Coordination polymer **2** was synthesized according to a published procedure. Elemental analysis (%) calcd for $C_{13}H_8NO_4Cu$: C 51.06, H 2.62, N 4.58; found C 50.62, H 2.45, N 4.60.

Gas adsorption measurements: Sorption isotherms were measured at 298 K on an FMS-BG (BEL inc.) automatic gravimetric adsorption measurement system with Rubotherm magnet coupling balance incorporated in a SUS steel pressure chamber. A known weight (200–300 mg) of the as-synthesized sample was placed in the aluminum sample cell in the chamber, and the sample was dried under high vacuum at 373 K for 5 h to remove the host water molecules. The adsorbate was dosed into the chamber, and the change in weight was monitored. After correction for buoyancy, the absorbed amount was determined.

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Glycopeptide Synthesis

Toward Fully Synthetic *N*-Linked Glycoproteins**

*Justin S. Miller, Vadim Y. Dudkin, Gholson J. Lyon, Tom W. Muir, and Samuel J. Danishefsky**

The structural and biological consequences of cellular protein modification through posttranslational glycosylation are central issues in the rapidly growing field of glycobiology.[1] The availability of homogeneous glycopeptides, both *O*-linked (serine, threonine, or tyrosine α-glycosides) and *N*-linked (asparagine β-glycosides), could greatly enhance insight into glycobiology.[2] It became our view that the prospect of total synthesis of homogeneous glycoproteins provides the best chance for gaining such access.

Numerous methods exist for glycopeptide synthesis: glycans have been introduced into peptides by means of amino acid "cassettes" with pendant protected saccharides,[3] through enzymatic manipulations of glycopeptides,[4] or by conjugation of fully elaborated, complex saccharides to short synthetic peptides.[5] Larger *O*-linked glycopeptides have been synthesized by using ligation techniques[6] such as expressed protein ligation.[7] Bertozzi and co-workers extended the scope of the "cassette" approach by applying native chemical ligation to the synthesis of a biologically active glycoprotein with two single-residue *O*-linked glycans.[8] Tolbert and Wong described the ligation of a 392-residue intein-generated peptide thioester and a dipeptide functionalized with a single *N*-acetylglucosamine residue.[7c] Using a different fragment condensation protocol, Hojo et al. reported the synthesis of a glycopeptide domain of Emmprin that contains an *N*-linked chitobiose unit, but the saccharide was not entirely stable to the conditions required for resin cleavage in their solid-phase synthesis.[9]

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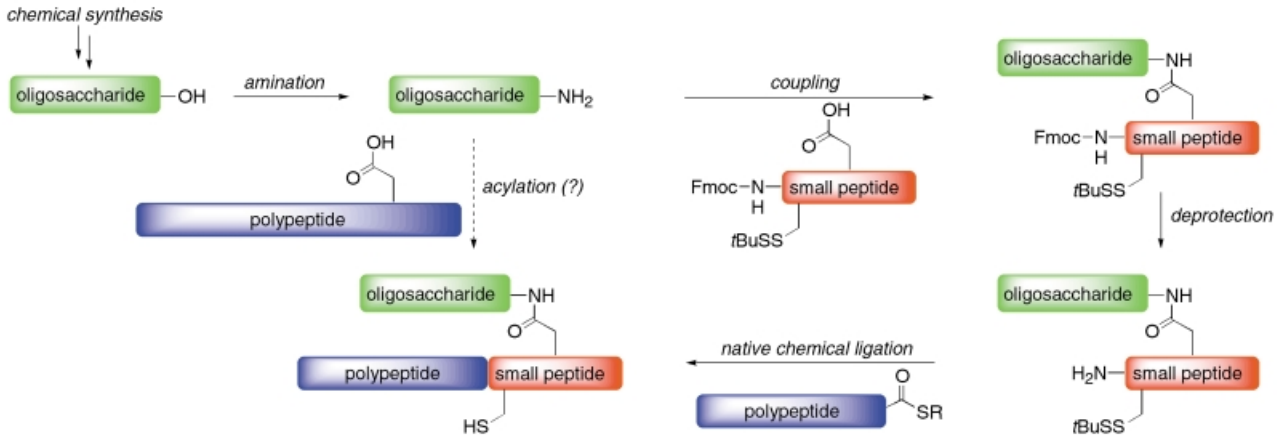
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author. Experimental details include the preparation of and mass spectral characteristics for **2**–**6, 8**, and **10**–**12**; and NMR spectra for **5, 6, 8**, and **10**.

