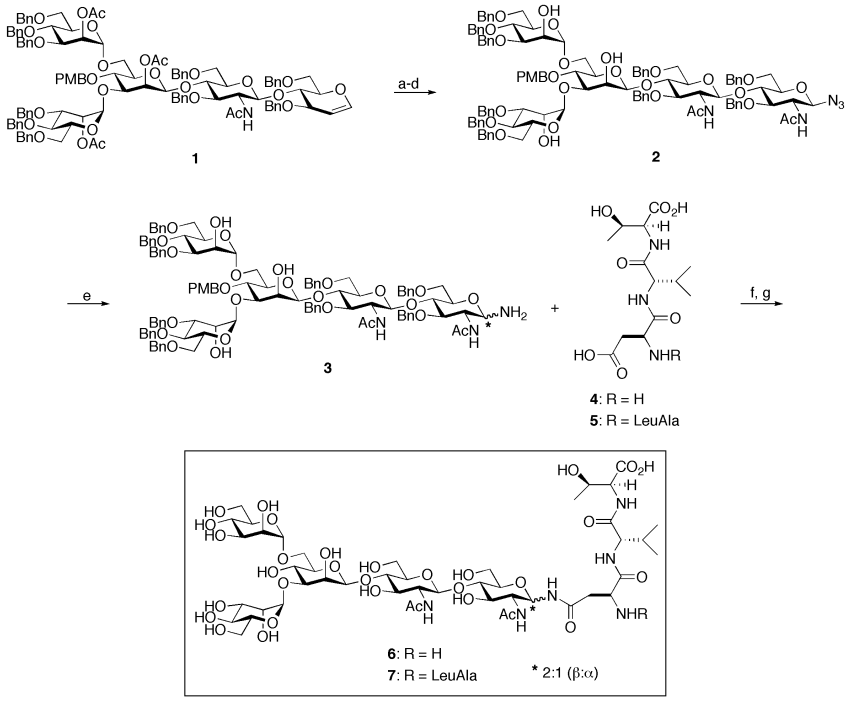
ing of multiple carbohydrate glycoforms, which cannot be readily separated through standard isolation procedures. For the purposes of rigorous biological investigation, it would be useful to have access to structurally homogeneous glycopeptides and even glycoproteins. It is in addressing this challenge that chemical synthesis has yet again revealed itself to be a remarkably powerful resource.

Long before we entered the arena of glycopeptide and glycoprotein synthesis, our laboratory had been involved in discovering and implementing methodology for the assembly of complex, structurally homogeneous carbohydrates. We started with our versatile and highly scalable paradigm of glycal assembly [7]. This method, outlined briefly in Scheme 1, can allow for the rapid elaboration of large and complex oligosaccharide domains, terminating in a glycal moiety. The terminal glycal, thus elaborated, can serve as a convenient handle for generating a new glycosyl donor to extend the domain still further. For building glycopolypeptides, the carbohydrate sector must be appended to the peptide either through an α-*O*-linkage to a serine (or threonine) residue or through a β-*N*-linkage to an asparagine (Asn) residue. Thus, as we started to investigate the applicability of our glycal assembly method to the synthesis of glycopeptides and glycoproteins, the first task would be the development of a means by which to append the fully synthetic carbohydrate sector to an amino acid—namely, an aspartate (Asp), which would thence become an asparagine residue.



Scheme 1 Logic of glycal assembly.

Our first effort to address the challenge of assembling homogeneous glycopeptides, drawing from our earlier efforts, is outlined in Scheme 2 [8]. Thus, the high-mannose pentasaccharide (**1**) was synthesized with heavy recourse to glycal assembly strategies. We had previously developed methods in our laboratory by which to achieve overall "acetamidoamination" of a terminal glycal olefin [9]. In line with our earlier disclosures, iodoanthracenesulfonamidation of **1**, followed by azidolytic turnstyle rearrangement, proceeded in good yield to afford, following protecting group manipulations, the azido glycan (**2**). At this stage, the hope was to effect the stereoselective reduction of the azide functionality. The realization of this goal presented a significant challenge. We were, in fact, well aware of the difficulties associated with the reduction of an anomeric azide of a carbohydrate in a complex setting, with maintenance of glycosidic integrity in the resultant anomeric amine. Competing processes might include anomerization of the initial azide or resultant amine. Also of concern was the possibility of *trans* acylation of the anomeric amine via the adjacent acetamido moiety. Nonetheless, on the basis of reports by Kunz and coworkers [10], which describe the use of the mild hydrogenation catalyst Raney nickel to effect reductions of saccharide substrates, we had been able to achieve stereoselective azide reductions in the context of simpler model systems. In the case at hand, however, all attempts to employ Raney nickel in the conversion of **2** to **3** led to disappointingly high levels of anomerization of the amino sugar. The anomeric mixture (**3**) could, in fact, be coupled to the Asp residue of a tripeptide (**4**) or of a



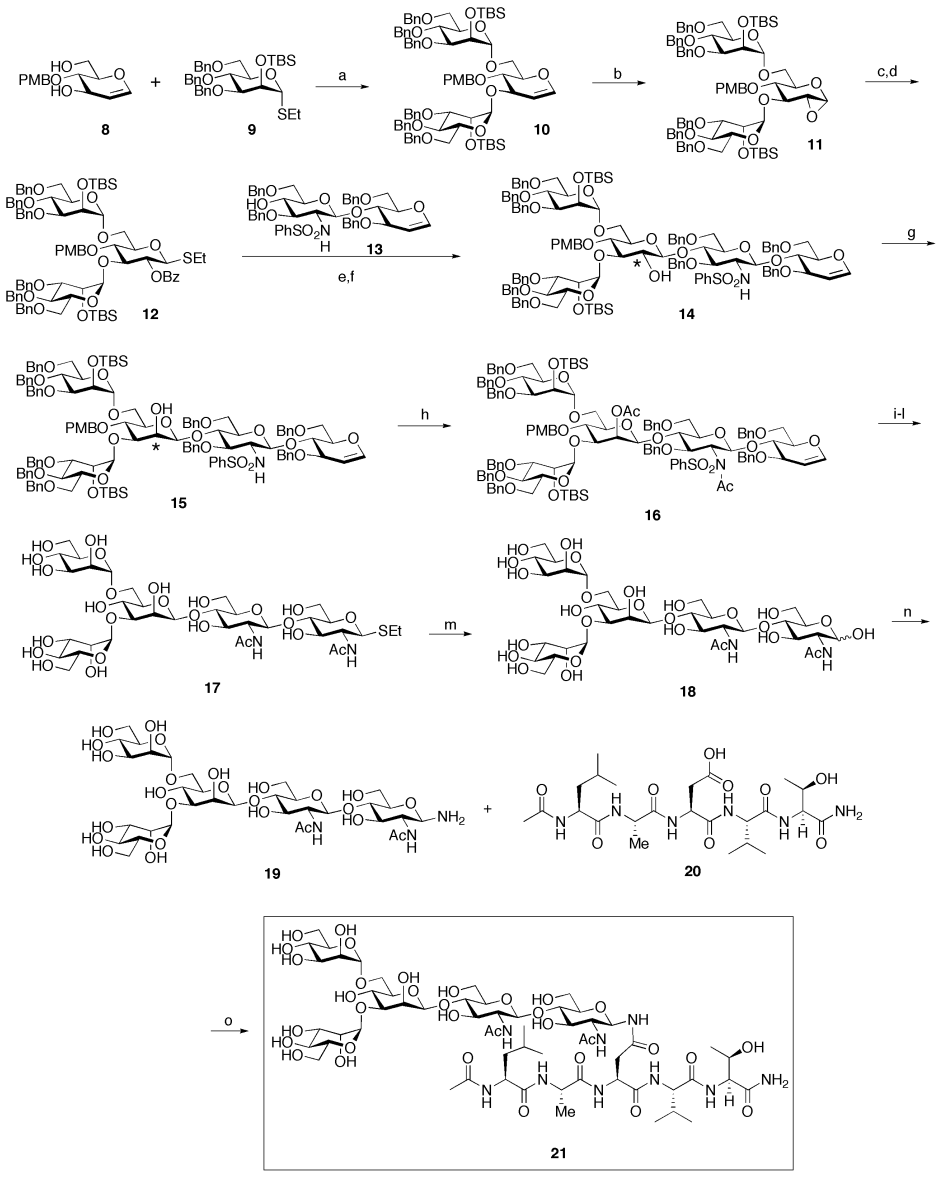
Scheme 2 First pentasaccharide glycopeptide synthesis. (a) $I(coll)_2ClO_4$, $AnthrSO_2NH_2$, 4A mol. sieves, then $TBAN_3$, 67 %; (b) Ac_2O, NEt_3, 86 %; (c) PhSH, DIPEA, 60 %; (d) NaOMe, MeOH, 94 %; (e) Ra-Ni, H_2; (f) IIDQ; (g) Pd/C, H_2, 80 % AcOH (aq.), 100 %.

pentapeptide (**5**) in respectable yields, to afford the glycopeptides **6** and **7** as 2:1 (β:α) anomeric mixtures (see asterisk). Clearly, we would have to develop a more stereoselective method for the advancement of the terminal glycal (**1**) to the coupling precursor anomeric amine (**3**).

Below, we recapitulate a stereospecific solution to the total synthesis of a complex carbohydrate *N*-linked to an Asp containing peptide [11]. Compound **10** was reached by two-fold α-mannosylation of **8**. At this stage, α-epoxidation of the glycal linkage was followed by thiolysis. Following protection of the C_2 hydroxyl as its benzoate (see **12**), the glucoside donor was coupled with lactosamine derivative **13**, to provide the β-glycosidic linkage. *Thus, the gluco-framework in the donating sugar had been used to ensure a β-linkage between the B and the C rings.* At this point, it was necessary to convert the α-benzoyloxy group in ring C from the glucose to the mannose stereochemistry. This was accomplished by an oxidation/reduction epimerization as shown (**14** → **15**). In this way, the proper stereochemistry of the mannose 5-mer was established.

Fortunately, a solution to the above-mentioned problem of the stereoselective installation of the β-anomeric amine was eventually identified. Thus, the high-mannose pentasaccharide glycal motif (**16**) was subjected to a standard iodosulfonamidation/ethane thiolate rearrangement sequence [12], as shown (Scheme 3), to afford, following global deprotection, the intermediate (**17**). NBS-mediated hydrolytic cleavage of the β-thioethyl moiety afforded the free reducing sugar (**18**). At this stage, through recourse to the enabling precedents of Lansbury [13] and Kochetkov [14], we were able to stereoselectively convert **18** to the β-amino anomer (**19**). The latter successfully underwent acylation with pentapeptide **20**, to provide the desired glycopeptide (**21**), as a *single stereoisomer* in 40 % yield.

