An Expeditious Route to GlcNAc-Cbz-Asn by Chemo-enzymatic Synthesis

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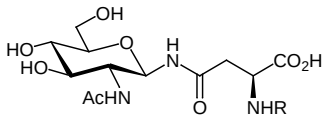
Abstract: A short-step route to GlcNAc-Cbz-Asn was developed. Treatment of GlcNAc in sat. aq. NH_4HCO_3 solution and subsequent electorodialytic desalting provided ammonia-free glycosylamine in large quantity. The product was coupled with Cbz-Asn α-isobutyl ester β-fluoride, and finally, the isobutyl ester was deprotected by enzyme-catalyzed hydrolysis under mild conditions.

Key words: amino acids, coupling, enzymes, glycosides, hydrolyses

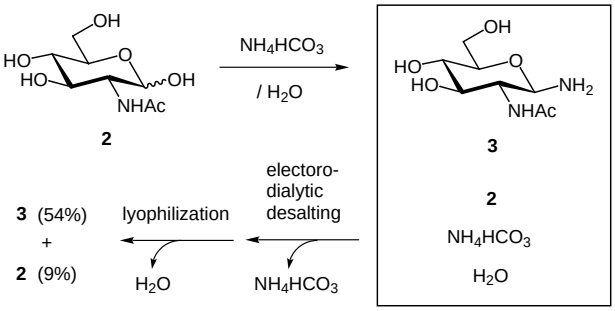
Increasing demand on *N*-acetylglucosaminylasparagine (**1a**) in the synthesis of glycoproteins and glycopeptides,[1] especially *endo*-β-*N*-acetylglucosaminidase-catalyzed chemo-enzymatic synthesis,[2] has prompted the development of expeditious routes to **1a** itself as well as the protected forms. Here we present a chemo-enzymatic approach to *N*-Cbz derivative **1b** (Figure 1), which does not depend on the so-far developed "glycosyl azide" intermediate.[3–7] Our scheme has the following two features: 1) as few synthetic steps as possible; 2) intermediates carrying the least protecting groups.



1a : R = H
1b : R = Cbz

Figure 1 GlcNAc-Asn and Cbz derivative.

First, glycosylamine **3** was prepared according to Kochetkov's report[8] (Scheme 1). There is a great advantage in Kochetkov's procedure in that the desired compound **3** is obtained in only one step from **2**. Indeed, treatment of **2** in a sat. aq. NH_4HCO_3 solution at 35 °C for 4 days reached an equilibrium of **3** and **2** in 86:14 ratio. This method of preparation is very simple; however, there remained a deleterious effect of the co-existing NH_4HCO_3 and H_2O, which show considerable nucleophilicity toward the amino acid acyl donor. Our initial attempts using vacuum pump-dried workup[9] only resulted in a very low yield of the subsequent coupling step.



Scheme 1 Synthesis of Ammonia-free Glycosylamine **3**.

This problem was cleanly solved by means of electrodialytic desalting. The concentration of NH_4HCO_3 was monitored by the measurement of electroconductivity, which lies in linear relationship as depicted in Figure 2.

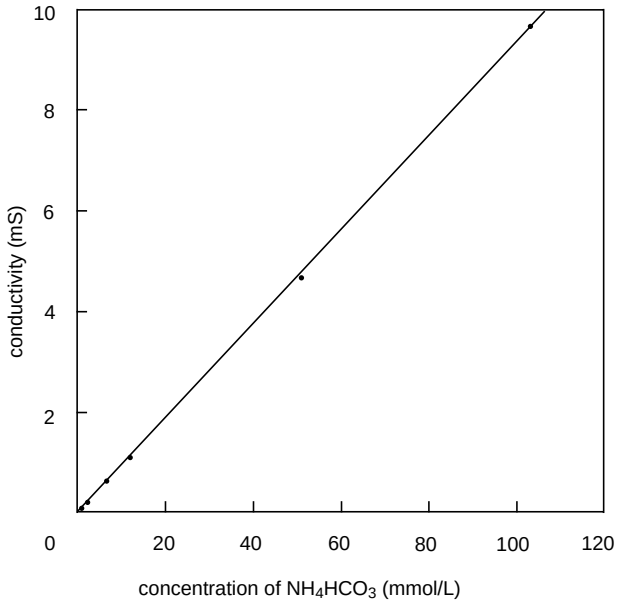


Figure 2 Calibration of NH_4HCO_3 in aq. solution.

