JOC*Article*

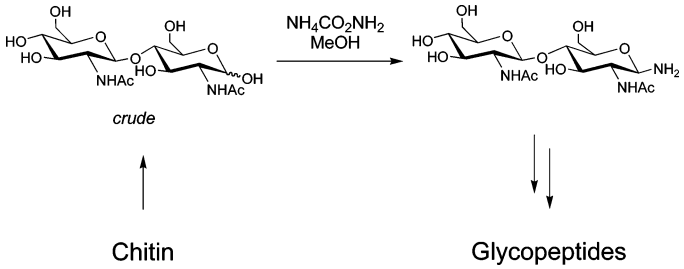
Improving Glycopeptide Synthesis: A Convenient Protocol for the Preparation of β-Glycosylamines and the Synthesis of Glycopeptides

Christian P. R. Hackenberger, Mary K. O'Reilly, and Barbara Imperiali*

Department of Chemistry and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

imper@mit.edu

Received December 16, 2004



Herein we apply a recently introduced protocol using ammonium carbamate in methanol to the amination of crude chitobiose leading to 1,β-aminochitobiose. This simple, one-step procedure allows a facile preparation of unstable glycosylamines in contrast to the commonly implemented ammonium bicarbonate based amination of water-soluble carbohydrates. The new amination protocol leads to an improved synthesis of the key chitobiosyl-asparagine building block for the SPPS of glycopeptides. The utility of the method is demonstrated with the synthesis of a 39-amino acid glycoprotein.

Significant effort has been devoted toward understanding the various roles of protein glycosylation, which is now known to modulate protein structure and to influence numerous biochemical and medicinally relevant processes.[1] Research in this area is, however, limited by the accessibility of homogeneous glycoproteins for study since the glycoconjugates isolated from natural sources commonly display considerable heterogeneity in the saccharide portion.[2] Therefore, the development of new synthetic routes toward glycoproteins, and glycoconjugates in general, continues to attract much attention.[3]

Carbohydrates in natural glycoproteins are attached to peptides through the oxygen of serine or threonine residues in *O*-linked glycoproteins or through the carboxamide nitrogen of asparagine in *N*-linked glycoproteins.[4] To access *N*-linked glycoproteins via chemical synthesis, particular emphasis has been placed on the preparation of β-glycosylamines, as these intermediates can be coupled to activated aspartic acid derivatives or peptides to establish a native *N*-linked glycopeptide linkage.[4b,5] Unfortunately, glycosylamines are unstable and prone to dimerization, hydrolysis, and isomerization.[6] These side reactions make the purification of glycosylamines extremely difficult and in general, separation steps must be carried out later in the synthesis on more complex intermediates.[7]

As part of the continuing effort to access quantities of glycopeptides and glycoproteins for biochemical and biophysical investigations, we now report the first example of a 1,β-aminochitobiose (**1**) synthesis directly from a crude chitobiose mixture derived from the enzymatic digestion of chitin with Chitinase (Scheme 1).[8] This

(1) (a) Varki, A. *Glycobiology* **1993**, *3*, 97−130. (b) Dwek, R. A. *Chem. Rev.* **1996**, *3*, 683−720. (c) Bertozzi, C. R.; Kiessling, L. L. *Science* **2002**, *291*, 2357−2364. (d) Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A. *Science* **2001**, *291*, 2370−2376.

(2) Helenius, A.; Aebi, M. *Science* **2001**, *291*, 2364−2369.

(3) (a) Grogan, M. J.; Pratt, M. R.; Marcaurelle, L. A.; Bertozzi, C. R. *Annu. Rev. Biochem.* **2002**, *71*, 593−634. (b) Arsequell, G.; Valencia, G. *Tetrahedron*: *Asymmetry* **1999**, *10*, 3045−3094.

(4) Bura, P.; Aebi, M. *Biochim. Biophys. Acta* **1999**, *1426*, 239−257.