Having identified a promising means by which to append a carbohydrate sector to an Asp residue of a short peptide fragment, we next sought to examine this methodology in the context of building a significantly more complex glycan system. We thus set for ourselves the challenge of synthesizing an

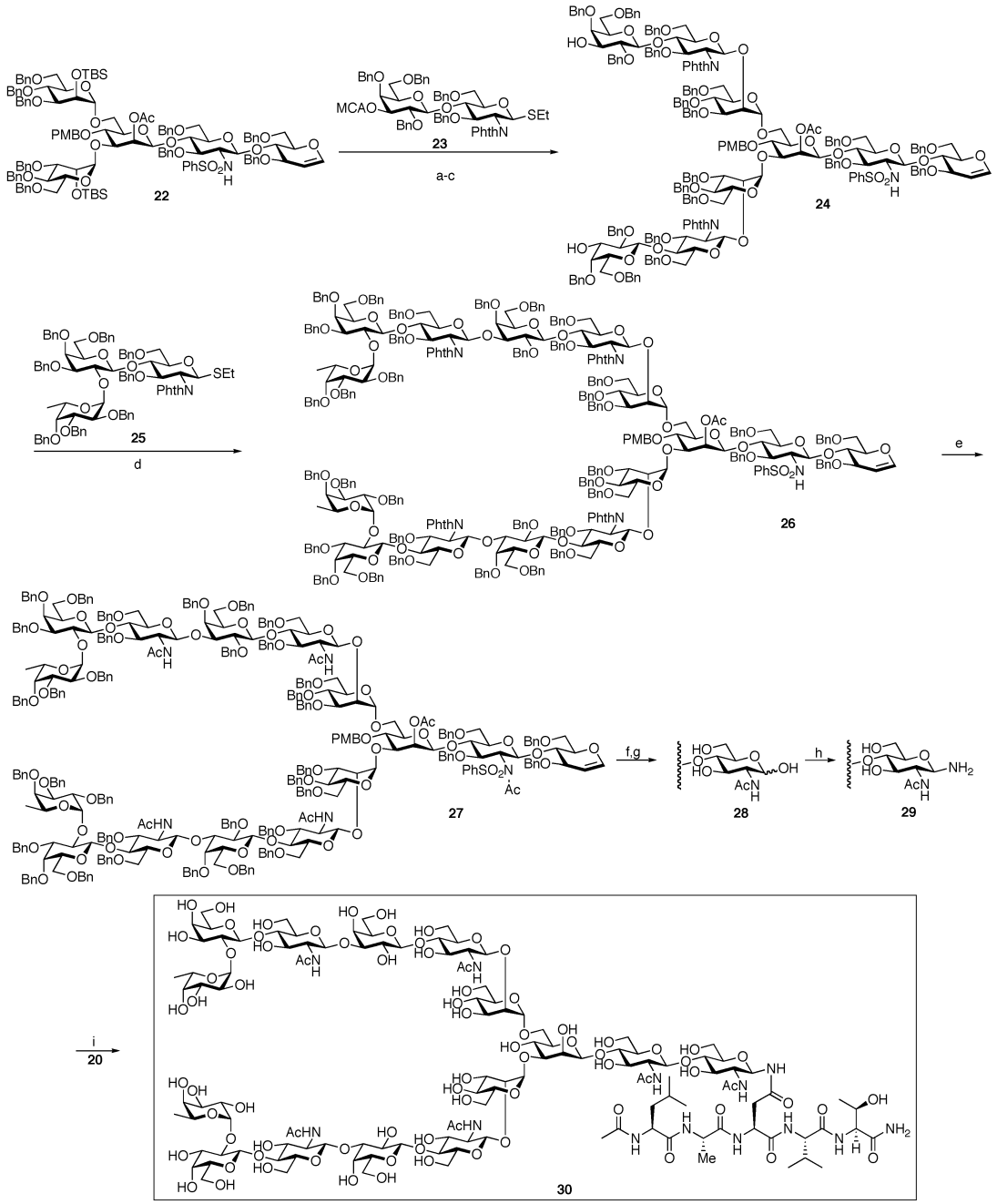


Scheme 3 Stereospecific synthesis of a pentasaccharide glycopeptide. (a) MeOTf, DTBP, DCM, 54 %; (b) DMDO, DCM, crude yield >99 %; (c) EtSH, TFAA, DCM, 76 %; (d) BzCl, pyridine, DCM, 90 %; (e) **13**, MeOTf, DTBP, DCM, 71 %; (f) $LiAlH_4$, Et_2O; (g) 1. Dess–Martin periodinane, DCM, 2. L-Selectride, THF; (h) Ac_2O, pyridine, DMAP, DCM, 82 % in four steps; (i) 1. IDCP, $PhSO_2NH_2$, DCM, 2. PhSH, LHMDS, DMF, 85 % in two steps; (j) TBAF, THF, 80 %; (k) 1. Na/NH_3, THF, 2. Ac_2O, pyridine, DMAP, 77 %; (l) NaOMe, MeOH, 86 %; (m) $HgCl_2$, $CaCO_3$, H_2O, 95 %; (n) NH_4HCO_3, H_2O, >99 %; (o) HOBt, HBTU, $^iPr_2NEt$, DMSO, 40 %.

N-linked, pentadecasaccharide glycopeptide construct, displaying a mature H-type 2 blood group properly linked at the reducing end of the determinant (**30**). At the time of these studies, no glycopeptide of this degree of complexity had been targeted for de novo chemical synthesis.

In the event, pentadecasaccharide **26** was prepared through a convergent, glycal assembly-based protocol [11b,15]. Thus, as seen, the removal of two appropriately-placed TBS protecting groups on pentasaccharide **22** served to expose two specific hydroxyl acceptor sites for two-fold introduction of

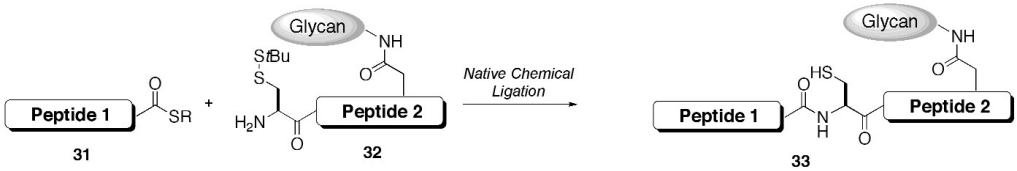
the *N*-acetyl lactosamine donor **23** (Scheme 4). Following appropriate management, two new acceptor sites in the lactosamine moieties of **24** were exposed. With this accomplished, two identical blood group



Scheme 4 Synthesis of a pentadecassacharide glycopeptide construct. (a) TBAF, THF, 77 %; (b) **23**, MeOTf, DTBP, DCM, 0 °C to rt, 62 %; (c) thiourea, $NaHCO_3$, EtOH, 99 %; (d) **25**, MeOTf, DTBP, DCM, Et_2O, 78 %; (e) 1. $NH_2CH_2CH_2NH_2$, EtOH, 2. Ac_2O, pyridine, DMAP, 85 % (two steps); (f) 1. IDCP, $PhSO_2NH_2$, DCM, 74 %; 2. LiHMDS, AgOTf, THF, H_2O, 63 %; (g) 1. Na/NH_3, THF; 2. Ac_2O, MeOH, 57 % (two steps); (h) NH_4HCO_3, H_2O, >99 %; (i) **20**, HOBt, HBTU, DIPEA, DMSO, 20 %.

determinant donors were inserted (**24** + **25** → **26**). In net terms, we had synthesized the fifteen-mer from an initial five-mer through the introduction of two lactosamines (two-mers) and then two H-type determinants (three-mers). The terminal glycal of the protected oligosaccharide (**27**) was upgraded to a hemiacetal through iodosulfonamidation, followed by base-mediated hydrolysis. Remarkably, we found the reducing end hemiacetal to be stable to global deprotection under dissolving metal reduction conditions. Thus, following exposure to sodium in liquid ammonia, the fully deprotected hemiacetal reducing sugar (**28**) was in hand. The latter was advanced to the β-situated anomeric amine (**29**) under the protocol developed for the synthesis of the pentasaccharide adduct (Scheme 3, above). As hoped, the anomeric amine (**29**) successfully underwent acylation with the Asp residue of pentapeptide **20**, to furnish the *N*-linked glycopeptide **30**, presenting the H-type 2 blood group specificity.

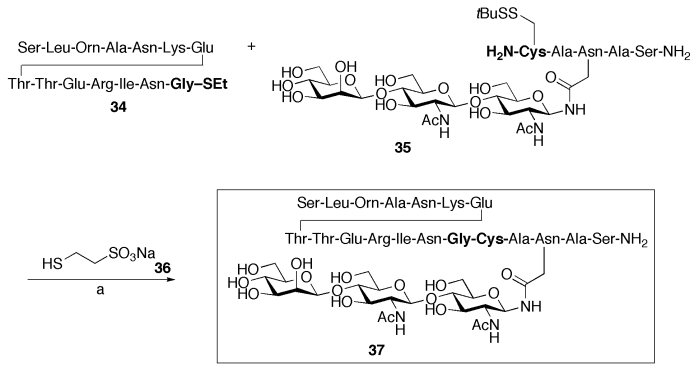
In establishing an efficient protocol by which to stereoselectively merge mature oligosaccharide and peptide domains, we had accomplished our first objective in the quest to begin to develop a menu of enabling methods for the synthesis of complex glycopeptides and glycoproteins. We next considered the synthetic implications of targeting constructs composed of much larger peptide fragments. While an ultimately convergent approach to such systems might conceivably involve the appendage of the mature glycan domain to the full peptide fragment in a single, climactic acylation event, such a strategy carries with it high risks in terms of synthetic practicality and flexibility. An alternative approach, outlined in Scheme 5, would first involve the merging of the glycan domain with a small peptide fragment, to afford a glycopeptide subunit (**32**) [16]. This intermediate would then be annealed with a longer peptide fragment (**31**) through native chemical ligation, to furnish the mono-glycosylated peptide adduct (**33**). This strategy would allow us to meet our primary requirement: that the precious, fully synthetic glycan be the limiting reagent in the sequence.



Scheme 5 Glycopeptide–peptide ligation strategy.

The viability of a ligation approach was demonstrated in the context of the trisaccharide pentapeptide **35** and the tetradecapeptide thioester **34** (Scheme 6). Following the native chemical ligation strategy pioneered by Kent and associates [17], the glycopeptide coupling partner (**35**), incorporating a *tert*-butyl disulfide-protected cysteine residue, was prepared through recourse to the glycal assembly and amination/aspartylation techniques described above. In the event, native chemical ligation with the polypeptide thioester (**34**) (itself synthesized on solid support through conventional methods) was performed in aqueous PBS in the presence of excess sulfonylethane-2-sulfonate (**36**). Upon global disulfide reduction with TCEP, the coupling partners smoothly underwent ligation to afford the fully unprotected glycopeptide adduct (**37**). Importantly, a single stereoisomeric adduct was observed, corresponding to the β-linked glycosylamide.

With the establishment of these important enabling protocols, a wide range of highly complex and biologically compelling glycopeptide target compounds now appeared to be, at least in principle, within the reach of de novo chemical synthesis. Hoping that progress would be possible, we identified some rather more complex targets to test the emerging skills of the laboratory. Moreover, we could envision application of these capabilities to problems of potential medical application. Specifically, our laboratory focused on: (1) the development of enhanced methods for the early diagnosis of prostate cancer, and (2) the development of novel carbohydrate-based HIV vaccines.



Scheme 6 Demonstration of a novel glycopeptide–peptide ligation protocol. (a) **36**, 0.2 M phosphate, 0.2 M NaCl, pH 7.4, 78 %.

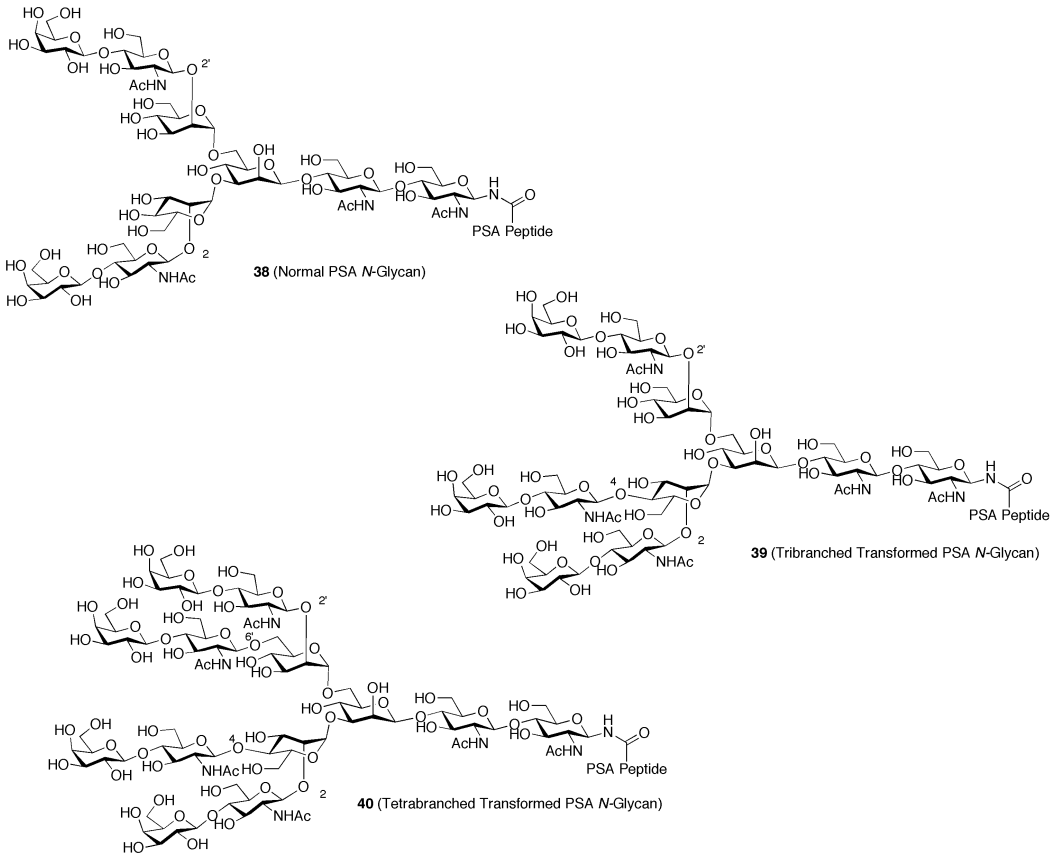
PROSTATE SPECIFIC ANTIGEN

The display of aberrant protein glycosylation patterns is recognized to be one of the indicative changes accompanying malignant transformation of a cell. As detailed elsewhere, we are actively engaged in a broad-based research program which seeks to exploit this unique phenotype of transformed cells for therapeutic benefit [18]. Thus, our fully synthetic carbohydrate-based tumor vaccine program seeks to induce a potent immune response against cancer cells over-expressing the relevant tumor-associated carbohydrates. Alternatively, in certain instances, one could envision the potential *diagnostic* benefit that might accrue from the ability to detect, at an early stage of cancer progression, the modified glycosylation patterns characteristic to a particular tumor type.