The electrodialysis was carried out with a membrane Ac-220-550 (Asahi Chemical Co.), which allows all ionized components with less than MW 220 to go through. Typical example of time course on the decrease of NH_4HCO_3 is shown in Figure 3. In the preparative-scale experiment,[10] 99.5% of the initial NH_4HCO_3 was removed. The subsequent lyophilization provided a mixture of **3** (54%)

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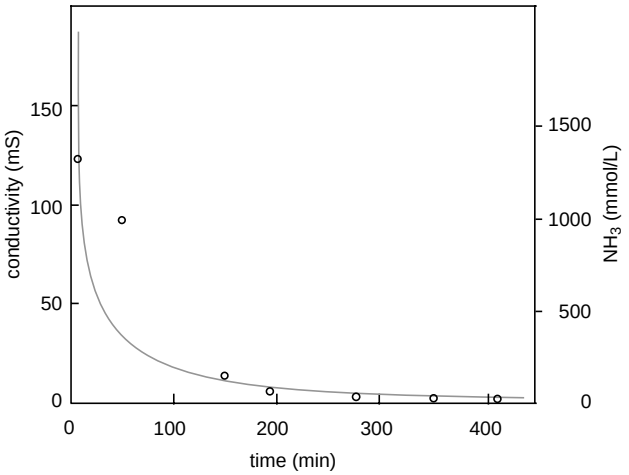


Figure 3 Electrodialytic Desalting of NH_4HCO_3 in the Reaction Mixture.

and **2** (9%). Through this step, no interchange between amine **3** and GlcNAc **2** was observed.

Next, a selectively protected (α-ester) form **5a**[11] of *N*-Cbz-L-aspartate was prepared according to the reported procedure via a cyclic oxazolidinone **6a**.[12] Activation of the acyl donor was accomplished by the conversion to acyl fluoride,[6] expecting enhanced affinity toward the nitrogen nucleophile,[13] glycosylamine **3**, since this amine is a free sugar and, moreover, contaminated with GlcNAc (**2**, ca. 9%). The acids **5a** and **6a** were treated with cyanuric fluoride[14] to give stable fluorides **5b** (92%)[15] and **6b** (90%), respectively.

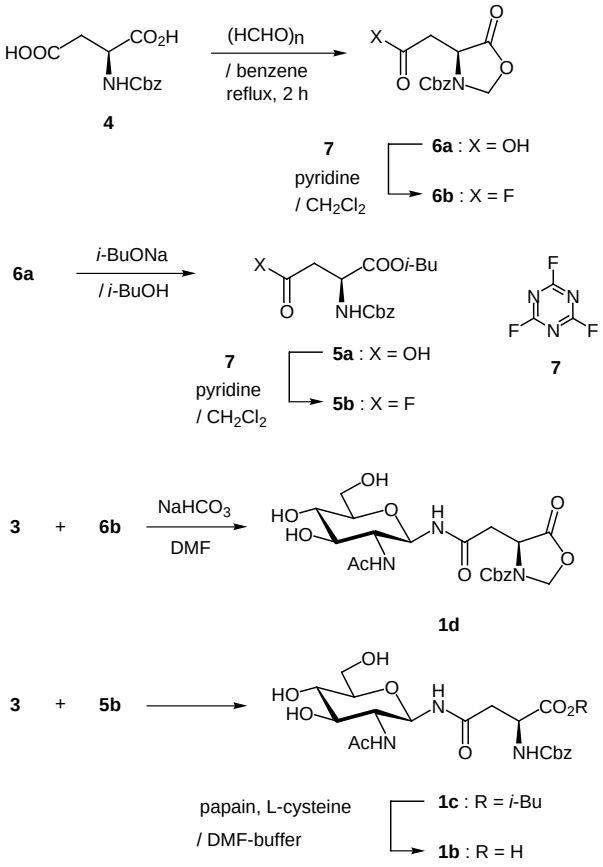
The coupling reactions were examined in the presence of $NaHCO_3$, as listed in Table. The complete dissolution of the glycosylamine **3** in a polar solvent such as DMF was essential for the efficient progress of the reaction, and the yield reached as high as 84%.[16] The great advantage of acyl fluoride indeed is the cleanness of the reaction product. In contrast, an attempted reaction between **3** and **5a** with a conventional *N*-hydroxysuccinimide protocol[8] provided the desired product; however, we faced many difficulties throughout the purification of such a polar polyhydroxy compound from the debris of the coupling reagents.