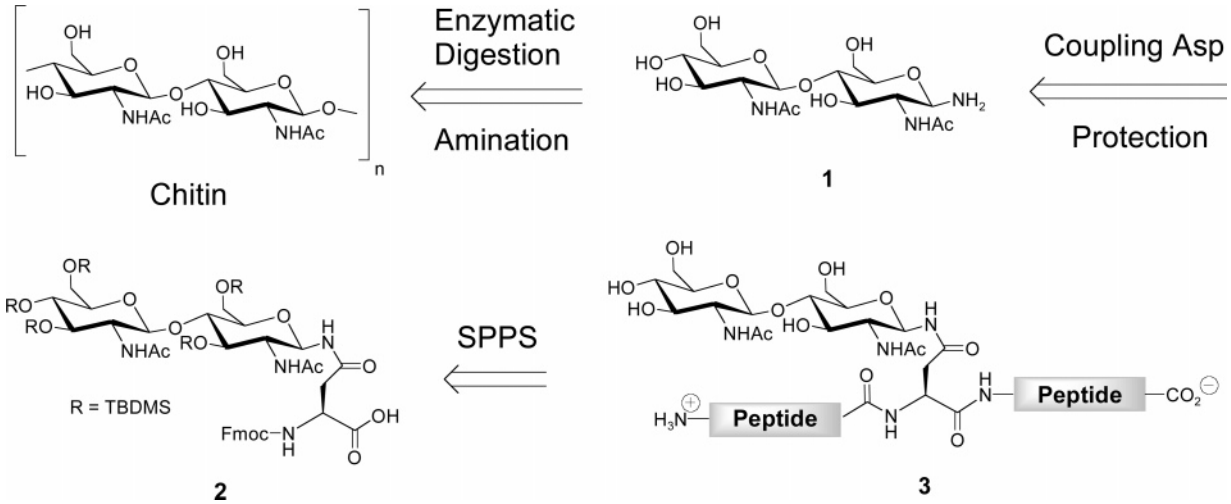
(5) For other examples to construct the carbohydrate N-linkage see: (a) Günther, W.; Kunz, H. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 1050−1051. (b) Handlon, A. L.; Fraser-Reid, B. *J. Am. Chem. Soc.* **1993**, *115*, 3796−3797. (c) Damkaci, F.; DeShong, P. *J. Am. Chem. Soc.* **2003**, *125*, 4408−4409. (d) Maunier, V.; Boullanger, P.; Lafont, D. *J. Carbohydr. Chem.* **1997**, *16*, 231−235. (e) Mizuno, M.; Muramoto, I.; Kobayashi, K.; Yaginuma, H.; Inazu, T. *Synthesis* **1999**, 162−165. (f) He, Y.; Hinklin, R. J.; Chang, J. Y.; Kiessling, L. L. *Org. Lett.* **2004**, *6*, 4479−4482.

(6) Likhoshertov, L. M.; Novikova, V. A.; Dervitskaja, V. A.; Kochetkov, N. K. *Carbohydr. Res.* **1986**, *46*, C1−C5.

(7) Takeda, T.; Utsuno, A.; Okamoto, N.; Ogihara, Y.; Shibata, S. *Carbohydr. Res.* **1990**, *207*, 71−79.

10.1021/jo047801v CCC: $30.25
Published on Web 03/31/2005

SCHEME 1. Route for the Synthesis of Glycopeptides Starting from Chitin

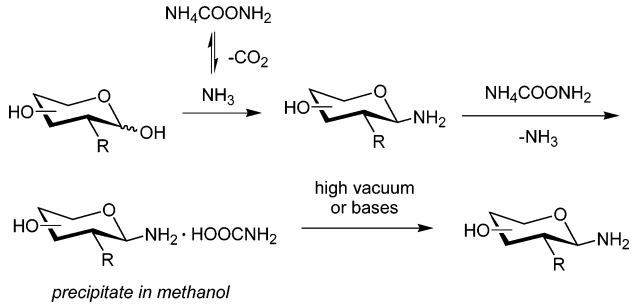


conversion allows a convenient six-step synthesis of the acid labile Fmoc-protected chitobiose amino acid **2** in gram quantities including two purification steps by column chromatography.[9] The building block **2** is then used directly in the solid-phase peptide synthesis (SPPS) of an *N*-linked glycopeptide modified with chitobiose **3**.