In this context, we took particular note of the need to develop improved methods for the diagnosis of prostate cancer. The state-of-the-art diagnostic methods for prostate cancer rely upon the evaluation of levels of prostate specific antigen (PSA). PSA is a 28 kDa glycoprotein which possesses a single site of N-glycosylation, and is secreted exclusively by the prostatic epithelium [19]. All currently available immunoassays are composed of antibodies which recognize only the peptide backbone of PSA [20]. However, ambiguities often arise in the interpretation of borderline PSA levels, rendering it difficult to distinguish between prostate cancer and benign prostatic hyperplasia [21].

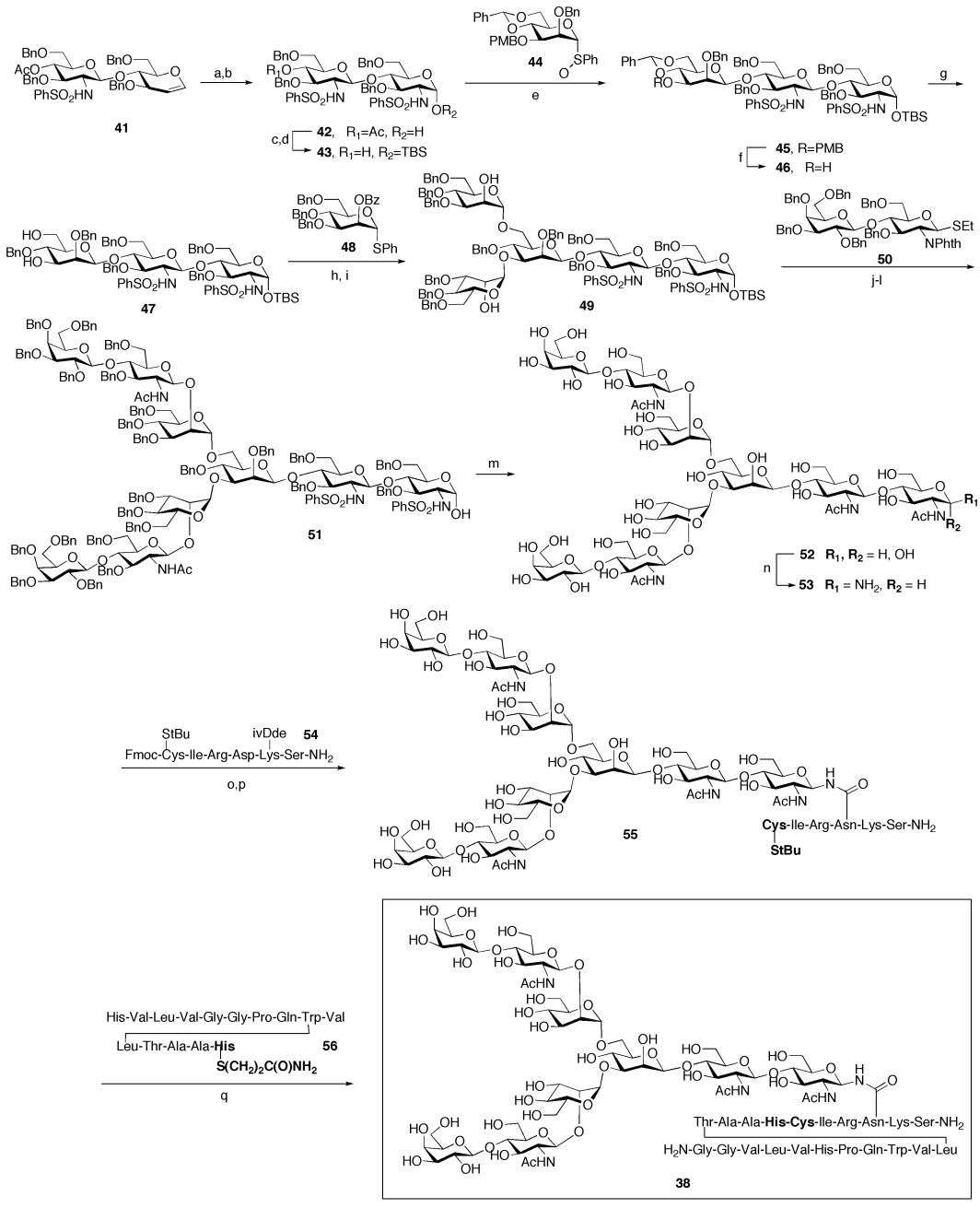
Of particular interest to our laboratory was the recent discovery that the PSA glycoproteins secreted by normal and transformed prostate cells differ appreciably in the degree of carbohydrate branching. Thus, the PSA produced by normal cells is only biantennary (cf. **38**, Scheme 7), while transformed prostate cancer cells may produce PSA glycoprotein presenting tri- or tetrabranched carbohydrate domains (cf. **39** and **40**) [22]. Because the antibodies only recognize the peptide backbone of the glycoprotein, current PSA detection methods are blind to such variations in the carbohydrate sector. Conceivably, an immunoassay that would provide information on the level of oligosaccharide branching could play an important role in the determination of the likely aggressiveness of the disease. Furthermore, a sufficiently sensitive assay might allow for the detection of malignant transformation at a very early stage of the disease, when serum PSA levels are not yet indicative.

In order to investigate this novel approach, we set out to prepare three different PSA glycopeptide fragments, incorporating normal (**38**) or transformed (**39** and **40**) carbohydrate domains. Seemingly, de novo chemical synthesis would be required in order to allow access to adequate levels of structurally homogeneous glycopeptide construct. We would then attempt to induce formation of antibodies, each of which would hopefully be at least selective for a single PSA glycoform.



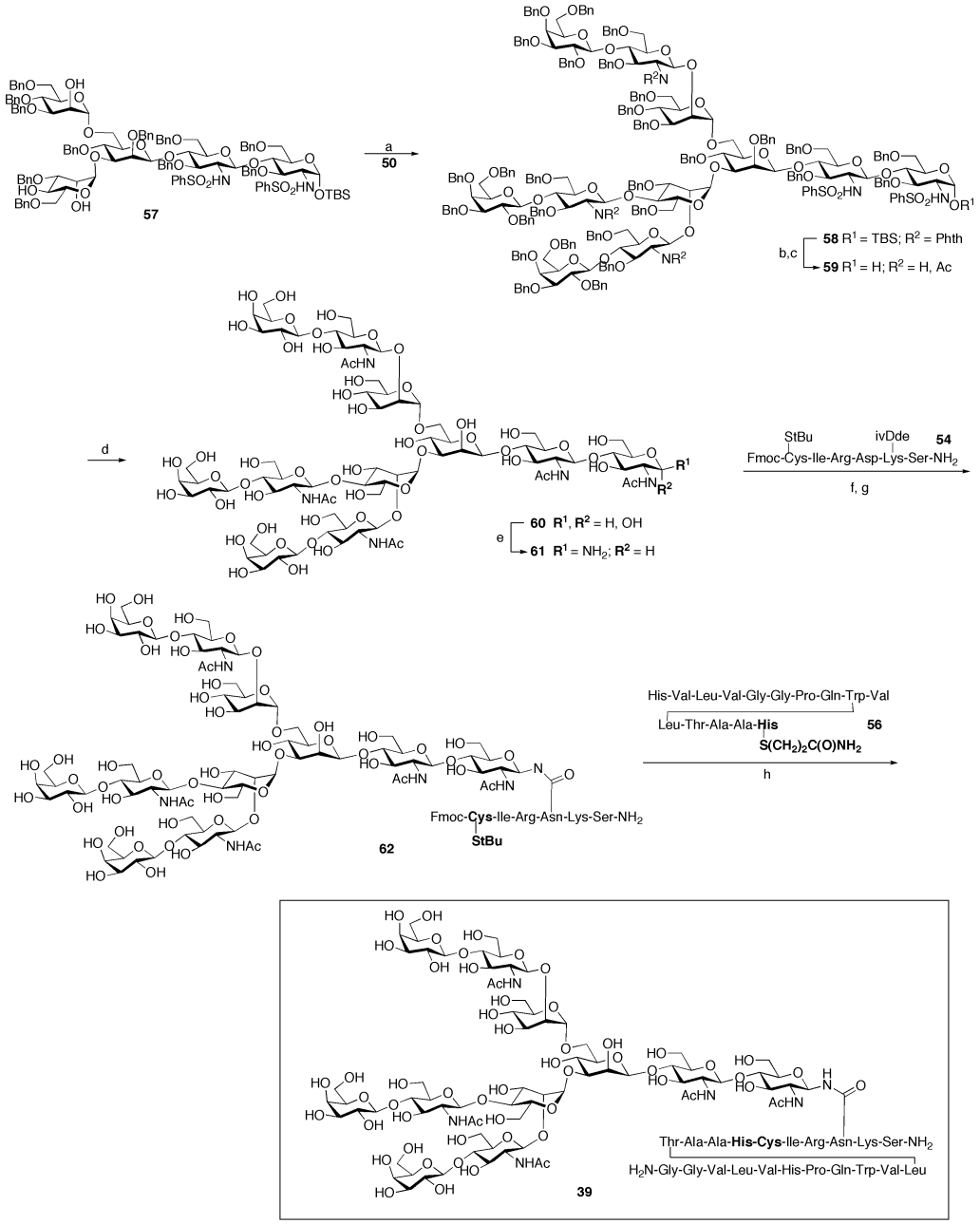
Scheme 7 Normal and transformed PSA glycans.

The synthesis of the normal, biantennary PSA glycopeptide (**38**) is outlined in Scheme 8 [23]. Thus, the nonasaccharide (**51**) was prepared through glycal assembly techniques. In designing this route, we sought to avoid the need for the technically demanding glucose to mannose transformation to adjust the stereochemistry in ring C, as had been required in our earlier synthesis of the pentasaccharide glycopeptide **21** (see Scheme 3, **14** → **15**). We also sought to remove the glycal functionality from ring A at an earlier point. It was felt that the presence of this labile glycal linkage throughout the course of the synthesis would tend to lower the yields of processes mediated by various Lewis acidic catalysts. Accordingly, a disaccharide glycal (**41**) was converted to the C_2 α-benzene sulfonamide/C_1 α-OTBS combination (cf. **43**) through well-established reactions in glycal assembly. At this stage, it proved possible to convert the disaccharide to trisaccharide **45** by direct mannosylation with **44**, using the advances pioneered by Kahne [24] and Crich [25]. Following exposure of the two hydroxy acceptor sites in ring C of trisaccharide **46**, two-fold α-mannosylation with donor **48** was accomplished (see pentasaccharide **49**). The synthesis continued with two-fold glycosylation of the two acceptor sites of **49** with lactosamine-derived donor **50**. This led eventually to **51**. Global Birch debenzylation once again proceeded without incident to provide **52**, with the reducing hemiacetal intact. Kochetkov amination provided the requisite β-glycosylamine, **53**, which was subsequently coupled with the hexapeptide **54** to afford, following removal of the Fmoc and ivDde protecting groups, the glycopeptide intermediate, **55**. At this stage, we were able to employ native chemical ligation with **56** to elongate the peptide fragment, as shown. Thus, through recourse to total synthesis, we were able to gain access to the normal PSA(27-47) glycopeptide fragment, presenting the homogeneous biantennary nonasaccharide (**38**). Analogous pro-