Table Coupling Reactions Between **3** and Acyl Fluorides.

Substrate	Solvent	Time (h)	Yield (5)
6b	H_2O–dioxane (1:5)	2.5	63
6b	CH_3CN	24	ND
6b	DMF	1	65
5b	H_2O–dioxane (1:3)	2.5	21
5b	dioxane	24	ND
5b	DMF	1	84

Finally, enzyme-catalyzed hydrolysis[17] under mild conditions as neutral pH was applied to deprotection of the α-ester, to avoid the decomposition of glycosyl amide and/or epimerization at the α-position of the asparagine moiety. *Bacillus licheniformis* protease (subsilisin, Sigma) showed very low activity; in turn, papain-catalyzed hydrolysis[18] worked well on the isobutyl ester to give **1b** in 67% isolated yield (Scheme 2).[19] The cyclic ester **1d**, however, was a poor substrate toward either enzyme.

In conclusion, a chemo-enzymatic short-step route to GlcNAc-Cbz-Asn **1b** was established, involving ammonia-free formation of *N*-acetylglucosaminylamine (**3**) in a preparative scale, followed by the coupling with acyl fluoride **5b**, and papain-catalyzed hydrolysis of the ester protective group of α-carboxyl group at the final step.



Scheme 2 Synthesis of **1b**.

Acknowledgement

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(10) GlcNAc (**2**, 40.4 g, 182.9 mmol) was dissolved in H_2O (220 mL) and saturated with NH_4HCO_3. The conversion of GlcNAc into glucosylamine **3** was determined by ^1H NMR spectroscopy by comparing anomeric protons. After stirring for 4 days at 35 °C, the conversion reached 86%, and the solution was diluted with H_2O (150 mL). The mixture was desalted by AC-220-550 on Asahi Chemical Micro Acylyzer S3. At the initial stage, the conductivity was 87.8 mS and after the desaltation at the 15 V (1.07 A), it reached 0.6 mS. NH_4HCO_3 was estimated to be 5.6 mmol/L based on the calibration linear as described in Figure 2. The yields of **3** and **2** were quantitatively estimated to be 54% and 9%, respectively, by ^1H NMR using methyl β-D-glucopyranoside as internal standard. The mixture was frozen and lyophilized to give a highly hygroscopic white powder (30.1 g). Again at this stage, the amounts of **3** and **2** in the solid were estimated to be 22.3 g (101 mmol) and 3.0 g (14 mmol), respectively. No decomposition of **3** was observed into **2** and ammonia through the lyophilization. ^1H NMR (400 MHz, D_2O) δ 4.16 (d, 1 H, *J* = 9.3 Hz), 3.85 (dd, 1 H, *J* = 2.1, 12.4 Hz), 3.69 (ddd, 1 H, *J* = 2.1, 4.6, 12.4 Hz), 3.62 (t, 1 H, *J* = 9.3 Hz), 3.52 (m, 1 H), 3.55-3.40 (m, 2 H), 2.07 (s, 3 H); ^{13}C NMR (100 MHz, D_2O) δ 175.26, 85.01, 77.63, 75.36, 70.90, 61.71, 57.21, 23.21; IR (KBr) 3352, 1652, 1558, 1375, 1047 cm^{-1}.