In principle, two different synthetic routes to glycosylamines have been explored recently. The carbohydrate amine functionality can be introduced after the hydroxyl group protection, which requires the use of organic solvents as reaction media. Examples of this approach include the conversion of glycosyl halides with ammonia,[10] azide[11] or the Burgess reagent,[12] and synthetic schemes based on glycals[13] or α-hydroxy nitriles.[14] The second route employs unprotected water-soluble carbohydrates and in this case the amination procedure is commonly carried out by the Kochetkov method, with use of a saturated solution of ammonium bicarbonate in water.[5,15] Applications of this strategy include complex carbohydrate syntheses pioneered by Danishefsky[16] as well as the previous route to building block **2**; the latter method implemented pure chitobiose as starting material for the synthesis of 1,β-aminochitobiose (**1**) before coupling with a preactivated aspartic acid derivative in an overall yield of 12%.[3b,9]

Recently, a new protocol for the selective amination of unprotected sugar derivatives was introduced by Likhosherstov, which used ammonium carbamate in methanol as the reactant and showed the general applicability for a series of mono- and disaccharide derivatives.[17] The major advantage of this procedure is the ease of isolation of the reaction product. The glycosylamine, formed by the reaction with ammonia that has been generated by dissociation of ammonium carbamate, is precipitated as a carbamic acid salt under the reaction conditions (Scheme 2). This salt formation prevents the hydrolysis and glycosylamine-dimer formation, as long as the amount of water is kept to a minimum. Finally, the free amine is generated by base treatment or under high vacuum. We envisioned that this protocol could also allow the purification of glycosylamines starting from a crude carbohydrate preparation.

SCHEME 2. Proposed Mechanism of the Carbamate-Mediated Amination of Sugar Derivatives

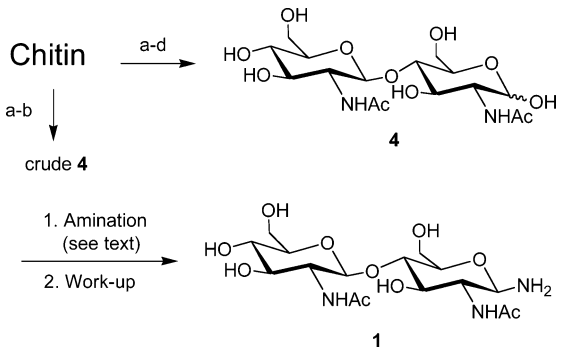


Results and Discussion

The investigations were initiated by evaluating amination of pure chitobiose (**4**) (Scheme 3), which was obtained from chitin by enzymatic digestion with Chitinase at pH 6.2 and subsequent recrystallization of the acetyl-protected carbohydrate.[18] Amination was carried out with ammonium carbamate for 24 h at 37 °C in methanol. The chitobiose amine-salt precipitated as a

(8) Terayama, H.; Takahashi, S.; Kuzuhara, H. *J. Carbohydr. Chem.* **1993**, *12*, 81−93.
(9) For a previous example of the synthesis of building block **2** see: Bosques, C. J.; Tai, V. W. F.; Imperiali, B. *Tetrahedron Lett.* **2001**, *42*, 7207−7210.
(10) Michael, F.; Frier, R.; Plate, E.; Hiller, A. *Chem. Ber.* **1952**, *85*, 1092−1096.
(11) Györdeák, Z.; Szilagyi, L.; Paulsen, H. *J. Carbohydr. Chem.* **1993**, *12*, 139−163.
(12) Nicolaou, K. C.; Snyder, S. A.; Nalbandian, A. Z.; Longbottom, L. A. *J. Am. Chem. Soc.* **2004**, *126*, 6234−6235.
(13) Danishefsky, S. J.; Bilodeau, M. T. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1380−1419.
(14) Dorsey, A. D.; Barbarow, J. E.; Trauner, D. *Org. Lett.* **2003**, *5*, 3237−3239.
(15) Lubineau, A.; Auge, J.; Drouillat, B. *Carbohydr. Res.* **1995**, *266*, 211−219.
(16) (a) Miller, J. S.; Dudkin, V. Y.; Lyon, G. J.; Muir, T. W.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2003**, *42*, 431−434. (b) Wang, Z.-G.; Zhang, X.; Live, D.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2000**, *39*, 3652−3656.
(17) (a) Likhosherstov, L. M.; Novikova, O. S.; Shibaev, V. N. *Dokl. Chem.* **2002**, *383*, 89−92. (b) Likhosherstov, L. M.; Novikova, O. S.; Shibaev, V. N. *Dokl. Chem.* **2003**, *389*, 73−76. (c) Likhosherstov, L. M.; Novikova, O. S.; Zheltova, A. O.; Shibaev, V. N. *Russ. Chem. Bull., Int. Ed.* **2004**, *53*, 709−713.