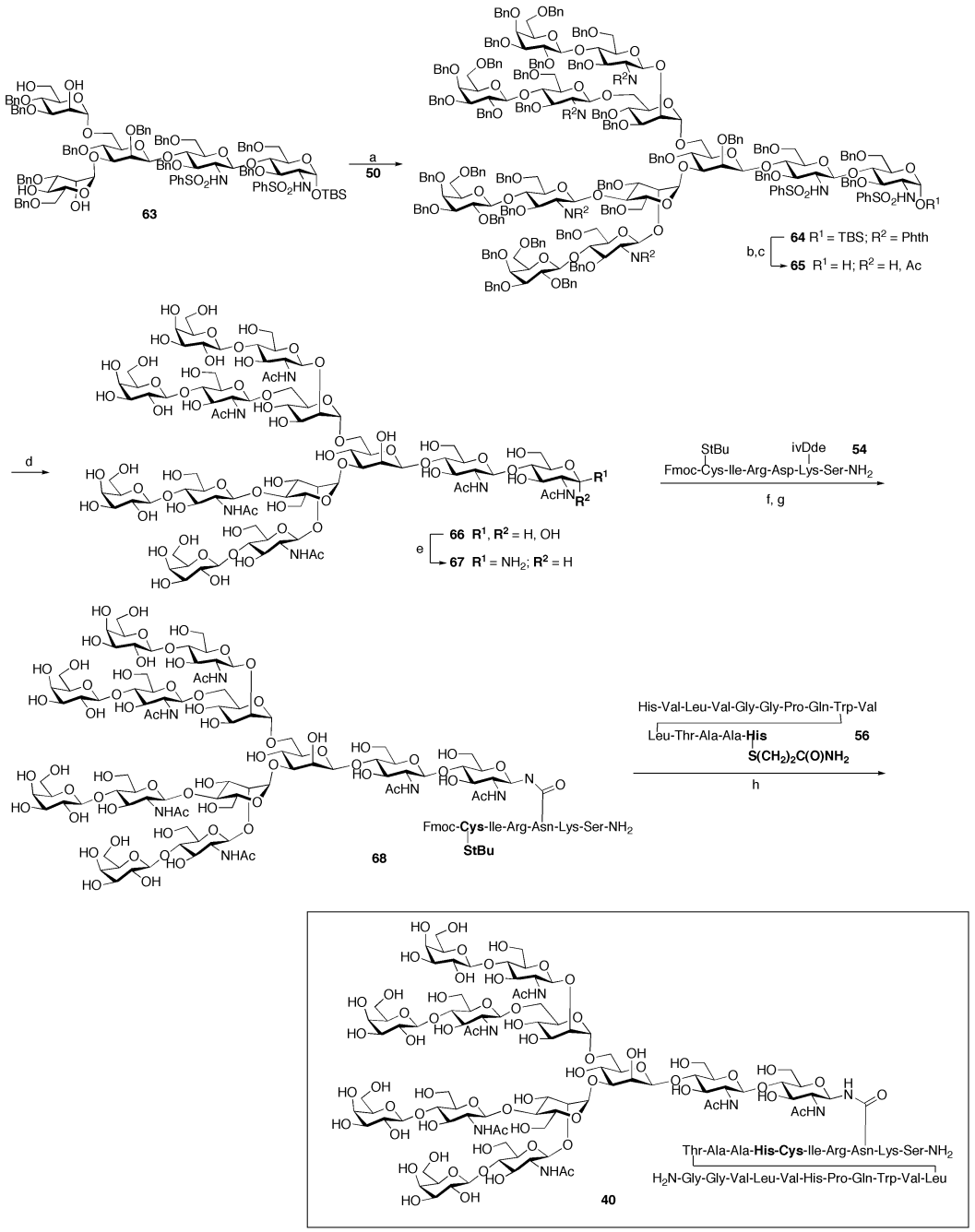


Scheme 8 Synthesis of the normal, biantennary PSA glycopeptide. (a) $I(coll)_2ClO_4$, $PhSO_2NH_2$; (b) Et_3N, H_2O/THF; (c) TBSOTf, 2,6-lutidine, CH_2Cl_2; (d) NaOMe/MeOH; 53–60 % for four steps; (e) i) Tf_2O, DTBMP, CH_2Cl_2, –78 °C, (ii) **44**, 85–91 % (β/α = 8/1); (f) CAN, MeCN/H_2O, 74 % for two steps; (g) Bu_2BOTf, BH_3 THF, 72 %; (h) **48**, $(BrC_6H_4)_3NSbCl_6$, MeCN, 74 %; (i) NaOMe/MeOH, 89 % for two steps; (j) MeOTf, DTBP, CH_2Cl_2, 60 %; (k) i. ethylenediamine, *n*-BuOH/toluene, 90 °C, ii. Ac_2O/py, iii. NaOMe/MeOH, 72 %; (l) TBAF/AcOH, THF, 76 %; (m) i. Na/NH_3, –78 °C, ii. Ac_2O, iii. NaOMe/MeOH, 65 %; (n) NH_4HCO_3/H_2O; (o) **54**, HATU, Hünig's base, DMSO, 61 % from **52**; (p) $(NH_2)_2$, piperidine, DMF, 62 %; (q) **56**, MES-Na, pH = 7.4

cedures were employed to synthesize both the tri- and tetrabranched transformed glycopeptide fragments (Schemes 9 and 10). In collaboration with the Scheinberg laboratory at Sloan-Kettering, we are currently attempting to produce and evaluate selective antibodies for each carbohydrate sector. These results will be disclosed shortly.



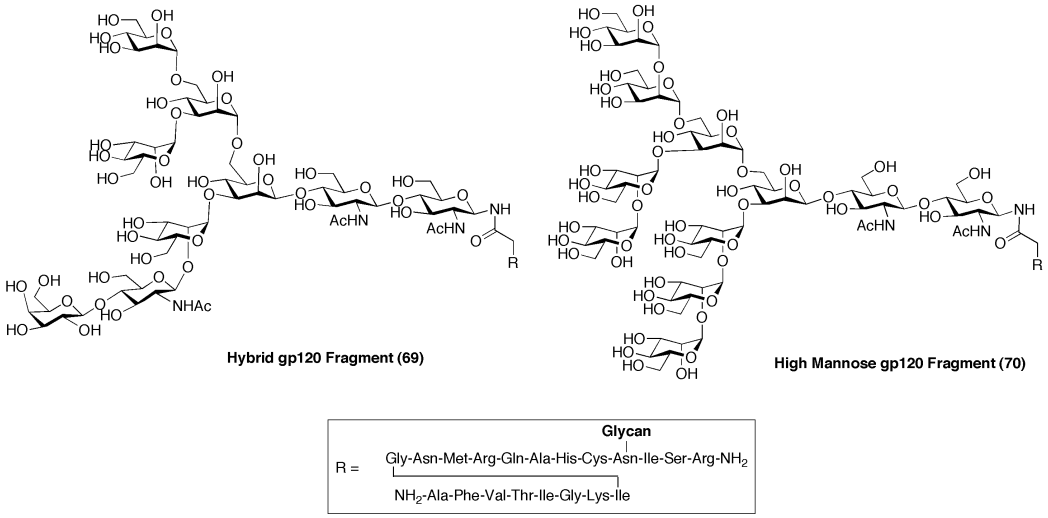
Scheme 9 Synthesis of the transformed, tribranched PSA glycopeptide. (a) **50**, MeOTf, DTBP, CH_2Cl_2, 41 %; (b) i. ethylenediamine, *n*-BuOH/toluene, 90 °C, ii. Ac_2O/py, iii. NaOMe/MeOH, 77 %; (c) TBAF/AcOH, THF, 95 %; (d) i. Na/NH_3, –78 °C, ii. Ac_2O, iii. NaOMe/MeOH, 94 %; (e) NH_4HCO_3/H_2O; (f) **54**, HATU, Hünig's base, DMSO, 25 % from **60**; (g) $(NH_2)_2$, piperidine, DMF, 66 %; (h) **56** MES-Na, pH = 7.4.



Scheme 10 Synthesis of the transformed, tetrabranched PSA glycopeptide. (a) **50**, MeOTf, DTBP, CH_2Cl_2, 19 %; (b) i. ethylenediamine, *n*-BuOH/toluene, 90 °C, ii. Ac_2O/py, iii. NaOMe/MeOH, 79 %; (c) TBAF/AcOH, THF, 97 %; (d) i. Na/NH_3, –78 °C, ii. Ac_2O, iii. NaOMe/MeOH, 81 %; (e) NH_4HCO_3/H_2O; (f) **54**, HATU, Hünig's base, DMSO, 25 % from **66**; (g) $(NH_2)_2$, piperidine, DMF, 52 % for three steps; (h) **56**, MES-Na, pH = 7.4, 65 %.

HIV VACCINES

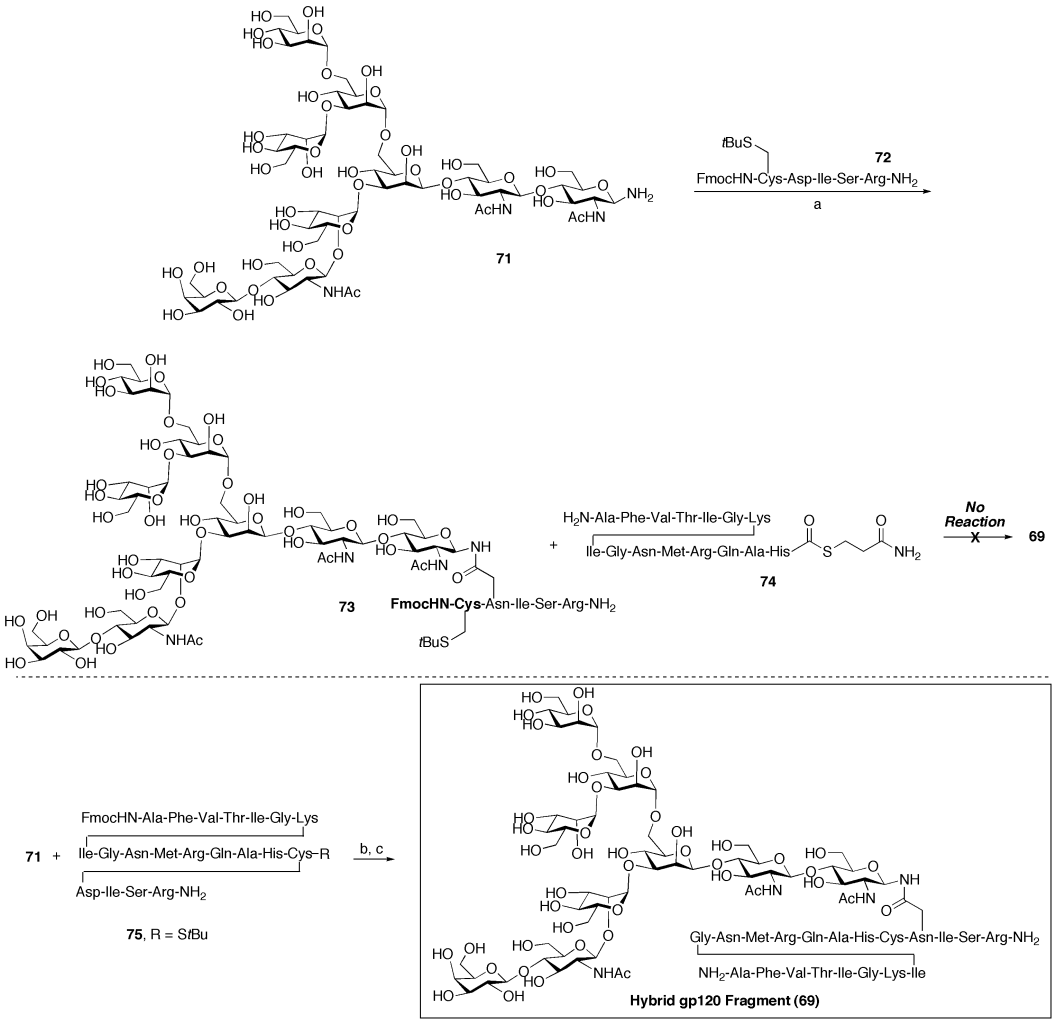
Despite ongoing and intensive efforts, the development of an effective vaccine against HIV has remained an elusive goal [26]. Even in vitro, the ability to demonstrate induction of broadly neutralizing antibodies is no small challenge. The failure to achieve success thus far may be attributed to the high rate of viral variation, as well as to the low immunogenicity of the protein sector of the viral surface envelope protein, gp120. Indeed, extensive glycosylation found on the surface of the gp120 glycoprotein may serve to effectively shield the polypeptide domain from recognition and attack by the immune system. The gp120 surface is coated with 24 different carbohydrate motifs, and, accordingly, we wondered whether these glycans could themselves be effectively targeted by a well-designed anti-HIV vaccine [27]. In this regard, we were encouraged that a number of gp120 glycans are highly conserved and are presented on the presumably more accessible outer side of the gp120 trimer. We took further note of some findings regarding the 2g12 antibody, one of the most potent anti-HIV antibodies currently known. Interestingly, 2g12 appears to bind to the hybrid- or high-mannose type carbohydrate domains of gp120 [28]. With this information in hand, we set, as our first objective, the synthesis of hybrid and high-mannose gp120 fragments (Scheme 11, **69** and **70**). Antibodies subsequently raised against each of these synthetic glycopeptides would hopefully serve as effective agents against the gp120 envelope protein of HIV.