Our goal was to set the stage for fully synthetic routes to complex glycoproteins. It would thus be necessary to harmonize *all* of the components of the undertaking. This includes building a complex glycodomain and incorporating it into a polypeptide setting. Herein we show how the pieces of the puzzle can be interfaced. In launching our program we took particular note of the work of Kochetkov and co-workers,[10] applied by Lansbury and co-workers,[5b] involving direct anomeric β-amination of unprotected saccharides followed by acylation with a peptide carboxylic acid.

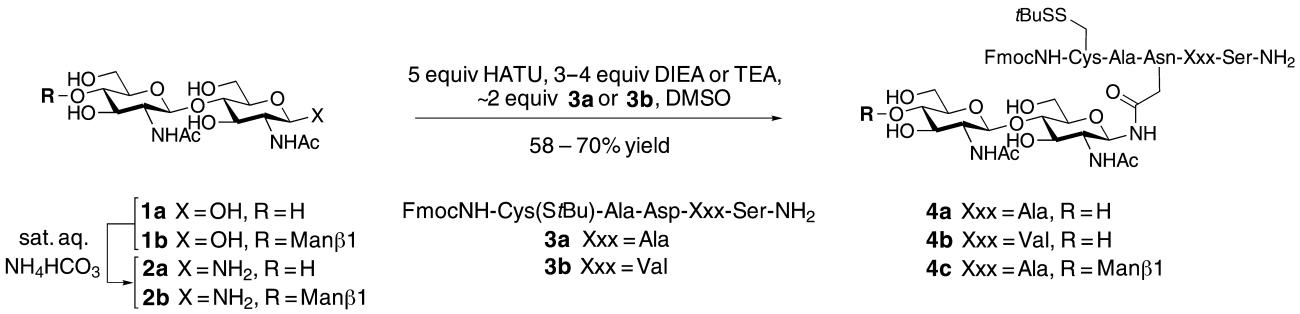
Our program focuses on natural *O*- and *N*-linkages as opposed to non-natural arrangements. Furthermore, as our oligosaccharides are assembled by total chemical synthesis,[11] there is, in principle, no limit to the structural complexity of the carbohydrate sectors of our glycopeptide targets, even as homogeneity is maintained.

The scenario for an ultimately convergent protocol involves merging fully mature oligosaccharide and polypeptide domains in one grand acylation event (dashed arrow, Scheme 1). For the moment, we favored the slightly less convergent, but in the end more practical and certainly more flexible, route shown in Scheme 1. In this case, the anomeric amine function of an oligosaccharide domain is acylated with a more manageable small peptide; native chemical ligation is then used to anneal this construct to a larger polypeptide segment. Critical for our long-term goals was the requirement that the precious, fully synthetic glycan be the limiting reagent in the chemical mergers. As shown in Scheme 1, this boundary condition has been attained.

We began our investigation as shown in Scheme 2. Treatment of known[12] unprotected saccharides **1a–b** (generated by total synthesis) with saturated aqueous ammonium hydrogen carbonate followed by lyophilization to a constant mass afforded glycosylamines **2a–b**. As a consequence of the known instability of anomeric glycosylamines and our desire to maximize yields, the resulting white powders were used without further purification or analysis, aside from mass spectrometry. The results of Kochetkov amination are well-documented,[13] and could in any case be confirmed by ^{1}H NMR coupling constants after peptide conjugation. Using optimized conditions developed for this purpose, glycosylamines **2a–b** were acylated with pentapeptides **3a** or **3b** by adding to the glycosylamine a twofold excess of peptide preactivated with HATU (5 equiv) and a tertiary amine (3–4 equiv) in DMSO. Upon completion of the reactions after only 2–4 h as monitored by analytical HPLC or LCMS, the reaction mixtures were purified by semipreparative HPLC. Two major side products were observed, showing molecular ions of 1 Da and 18 Da less than the starting aspartate-containing peptides. These are consistent with conversion of Asp into Asn through acylation of spurious ammonia and aspartimide formation as shown in Scheme 3,[14] which several other authors also note and seek to avoid by various methods. Though these processes are competitive with glycopeptide formation in terms of rate, their products are solely peptide-derived. Thus an excess of peptide starting material avoids most losses caused by these processes, even with an Ala residue immediately towards the C-terminus of the activated



Scheme 1. Convergent approach to *N*-linked glycopeptides. Fmoc = 9-fluorenylmethoxycarbonyl.