SCHEME 3. General Route for the Amination of Chitobiose[a]



[a] Reagents and conditions: (a) HCl, sonication; (b) Chitinase, pH 6.2, phosphate buffer; (c) Ac_2O, NaOAc; (d) NaOMe, MeOH.

white solid, which was transformed into the corresponding β-chitobioseamine (**1**) after brief drying under high vacuum in 76% yield and high purity (see the Experimental Section for details). Under these conditions, none of the corresponding α-anomer was detected. Longer reaction times did not improve the yield, whereas shorter reaction reduced the yield significantly.

Next, amination of the *crude* chitobiose enzymatic digestion product, instead of pure **4**, was investigated to evaluate whether this method would afford pure β-chitobioseamine, since soluble byproducts could be removed by simple filtration (Scheme 3). To test this hypothesis, we directly compared ammonium carbamate and ammonium bicarbonate as reagents for the amination of crude chitobiose. We found that the ammonium carbamate protocol leads to the formation of pure 1,β-aminochitobiose (**1**), which was confirmed by ^{1}H NMR analysis of the anomeric α-hydrogens and the amide methyl groups (Figure 1, bottom). In contrast, treatment of crude chitobiose with ammonium bicarbonate and concentration of the aqueous reaction mixture results in an inseparable mixture of compounds that was not further characterized (Figure 1, top). Further optimization studies into the carbamate amination of crude chitobiose revealed that reaction at 37 °C for 16 h with 4 equiv of ammonium carbamate are the optimal reaction conditions, with a yield of 69%.

After the successful optimization of the glycosylamine synthesis, we concentrated on improving the preparation of the Fmoc-amino acid building block **2** for the SPPS of glycopeptides. This building block includes the fully TBDMS-protected chitobiose attached to aspartic acid via a β-glycosyl amide linkage.[9,19] The acid-labile building block **2** has proven to be advantageous for glycopeptide SPPS in comparison to the acetyl protected analogue, as the protecting groups can be removed during the resin