Scheme 11 Hybrid and high-mannose gp120 fragments.

We emphasize once more the central role that chemical synthesis must play in this research program. Indeed, despite the undeniable challenges associated with the de novo synthesis of such complex constructs, it is our judgment that total synthesis represents the most promising means by which to obtain adequate levels of these glycopeptides in structurally homogeneous form for rigorous evaluation and use. Furthermore, through total synthesis, we retain the flexibility necessary to target a diverse range of glycopeptide congeners.

In designing our synthetic glycopeptide construct, we elected to synthesize the $gp120^{316\text{-}335}$ peptide fragment, encompassing Asn 332, which displays the hybrid or high-mannose oligosaccharide domains. The preparation of the hybrid gp120 glycopeptide fragment (**69**) is outlined in Scheme 12 below [29]. Nonasaccharide (**71**) was prepared through the glycal assembly and Kochetkov amination methods described above. At the planning stages, we proposed to first assemble the $gp120^{331\text{-}335}$ substructure (cf. **73**). This short glycopeptide fragment, possessing a Cys 331 residue, would subsequently be

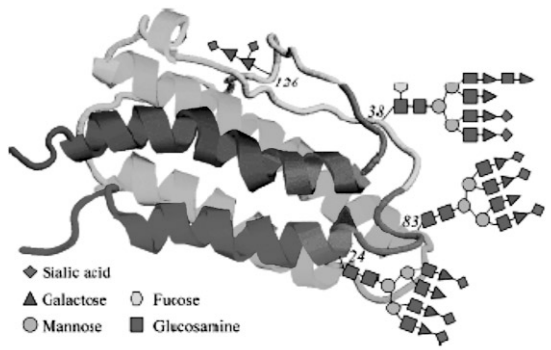


Scheme 12 Synthesis of the hybrid gp120 fragment. (a) 1. HATU, DIPEA, DMSO; 2. NH_2NH_2, piperidine, DMF, 30 %; (b) 1. HATU, DIPEA, DMSO; 2. NH_2NH_2, piperidine, DMF, 20 %; (c) Mes-Na, DMF, DIPEA, H_2O, 95 %.

ligated with a longer peptide fragment. In the event, however, although aspartylation proceeded smoothly to furnish the glycopeptide, **73**, the subsequent native chemical ligation with peptide **74** could not be achieved under any conditions evaluated. Ultimately, we were forced to append the entire peptide fragment to the carbohydrate domain in a single acylation event (see coupling of **71** and **75**). This transformation, while successful, proceeded in a not surprisingly modest (20 %) yield. Nonetheless, we were able to employ this coupling strategy to gain access to the hybrid gp120 glycopeptide fragment (**69**). A similar procedure was employed for the synthesis of the high mannose gp120 fragment (**70**) [30]. In summary, although our synthetic goals were ultimately attained, the failure of the ligation to proceed as hoped was a setback. More recent investigations of this phenomenon reveal that the nature of the amino acid sequence and the positioning of the carbohydrate is likely an important factor in determining the facility with which the subsequent native chemical ligation may occur.

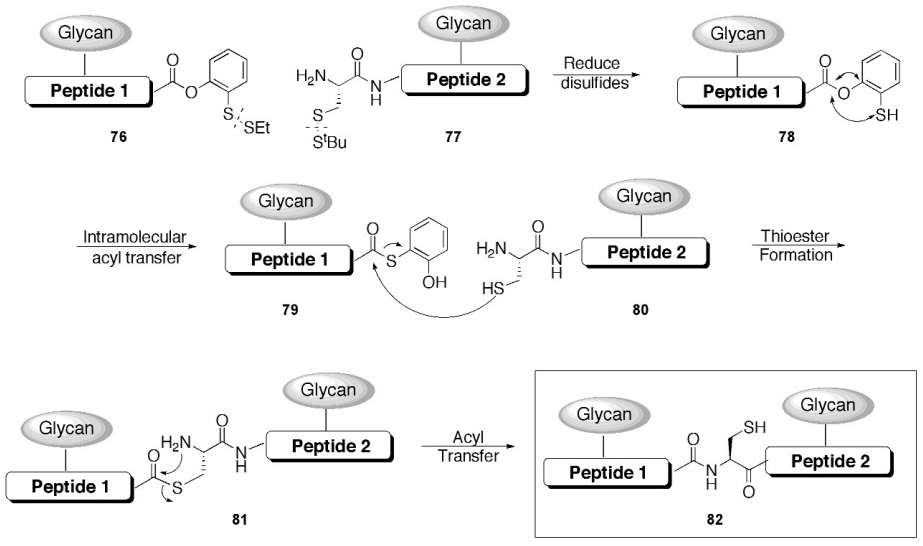
ERYTHROPOIETIN

With our completion of the syntheses of the normal and transformed PSA glycopeptides as well as the hybrid and high-mannose type gp120 glycopeptides, we had established the ability of our synthetic methodologies to provide access to significant quantities of biologically relevant, structurally complex glycopeptide fragments. Having met our early-stage objectives, we soon set our sights on a much more complicated and daunting target molecule: *erythropoietin alpha* (Scheme 13). Erythropoietin alpha (EPO), a heterogeneous 166-residue glycoprotein containing four sites of glycosylation (one *O*-linkage at Ser^{126} and three *N*-linkages at Asn^{24}, Asn^{38}, and Asn^{83}) [31], is a medicinally important agent which is widely used in the treatment of anemia. The critical role that the carbohydrate domains play in conferring efficacy and stability to the glycoprotein has been well documented. However, a rigorous comparison of the therapeutic value of various glycoforms of EPO has thus far been significantly hindered by the difficulties associated with isolating homogeneous EPO from natural sources [32]. Certainly, the complexities associated with attempting to synthesize, de novo, a multiply glycosylated protein such as EPO cannot be understated. Nonetheless, we recognized in EPO the potential for chemical synthesis to critically assist in addressing highly relevant biological questions. Furthermore, we were virtually compelled to attempt a total synthesis of EPO simply for the sheer chemical challenges that would surely arise. Indeed, as will be seen, our ongoing synthetic EPO program has presented a number of opportunities to expand upon the scope of known glycopeptide methodologies. In addition to enabling our synthesis of EPO, we expect that these advances will prove useful in the synthesis of a range of other glycopeptide and glycoprotein targets.



Scheme 13 EPO alpha.

In order to realize our overarching objective; i.e., the total synthesis of homogeneous multiply glycosylated EPO, we would first need to establish a means by which to efficiently ligate two glycopeptide fragments, each possessing a different oligosaccharide domain (Scheme 14). As described above, we had earlier developed the capacity to extend the peptide chain of a singly glycosylated peptide through a glycopeptide–peptide ligation protocol (cf. Scheme 5). A direct extension of this previously described method to encompass the coupling of two glycopeptide fragments was not considered to be a practical solution to this challenge. Thus, under our glycopeptide–peptide native chemical ligation (NCL) technique, the peptide fragment is equipped with a preformed thioester functionality. In envisioning an analogous glycopeptide–glycopeptide ligation, we were concerned about the practical difficulties that would ensue in attempting to gain access to a preformed glycopeptide thioester through convergent means. As an alternative, we considered the possibility of installing a *latent* activating moiety on one of the glycopeptide fragments. Thus, as portrayed in Scheme 14, two differentiated glycopeptide units would be assembled [33]. One fragment (**76**) would be equipped with a *C*-terminal phenolic ester possessing an *ortho* disulfide moiety, while the other would incorporate a protected,

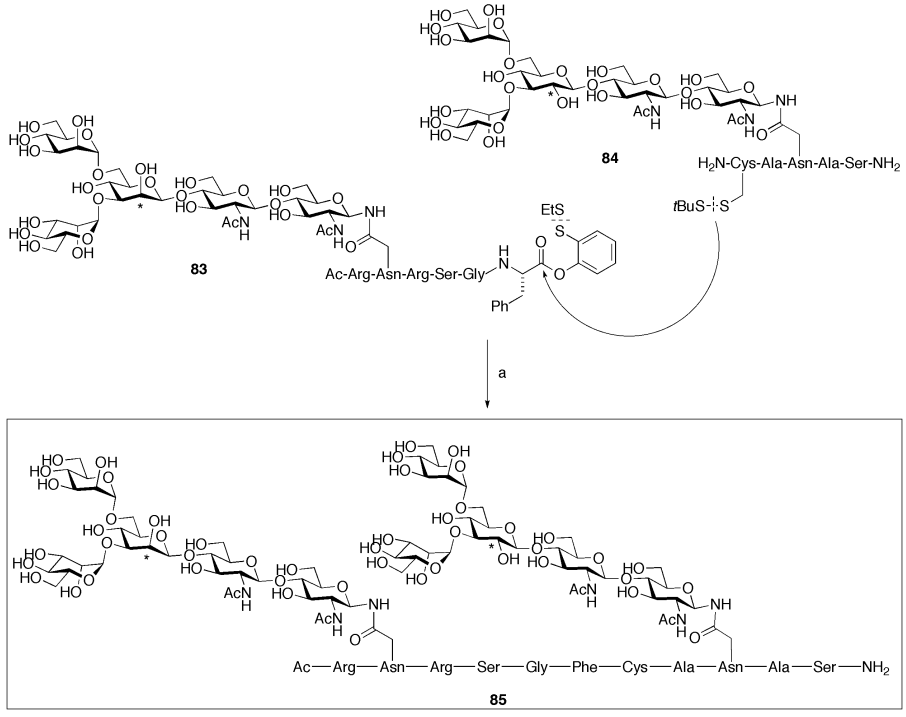


Scheme 14 Strategy for a novel glycopeptide–glycopeptide ligation.

N-terminal cysteine residue (**77**). We envisioned that, upon simultaneous reduction of the two disulfides, the phenol of fragment **78** might undergo intramolecular $O \rightarrow S$ migration. This intermediate thioester was expected to be sufficiently activated to allow intermolecular thioester formation by the free cysteine residue of glycopeptide **80**. Finally, following intramolecular acyl transfer, a bidomainal glycopeptide, incorporating two differential sites of glycosylation, would be in hand. We note that, although we suspected that the phenolic ester (**78**) could indeed undergo intramolecular acyl transfer to some degree, to generate the thioester (**79**), such an event need not necessarily be critical to the success of the ligation. Indeed, one could imagine that the presence of a free *ortho* benzene thiol function might well serve to significantly activate the phenolic ester toward intermolecular acyl transfer. Furthermore, we anticipated that, even if $O \rightarrow S$ migration would occur, the thioester intermediate would likely exist in an unfavorable yet dynamic equilibrium. The mechanistic underpinnings of this transformation would later be examined in some detail (see below).

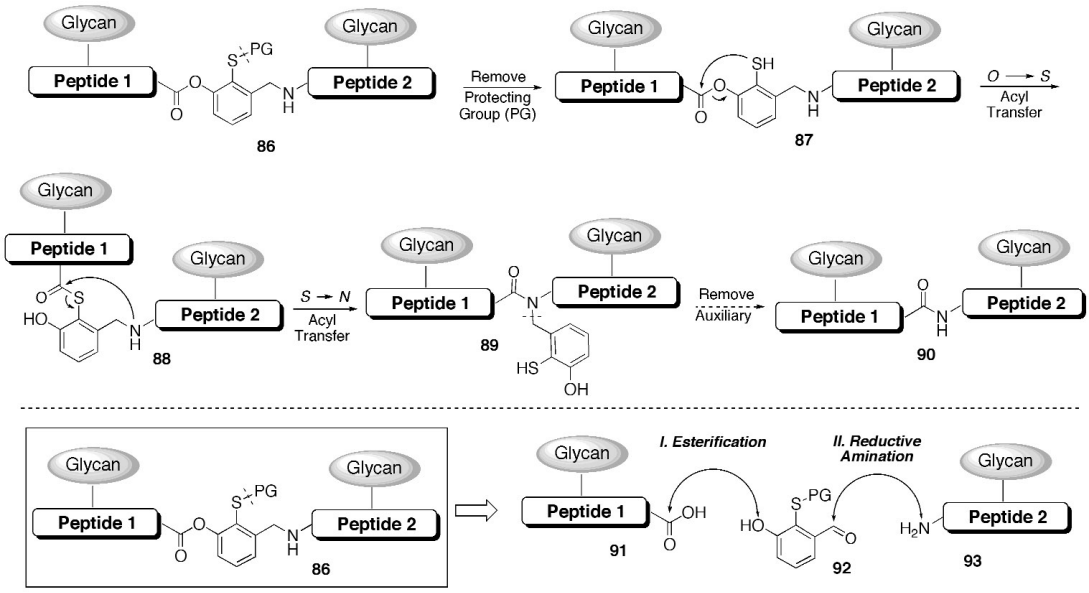
Indeed, the rationale behind this proposed native chemical ligation protocol was borne out experimentally. Two glycopeptide fragments were prepared through standard techniques. One fragment (**83**) was equipped with an *ortho* disulfide phenolic ester, while the other (**84**) presented a protected *N*-terminal cysteine residue (Scheme 15). Following Mes-Na mediated disulfide reduction, the glycopeptide fragments smoothly underwent ligation to provide the polypeptide adduct (**85**), incorporating two different carbohydrate sectors. This cysteine-based native chemical ligation strategy has been successfully extended to the syntheses of a range of doubly glycosylated polypeptides.