Scheme 2. Glycan preparation and peptide conjugation. HATU = *N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]-pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate, DIEA = diisopropylethylamine, TEA = triethylamine, DMSO = dimethyl sulfoxide.

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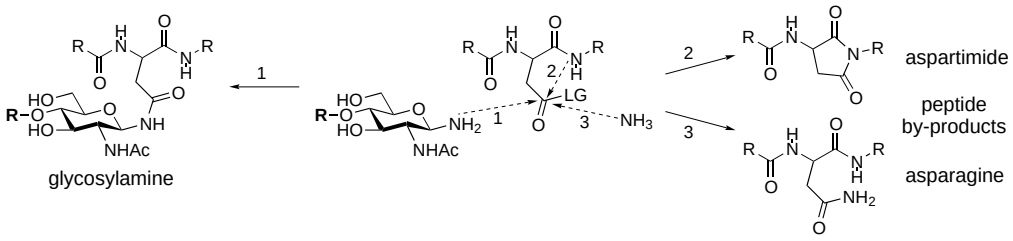


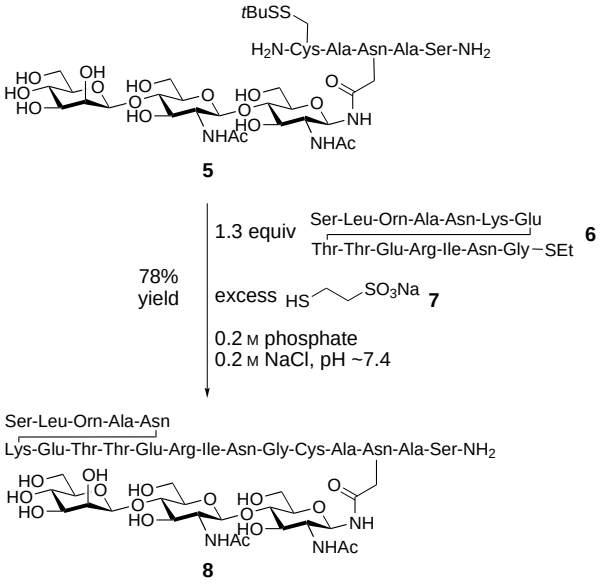
Scheme 3. Peptide byproducts of aspartate acylation.

Asp.[15] The yields of the combined amination and acylation products **4a–c** upon isolation were in the range of 58 to 70% based on starting glycan,[16] representing a significant improvement over the best yields previously reported.

Deprotection of Fmoc-glycopeptide **4c** with 20% piperidine in DMF followed by purification by HPLC afforded free glycopeptide **5** as a cysteine thiol *tert*-butyl disulfide in 68% yield. Additional products were observed with molecular ions identical to that of the desired material, perhaps as a result of epimerization of cysteine or the anomeric amide. The purified, isolated material at this stage was characterized by ^{1}H NMR spectroscopy, electrospray ionization (ES) MS, and liquid chromatography (LC) MS as a single isomer with an anomeric ^{1}H NMR shift ($\delta = 5.04$) and coupling constant ($J =$ 9.6 Hz) indicating the presence of a β-linked anomeric glycosylamide, thus validating the results of the Kochetkov–Lansbury amination.

We next extended glycopeptide **5** through native chemical ligation on a sizable (~15 mg) scale as shown in Scheme 4. As an independent test of the methodology, we synthesized tetradecapeptide thioester **6** employing the Fmoc/*t*Bu solid-phase peptide synthesis method recently reported by Hilvert and co-workers.[17] After automated peptide synthesis on a PEG-type Wang resin,[18] cleavage with trimethylaluminum and ethanethiol in dichloromethane afforded the desired thioester along with several (presumably Glu side chain) thioester derivatives. We noted a significant improvement in the purity of our peptide when the cleavage was quenched by filtration of the cleavage mixture (to remove resin) into a stirred mixture of trifluoroacetic acid, water, and phenol over an ice bath rather than pouring the entire cleavage reaction mixture into the TFA mixture at room temperature; in fact, we observed no side chain thioesters at all when the cleavage was quenched as described.