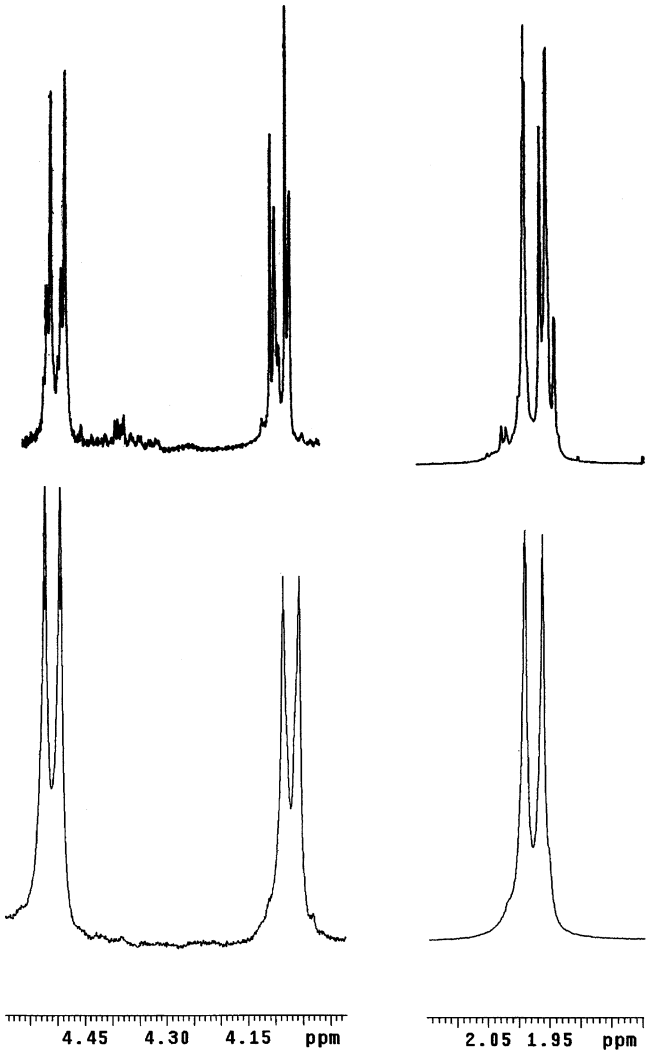


FIGURE 1. ^{1}H NMR spectra in D_2O of 1,β-aminochitobiose (**1**) obtained from ammonium bicarbonate amination (top) and ammonium carbamate amination (bottom). Spectra to the left display the anomeric hydrogens, while spectra to the right represent the acetamide-methyl group hydrogens.

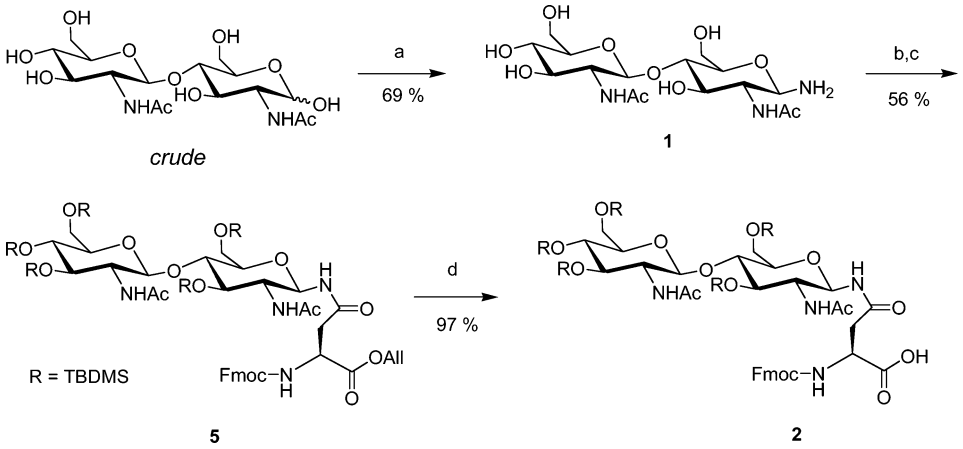
cleavage in a single synthetic step by using standard TFA-based cleavage cocktails.[19d,e] Additionally, aggregation, which occurs frequently upon treatment of unprotected peptides with harsh basic conditions, is minimized in the final acidic treatment.[9]

The final optimized sequence is summarized in Scheme 4. Coupling of the chitobioseamine **1** was performed under HBTU/HOBt conditions in DMF with use of 2 equiv of chitobioseamine and 1 equiv of Fmoc-Asp-OAll. The resulting unprotected amino acid derivative was precipitated from ethyl acetate, isolated by filtration, and directly used without further purification in the next protection step. TBDMS-protection to yield the protected amino acid **5** was performed with 16 equiv of TBDMS−OTf, which were added over 24 h in three portions. It was essential to implement the silyl triflate because the less reactive silyl-based electrophiles, such as TBDMS-chloride or bromide, did not lead to complete protection of the building block. Final deprotection of the allyl ester and purification by size exclusion chromatography yielded building block **2** in 56% overall yield based on the amount of Fmoc-amino acid used in this transformation.

(18) *Crude* chitobiose contains a mixture of α- and β-anomers of chitobiose as well as amounts of GlcNAc monosaccharides as analyzed by ^{1}H NMR spectroscopy and HPLC of fluorescently labeled substrates (see the Supporting Information for details).