While this newly developed, cysteine-based glycopeptide–glycopeptide NCL strategy would surely play a key role in our eventual synthesis of EPO, an examination of the primary amino acid sequence of EPO revealed a limitation of our current methodological capacities. Thus, an ultimately convergent route to EPO would involve the separate preparation of four glycopeptide fragments of relatively equal size, each of which would present one of the EPO carbohydrate domains. These four glycopeptide sectors would then be iteratively coupled through NCL to afford the fully glycosylated protein. In considering this perspective, we took note of the relative paucity of cysteine residues on the EPO backbone. In fact, EPO possesses only four cysteine residues, which are positioned in such a way that they do not partition the protein into four roughly equally sized carbohydrate-bearing domains. Thus, for practical reasons, it would be necessary to develop a non-cysteine-based glycopeptide ligation protocol that could be used in concert with our newly developed cysteine-based strategy.



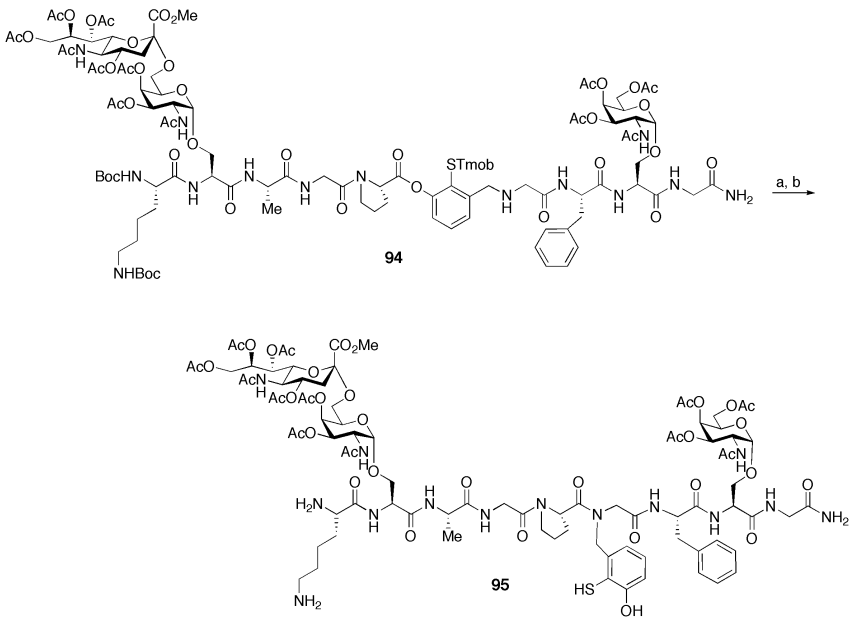
Scheme 15 Demonstration of a novel glycopeptide–glycopeptide ligation protocol. (a) 0.2 M NaCl, pH ~7.4, excess Mes-Na, 75 %.

In the course of mechanistic studies of the cysteine-based NCL strategy, we had observed that, following disulfide reduction, the *ortho*-thiol phenolic ester apparently undergoes *complete* intramolecular acyl transfer to afford the thioester intermediate (cf. **78** → **79**, Scheme 14). On the basis of this surprising finding, we began to consider a cysteine-free ligation strategy that would exploit the intermediacy of the glycopeptide thioester. Our proposed ligation, briefly outlined in Scheme 16, would commence with a substrate (**86**), wherein the two glycopeptide fragments are positioned in a *meta* arrangement upon a benzylic framework [34]. A protected thiol would reside between (*ortho* to) the two glycopeptide fragments. It was envisioned that thiol deprotection would set the stage for an $O \rightarrow S$ acyl transfer (**87** → **88**). The resulting thioester would be positioned for intramolecular $S \rightarrow N$ acyl transfer with the amine of the second glycopeptide, to afford, following auxiliary removal, a cysteine-free ligated glycopeptide (**90**). Through this strategy, we hoped to harness the benefits of intramolecularity while relieving our dependency on the *N*-terminal cysteine residue, which, in our original strategy, had served the critical function of transiently joining the two glycopeptide fragments. The unimolecular substrate (**86**) would be synthesized as shown in Scheme 16, through a three-component coupling of the two glycopeptides (**91** and **93**) with the phenolic aldehyde **92**.



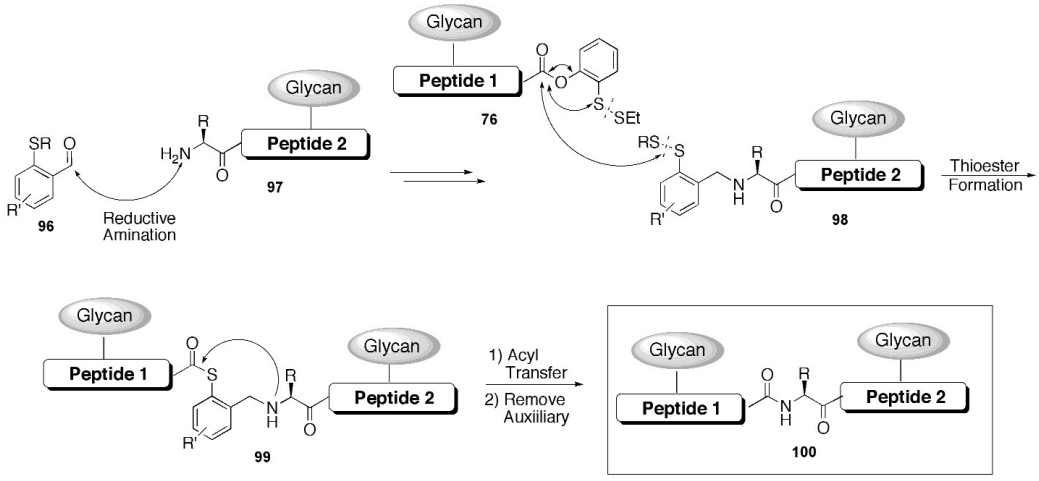
Scheme 16 Strategy for a cysteine-free glycopeptide–glycopeptide ligation.

In the event, we were pleased to find that, following thiol deprotection, the unimolecular substrate **94** did indeed undergo the hoped-for ligation, to provide the bidomainal glycopeptide **95** in 72 % yield (Scheme 17). The ultimate applicability of this transformation to the merger of EPO glycopeptide fragments has not yet been realized, due to our inability to remove the resultant phenolic auxiliary. In the meantime, an alternative and highly useful cysteine-free ligation strategy has been developed.



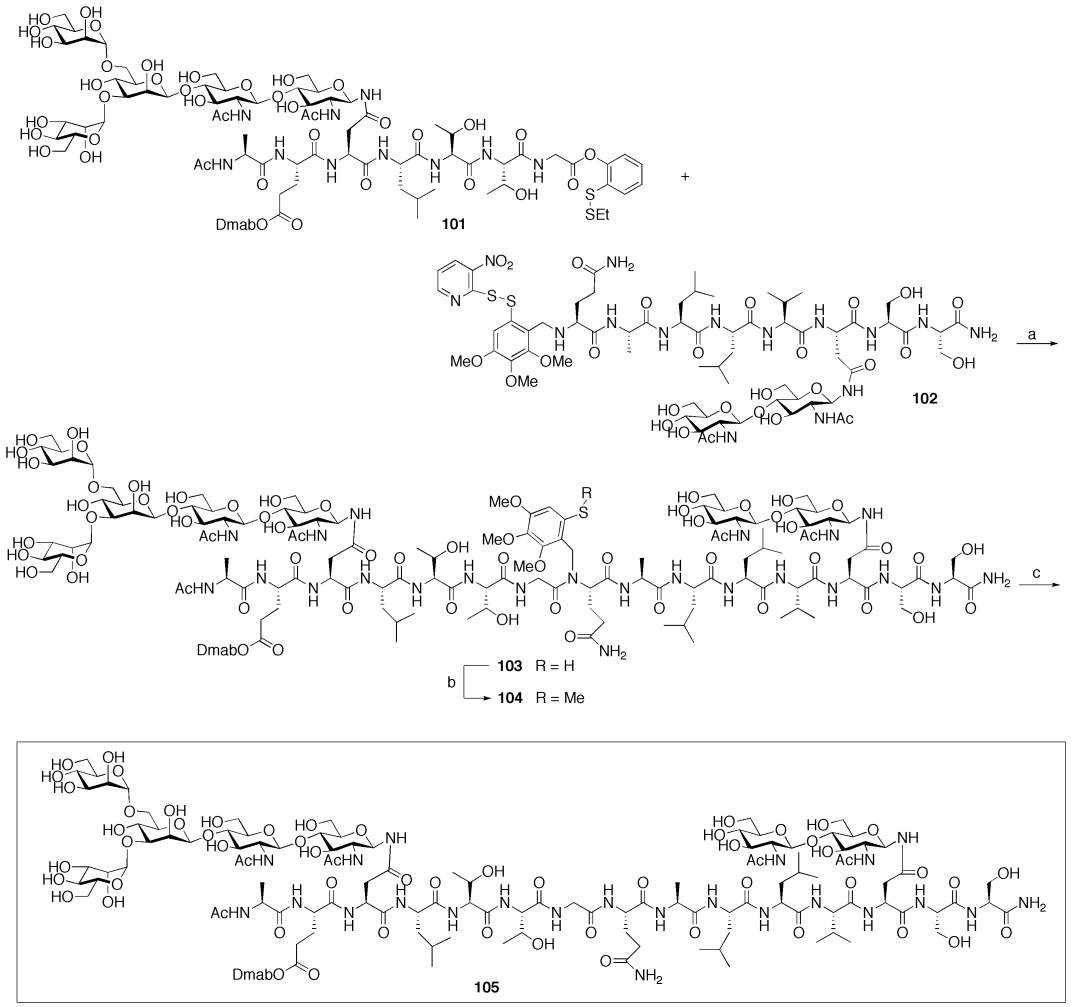
Scheme 17 Demonstration of a cysteine-free glycopeptide–glycopeptide ligation protocol. (a) 50 % TFA/0.5 % TIPS, CH_2Cl_2, 35 min; (b) MeOH, NaH_2PO_4, 10 h, 72 % (two steps).

The principles underlying our cysteine-free NCL strategy are based heavily upon those which informed our cysteine-based ligation protocol. The *N*-terminal cysteine residue had served the critical function of providing a temporary linkage between the two glycopeptide units, thus facilitating the subsequent ligation. As outlined in Scheme 18, we hoped to install, on the *N*-terminal glycopeptide moiety, a thiol auxiliary which would similarly serve to temporarily engage the two fragments, thus bringing the reactants into suitable proximity to undergo the requisite acylation [35]. According to this strategy, the *N*-terminus of one glycopeptide would be equipped with a protected thiobenzene auxiliary through reductive amination, as shown (cf. **97** + **96** → **98**). The second glycopeptide unit (**76**) would present the *ortho* disulfide phenol functionality employed in the cysteine-based ligation protocol. The hope was that, following disulfide reduction and $O \rightarrow S$ migration of **76**, the free thiol of the *N*-terminal glycopeptide substrate would undergo thioester formation to provide the unimolecular intermediate (**99**), which would be appropriately positioned for acyl transfer to furnish, following auxiliary removal, the bidomainal glycopeptide adduct (**100**).