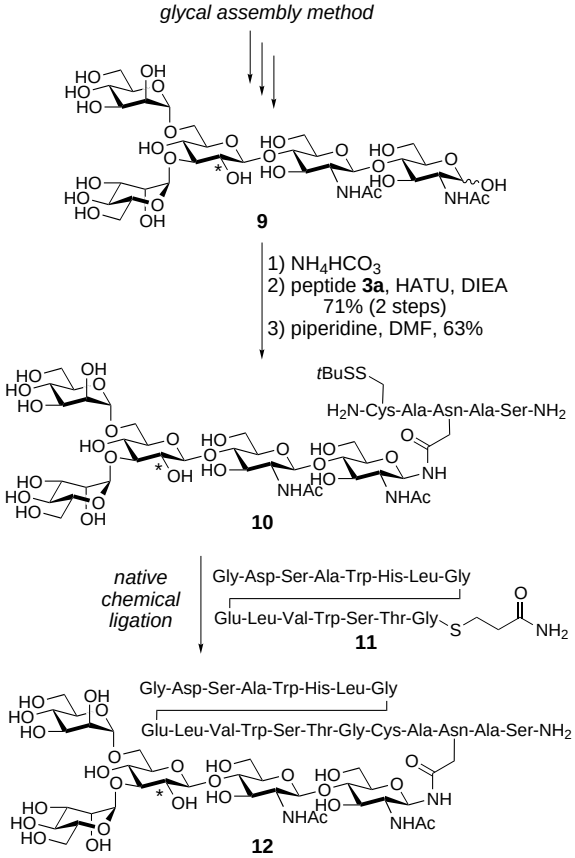
Ligation of **5** and **6** was carried out in aqueous PBS, 0.2 M in both saline and phosphate, pH ~7.4, in the presence of excess sulfanylethane-2-sulfonate (**7**) as illustrated in Scheme 4. Global disulfide reduction with the water-soluble phosphane TCEP[19] followed by semipreparative HPLC afforded the desired, fully unprotected glycopeptide **8** in 78% yield based on starting glycopeptide. Characterization of glycopeptide **8** by ESMS, LCMS, and ^{1}H and ^{13}C NMR spectroscopy in D_2O (Supporting Information) was consistent with a single compound containing a β-linked glycosylamide.



Scheme 4. High-yielding ligation of an *N*-linked glycopeptide.

As an example of the power of this method for complex glycopeptide synthesis, we employed pentasaccharide **9** (Scheme 5), prepared by chemical synthesis.[11] The compound differs from a characteristic high mannose pentasaccharide at one of its 25 stereogenic centers (asterisk, Scheme 5).[20] Amination followed by our peptide acylation conditions with pentapeptide **3a** and Fmoc removal yielded pentasaccharide glycopeptide **10** as a single, β-linked isomer, confirmed by HPLC and ^{1}H NMR spectroscopy ($\delta =$ 5.01 ppm, $J = 9.6$ Hz). Native chemical ligation with **10** and excess pentadecapeptide thioester **11** synthesized by Boc chemistry[21] afforded glycopeptide **12** as evidenced by HPLC and ESMS, again demonstrating proof of principle.

In summary, we have presented a highly convergent route for the production of substantial quantities of homogeneous glycopolypeptides. In this effort we retain the full flexibility accruing from total chemical synthesis of the oligosaccharide (see compound **9**). Of course, the same flexibility is also retained in the polypeptide.[22] Although unexpected difficulties will no doubt be encountered, we presently see no insuperable boundary to progression towards fully synthetic,



Scheme 5. Native chemical ligation of a pentasaccharide glycopeptide and a pentadecapeptide. DMF = *N*,*N*-dimethylformamide.

homogeneous, complex glycoproteins. Applications of the advances recorded above to critical biological goal systems are well underway, and will be described in due course.

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