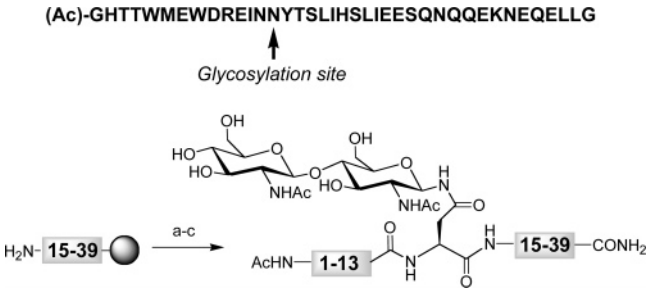
(19) For examples of SPPS of glycopeptides using the building block approach see: (a) Jobron, L.; Hummel, G. *Angew. Chem., Int. Ed.* **2000**, *39*, 1621−1624. (b) Christiansen-Brams, I.; Meldal, M.; Bock, K. *J. Chem. Soc., Perkin Trans. 1* **1993**, 1461−1471. (c) Meinjohanns, E.; Meldal, M.; Paulsen, H.; Dwek, R. A.; Bock, K. *J. Chem. Soc., Perkin Trans. 1* **1998**, 549−560. (d) Holm, B.; Linse, S.; Kihlberg, J. *Tetrahedron* **1998**, *54*, 11995−12006. (e) Broddefalk, J.; Bergquist, K.; Kihlberg, J. *Tetrahedron* **1998**, *54*, 12047−12070.

SCHEME 4. Optimized Synthesis of the Building Block 2[a]



[a] Reagents and conditions: (a) $NH_4CO_2NH_2$, MeOH; then high vacuum; (b) Fmoc-Asp-OAll, HBTU, HOBt, DIPEA, DMF; (c) TBDMS-OTf, DMAP, pyridine; (d) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2.

SCHEME 5. SPPS of the Glycosylated C-Peptide Analogue G1-G39[a]



[a] Reagents and conditions: (a) **2**, PyAOP, collidine, CH_2Cl_2; (b) Fmoc-Xaa-OH, HBTU, HOBt, DIPEA, DMF; (c) Cleavage cocktail K (TFA, phenol, EDT, PhSMe, TIS, H_2O).

Having established a convenient route toward the acid labile building block **2**, this derivative was used in the preparation of a complex *N*-linked glycopeptide. We chose a glycosylated C-peptide analogue as our synthetic target, which, in the unglycosylated form, plays an important role in the inhibition of the HIV membrane fusion process by binding tightly to the viral envelope glycoprotein gp41.[20] The C-peptide consists of 39 amino acids and includes a natural glycosylation sequon (NYT) from positions 14 to 16.[21] The C-peptide sequence is displayed in Scheme 5 with the glycosylation site as indicated.

The peptide Y15-G39 was synthesized on a Tenta-Gel resin, using standard Fmoc SPPS conditions with HOBt/HBTU activation.[22] Next, 2 equiv of the building block **2** were coupled manually under PyAOP/collidine activation in CH_2Cl_2. PyAOP/collidine was also used for the coupling of the next two amino acids before the conditions were reverted to HOBt/HBTU activation for the remaining 11 amino acid couplings. The *N*-terminal deprotected peptide G1-G39 was capped with an acetyl group and cleaved from the resin with the cleavage cocktail K.[23] HPLC analysis of the crude peptide product reveals the successful utilization of the new chitobiose building block in the glycopeptide synthesis. The crude peptide was purified by preparative HPLC, and analyzed by ESI-MS and MALDI (Figure 2).

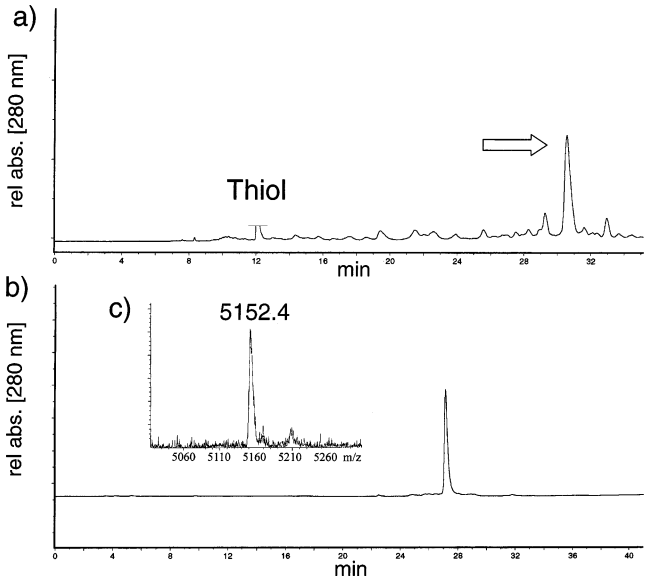


FIGURE 2. Synthesis of the glycosylated C-peptide G1-G39 (indicated by arrow): (a) HPLC spectrum of crude SPPS product after resin cleavage (constant flow: 5 min at 7% CH_3CN, 15 min at 20% CH_3CN; gradient 1: 45% to 53% CH_3CN over 20 min; gradient 2: 53% to 95% over 5 min); (b) HPLC spectrum of purified glycopeptide (gradient: 7% to 95% CH_3CN over 35 min); and (c) MALDI analysis (inset).