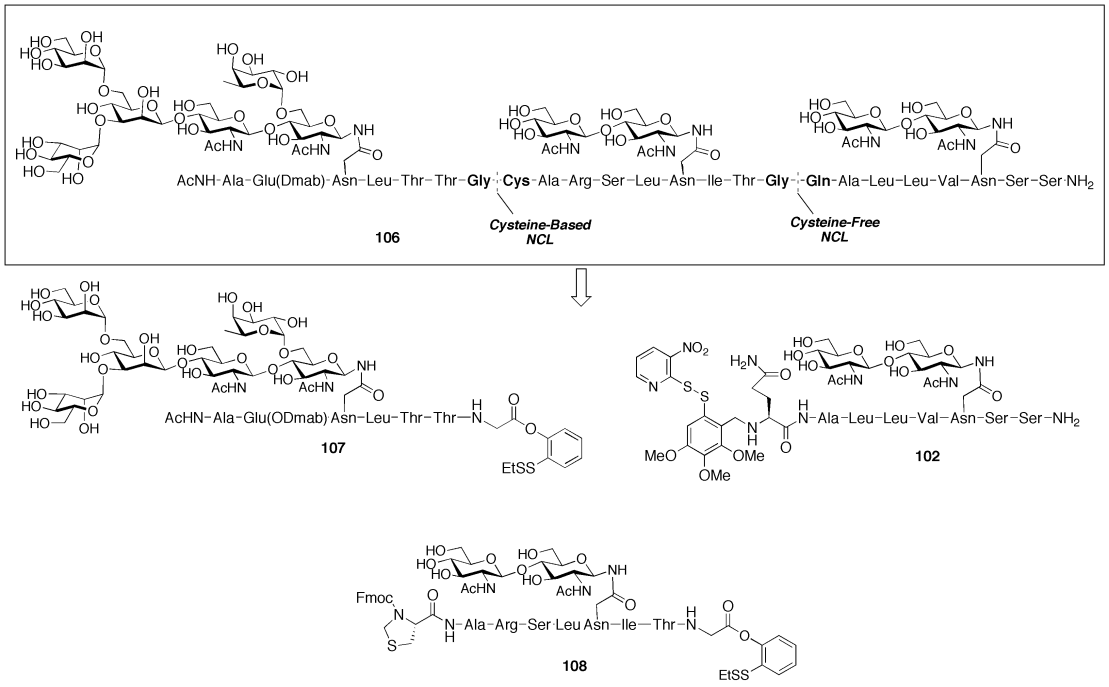
Scheme 18 Strategy for a widely useful cysteine-free glycopeptide–glycopeptide ligation.

Happily, this proposed cysteine-free ligation sequence was indeed reduced to practice, even in complex settings. Thus, glycopeptide units **101** and **102** were prepared through standard glycal assembly/aspartylation methods and were equipped with the appropriate *C*-terminal and *N*-terminal auxiliaries (Scheme 19). Following TCEP-mediated disulfide reduction, the two units readily underwent the hoped-for ligation to provide the glycopeptide adduct (**103**). An efficient two-step auxiliary removal procedure was developed, wherein the free thiol of the auxiliary was first equipped with a methyl group (**104**). Following exposure to TFA, the auxiliary was cleaved to provide the ligation adduct (**105**), as shown. While our version of non-cysteine-based chemical ligation has shown considerable promise and can be integrated into an EPO synthesis, there is a limitation which should be plainly stated. In the last S → N migration (cf. **99** → **100**), difficulties arise if the *C*-terminal and the *N*-terminal amino acids contain significant substitutions in the side chain. The system works well when at least one of the components is a glycine or even an alanine. However, much lower yields are encountered in this last acyl transfer when both amino acids contain branched side chains or extensive functionality.



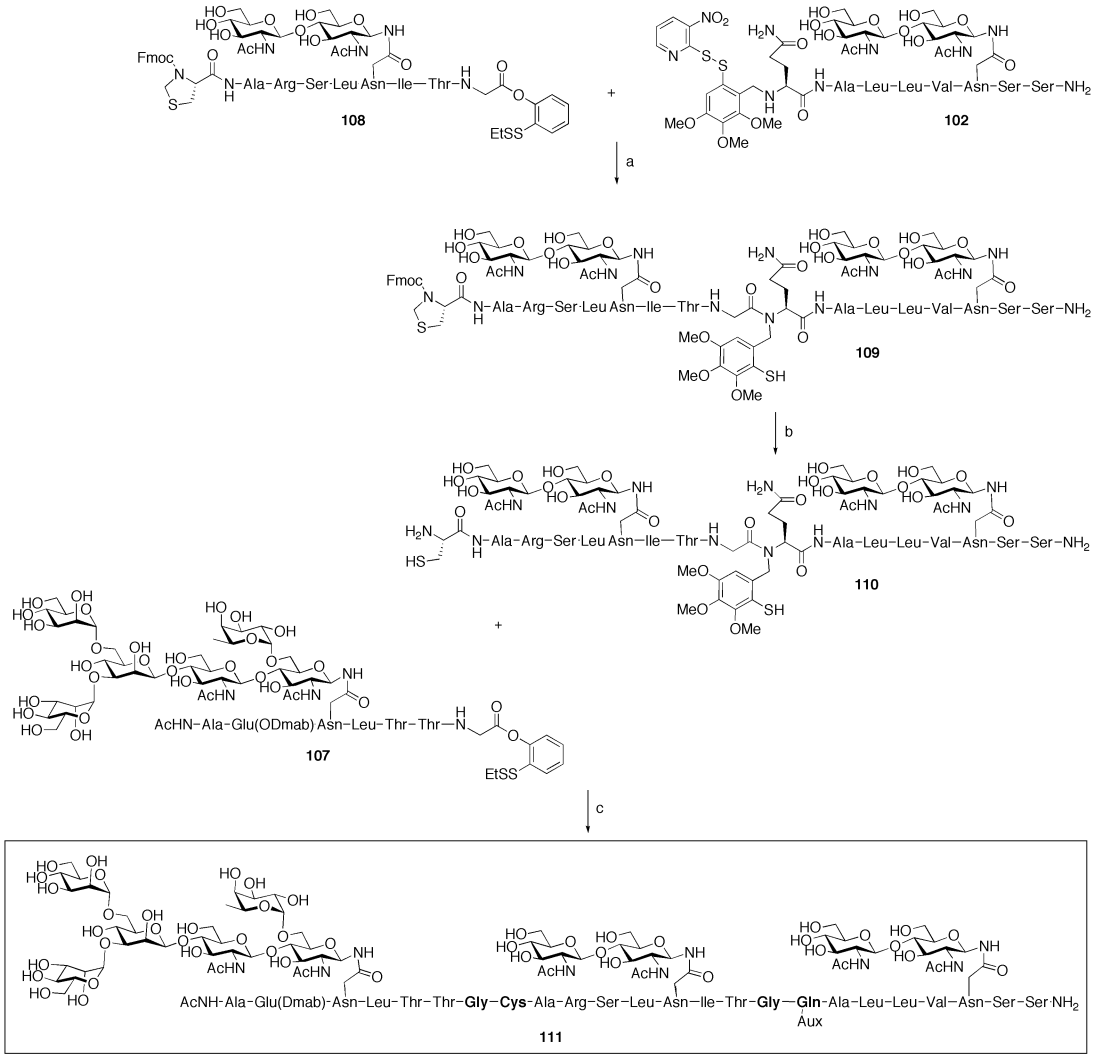
Scheme 19 Demonstration of a novel cysteine-free glycopeptide–glycopeptide ligation. (a) TCEP, DMF, Na_2HPO_4, 32 ºC, 54 %; (b) methyl *p*-nitrobenzene sulfonate; (c) 95 % TFA.

With powerful cysteine-based and cysteine-free ligation techniques in hand, it would now be necessary to evaluate the feasibility of employing these methodologies in tandem in order to gain access to longer peptide fragments incorporating more than two sites of glycosylation. With the long-term goal of synthetic EPO in mind, we designed a model peptide (**106**), incorporating three sites of glycosylation (Scheme 20). We would attempt to synthesize this glycopeptide through a combination of cysteine-based and cysteine-free NCL methods, thereby demonstrating reiterative ligation. Thus, glycopeptides **102**, **107**, and **108** would be prepared through the techniques developed and honed in our laboratory over the course of many years. The Gly-Gln junction would be established through cysteine-free NCL between **102** and **108**. Next, following the deprotection of the *N*-terminal cysteine residue, the bifunctional glycopeptide would be ligated with fragment **107** according to our previously established cysteine-based NCL protocol.



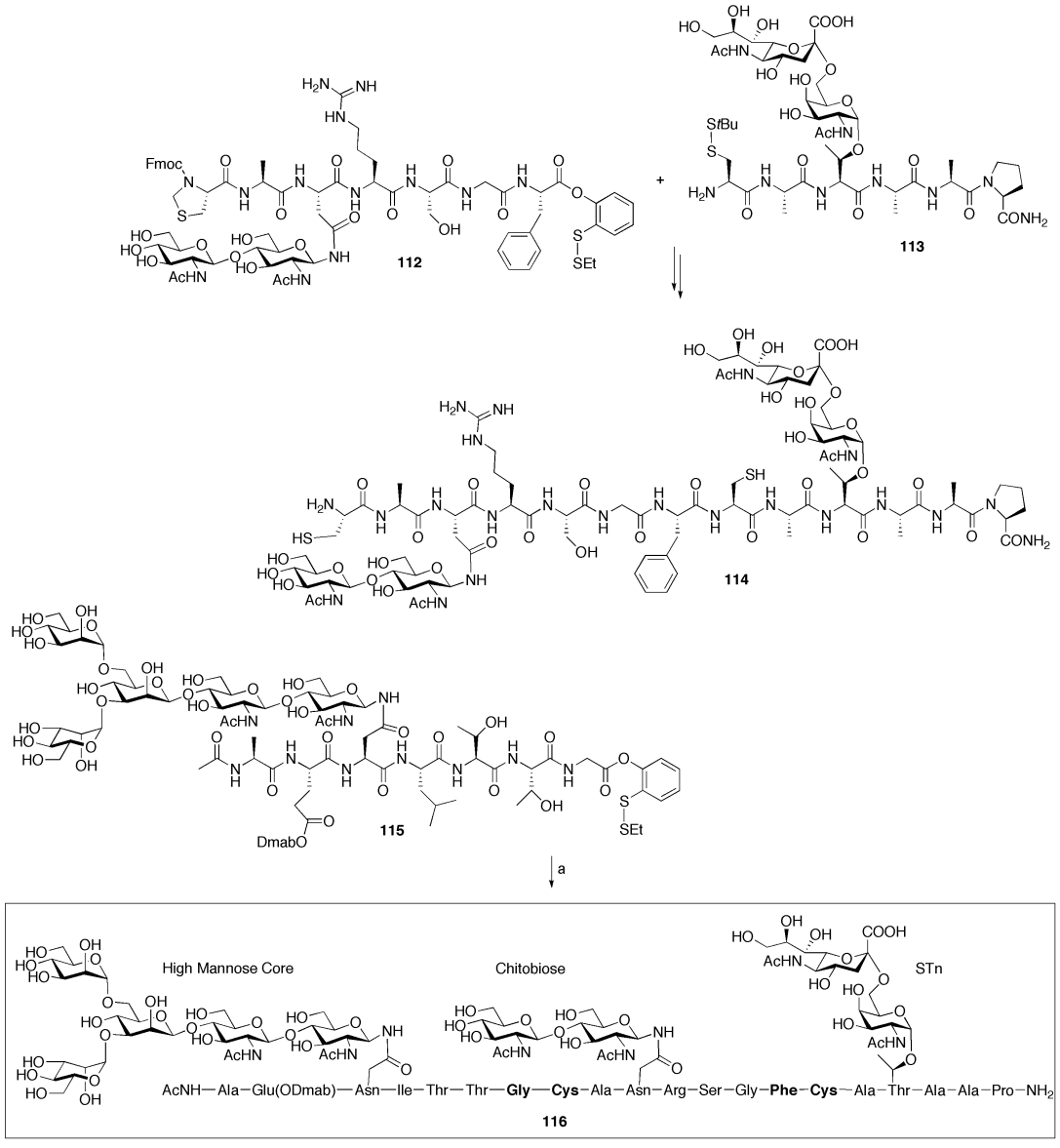
Scheme 20 Reiterative, cysteine-based and cysteine-free NCL strategy.

In the event, the reiterative coupling of glycopeptide fragments **102**, **107**, and **108** proceeded without incident (Scheme 21). Thus, **102** and **108** underwent cysteine-free NCL to provide the bifunctional glycopeptide **109** in 58 % yield. The *N*-terminal cysteine residue of **109** was unmasked, as shown, and the resultant fragment (**110**) readily underwent cysteine-based NCL with **107** to provide the trifunctional glycopeptide **111** in 57 % yield. We note that, in a separate study, we had successfully prepared a triply glycosylated peptide through a reiterative cysteine-based protocol (Scheme 22) [36].