In summary, we have developed an efficient synthesis of glycopeptides bearing the native amide linkage, starting from simple and inexpensive starting materials. The major advantage of this route is the straightforward isolation and purification of pure glycosylamines, which are finally converted into a building block for the SPPS

(20) (a) Root, M. J.; Kay, M. S.; Kim, P. S. *Science* **2001**, *291*, 884−888. (b) Root, M. J.; Hamer, D. H. *PNAS* **2003**, *100*, 5016−5021.

(21) For the SPPS of a glycopeptide-thioester with a 26 amino acid sequence see: (a) Hojo, H.; Watabe, J.; Nakahara, Y.; Ito, Y.; Nabeshima, K.; Toole, B. P. *Tetrahedron Lett.* **2001**, *42*, 3001−3004. (b) Hojo, H.; Haginoya, E.; Matsumoto, Y.; Nakahara, Y.; Nabeshima, K.; Toole, B. P.; Watanabe, Y. *Tetrahedron Lett.* **2003**, *44*, 2961−2964.

(22) We acknowledge Professor Michael J. Root for the SPPS of the peptide Y15-G39.

(23) (a) King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Peptide Protein Res.* **1990**, *36*, 255−266. (b) Huang, H.; Rabenstein, D. L. *J. Pept. Res.* **1999**, *53*, 548−553.

of glycopeptides. This synthetic route greatly increases the accessibility of homogeneous *N*-linked glycopeptides for biochemical and biophysical investigations. We are currently using this methodology to access large peptide fragments for the semi-synthesis of glycoproteins via native chemical ligation for protein folding studies as well as for other biochemical studies.

Experimental Section

General. Solvent purification, suppliers, and instruments, as well as full synthetic protocols for the synthesis of pure and crude chitobiose and building block **2**, are described in the Supporting Information.

Synthesis of 1,β-Aminochitobiose (1) via Amination of Crude Chitobiose with Ammonium Carbamate. In a flask equipped with a reflux condenser, 1 equiv of the crude chitobiose and 4 equiv of ammonium carbamate were suspended in methanol (5 mL/mmol of chitobiose). The suspension was stirred for 16 h at 37 °C until the precipitate became white and fluffy and TLC analysis (silica, 4:3:2 ethyl acetate/MeOH/water; CAM and 10% H_2SO_4 detection; R_f(unprotected chitobiose) = 0.73; R_f(**1**) = 0.33) indicated no further amination product. The mixture was stirred for 1 h at 0 °C, and the precipitate was filtered, washed twice with cold methanol and dried at high vacuum for no more than 10 s to avoid decomposition of the aminosugar. The resulting white solid was directly used for analysis or for further synthetic transformations.

^{1}H NMR (500 MHz, D_2O) δ (ppm) 4.50 (1 H, d, J = 8.5 Hz, H-1′), 4.12 (1 H, d, J = 8.5 Hz, H-1), 3.90−3.40 (13 H, m), 2.04 (3 H, s, NAc), 2.01 (3 H, s, NAc).

MS (ESMS, [MH^+]) 424.1 (obsd), 424.4 (calcd).

Acknowledgment. This work was funded by the NIH (GM 39334). C.P.R.H. acknowledges a postdoctoral fellowship from the German Academic Exchange Service (DAAD). We thank Dr. Mark Nitz, Dr. Carlos Bosques, and Dr. Eugenio Vazquez for valuable discussions and advice and Professor Michael J. Root for the gift of the resin bound peptide intermediate Y15-G39.

Supporting Information Available: ^{1}H NMR spectra of amination products, HPLC analysis of crude chitobiose, full experimental details, and analysis for the building block **2** synthesis and the SPPS of the glycosylated C-peptide analogue. This material is available free of charge via the Internet at http://pubs.acs.org.

JO047801V