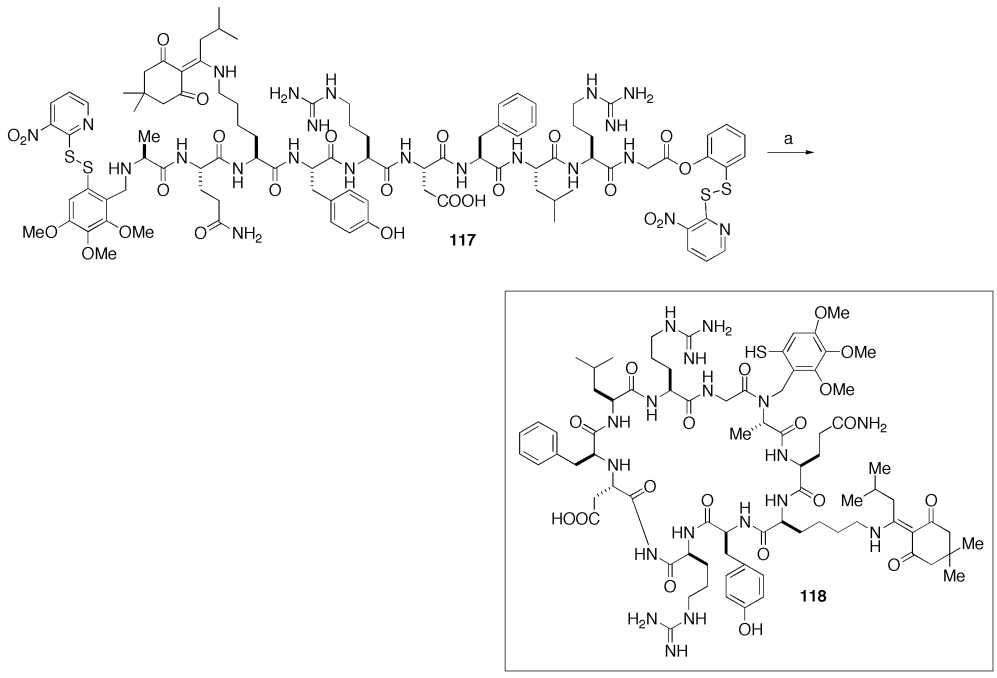


Scheme 21 Synthesis of the tridomainal glycopeptide **111**. (a) TCEP, DMF, Na_2HPO_4, 58 %; (b) (i) 10 % morpholine in DMF; (ii) 0.4 M $MeONH_2$•HCl, 60 %; (c) MesNa, TCEP, PBS (pH = 8.0), 57 %.

Although our focus throughout this program has been on the exploration of methods to enable the synthesis of homogeneous EPO, these newly developed techniques are expected to have broad implications which will extend to other areas of peptide and glycopeptide synthesis. We digress briefly to note that our cysteine-free ligation methodology has been found to be effective in the synthesis of a biologically relevant class of compounds: cyclic peptides. In biological settings, cyclic peptides often exhibit enhanced specificity, activity, and stability in comparison with their linear counterparts. Traditional strategies for cyclic peptide formation typically rely on macrolactam or disulfide formation; however, recent disclosures by Tam and coworkers reveal the possibility of accessing these important motifs through NCL [37]. Given the scarcity of cysteine residues in nature, the application of our cysteine-free NCL technique to cyclic peptides would be of particular import. Indeed, as outlined in Scheme 23, this newly developed methodology could be extended to the synthesis of a cyclic peptide [38]. Thus, peptide **117**, equipped with both the *C*-terminal phenolic moiety and the *N*-terminal auxiliary, readily underwent intramolecular ligation to provide the cyclic peptide (**118**) in 78 % yield.

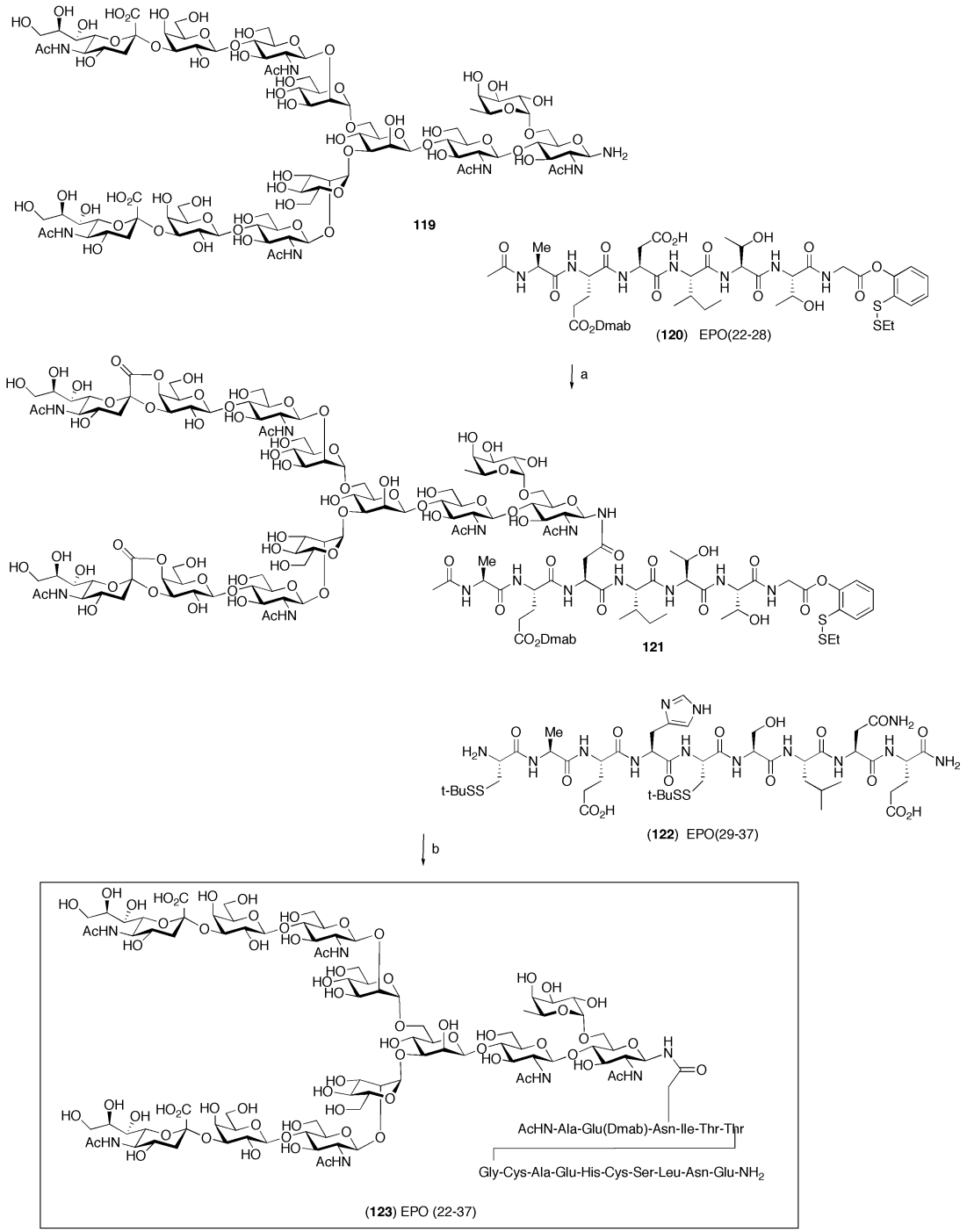


Scheme 22 Synthesis of tridomainal glycopeptide **116**. (a) MesNa, TCEP, PBS, DMF.

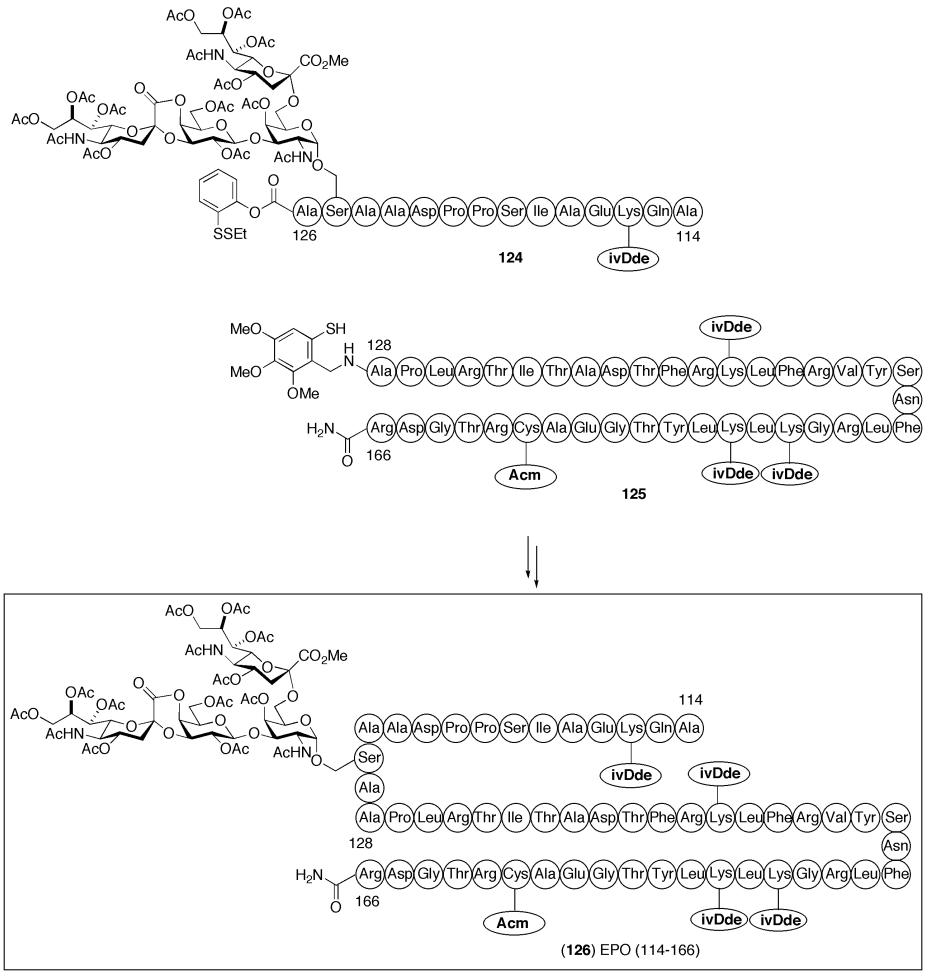


Scheme 23 Synthesis of a cyclic peptide through non-cysteine-based ligation. (a) TCEP, Na_2HPO_4, DMF, 78 %.

With the development of novel cysteine-based and cysteine-free glycopeptide ligation techniques, and with the establishment of a reiterative protocol by which to gain access to multiply glycosylated polypeptides, we have achieved several of the central methodological capabilities that will be required for the de novo synthesis of homogeneous EPO. While many challenges surely lay ahead, we have now reached a point where it seems that this paramount goal can be realized. Toward this end, we recently disclosed the assembly of two of the four glycopeptide domains of EPO. Thus, as summarized in Scheme 24, we have accomplished the synthesis of Ala^{22}–Glu^{37}, presenting the biantennary dodecamer at Asn^{24} (**123**) [39,40]. Scheme 25 summarizes the synthesis of the Ala^{114}–Arg^{166} fragment, incorporating the requisite glycophorin at Ser^{126} (**126**) [41]. Further progress has since been made toward the completion of the EPO synthesis, and results will be reported in due course.



Scheme 24 Synthesis of the Ala^{22}–Glu^{37} sector of EPO. (a) HATU, iPr_2NEt, DMSO, 40 %; (b) PBS, 1 % PhSH, TCEP, 56 %.



Scheme 25 Synthesis of the Ala^{114}–Arg^{166} sector of EPO.

CONCLUSIONS

In summary, we have charted the extensive progress made in what we call "bridging the gap" between synthetically accessible, traditional pharmacologically user-friendly, small molecules, and the emerging and exciting world of biologics with their rather well-defined molecular targets and the exquisite specificity of their noncovalent modes of molecular recognition. It is a certainty that the barrier between these two types of pharma agents is rapidly giving way to new facts on the ground in which the magic of biology can be combined with the capacity of organic synthesis to bring into being virtually any reasonable structure of which the mind can conceive. It is to be expected that, in the years ahead, much will be learned and gained from this type of programmatic synergy.

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