(11) Ester **5a** was obtained from oxazolidinone **6a**[12] with sodium isobutoxide. The alkoxide solution prepared from isobutyl alcohol (37.8 mL) and sodium (0.83 g, 36.2 mmol) was added dropwise into the solution of **6a** (10.1 g, 36.2 mmol) in isobutyl alcohol (55 mL) at 65 °C with stirring. After the stirring was continued for 3 h, AcOH (10 g) was added to the reaction mixture. Conventional work up procedure and subsequent silica gel column chromatography of the residue [$CHCl_3$–MeOH–AcOH (90:3:2)] gave **5a** (5.5 g, 47% yield). Recrystallization from *n*-hexane–Et_2O afforded fine colorless needles. Mp 65.0–66.0 °C [lit.[12] mp 68–69 °C]; $[\alpha]_D^{22}$ -4.4 (c 1.3, AcOH); $[\alpha]_{435}^{22}$ -10.3 (c 1.3, AcOH) [lit.[12] $[\alpha]_{427}^{22}$ -11.8 (c 1.3, AcOH)]; ^1H NMR (400 MHz, $CDCl_3$) δ 7.28 (m, 5 H), 5.71 (d, 1 H, *J* = 8.3 Hz), 5.06 (m, 2 H), 4.58 (ddd, 1 H, *J* = 3.9, 4.4, 8.3 Hz), 3.92 (d, 2 H, *J* = 6.8 Hz), 3.04 (dd, 1 H, *J* = 3.9, 17.1 Hz), 2.85 (dd, 1 H, *J* = 4.4, 17.1 Hz), 1.86 (m, 1 H), 0.83 (d, 6 H, *J* = 6.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.83, 170.36, 155.89, 135.84, 128.40, 128.01, 127.96, 71.98, 67.23, 50.24, 36.46, 27.63, 19.06; IR (KBr) 3350, 3127, 2954, 1761, 1727, 1692, 1280, 1224, 1063 cm^{-1}. Its NMR spectrum was in good accordance with that reported previously.[12] No β-ester was detected. In contrast, the ring-opening reaction[20] of a cyclic anhydride of *N*-Cbz-aspartate with isobutyl alcohol gave a mixture of α-ester and β-ester (ca. 10:1, δ 3.92 for α-ester and 3.86 for β-ester). Attempts for enzyme-catalyzed selective hydrolysis[21] of diethyl *N*-Cbz-aspartate or ethyl *N*-Cbz-asparagine gave no fruitful results, such as the formation of a diacid, or entirely no reaction.

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(15) Mp 37.5-40.0 °C (colorless fine needles from CH_2Cl_2–hexane); ^1H NMR (400 MHz, $CDCl_3$) δ 7.37 (m, 5 H), 5.72 (d, 1 H, *J* = 7.3 Hz), 5.14 (d, 1 H, *J* = 12.2 Hz), 5.10 (d, 1 H, *J* = 12.2 Hz), 4.67 (ddd, 1 H, *J* = 2.4, 4.8, 7.3 Hz), 3.98 (d, 2 H, *J* = 6.8 Hz), 3.23 (dd, 1 H, *J* = 2.4, 18.1 Hz), 3.13 (dd, 1 H, *J* = 4.8, 18.1 Hz), 1.95 (m, 1 H), 0.92 (d, 6 H, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.90 (d, *J* = 357 Hz), 169.21, 155.65, 135.67, 128.44, 128.20, 128.01, 72.43, 67.38, 49.98, 35.14 (d, *J* = 52 Hz), 27.64, 18.93; IR (KBr) 3341, 2961, 1840, 1727, 1691, 1533, 1294, 1269, 1096 cm^{-1}.

(16) To a solution of **3** (782.9 mg, 2.42 mmol) in anhydrous DMF (15 mL) was added $NaHCO_3$ (383 mg, 4.56 mmol) and fluoride (**5b**, 872.8 mg, 2.94 mmol) at room temperature. After stirring for 2 h, the reaction mixture was filtered through a Celite pad and washed with 1,4-dioxane. The filtrate was evaporated and purified by silica gel column chromatography. Elution with $CHCl_3$–MeOH (5:1) afforded **1c** (1.07 g, 84%). Mp 195.0–196.5 °C; $[\alpha]_D^{25}$ +6.9 (c 0.81, MeOH); ^1H NMR (400 MHz, CD_3OD) δ 7.28 (m, 5 H), 5.09 (d, 1 H, *J* = 12.2 Hz), 5.05 (d, 1 H, *J* = 12.2 Hz), 4.93 (d, 1 H, *J* = 9.8 Hz), 4.58 (dd, 1 H, *J* = 4.9, 7.3 Hz), 3.88 (d, 2 H, *J* = 6.8 Hz), 3.81 (dd, 1 H, *J* = 1.5, 12.2 Hz), 3.73 (t, 1 H, *J* = 9.8 Hz), 3.64 (ddd, 1 H, *J* = 0.9, 3.4, 12.2 Hz), 3.44 (m, 1 H), 3.30 (m, 2 H), 2.79 (dd, 1 H, *J* = 4.9, 16.0 Hz), 2.68 (dd, 1 H, *J* = 7.3, 16.0 Hz), 1.90 (s, 3 H), 1.89 (m, 1 H), 0.90 (d, 6 H, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 175.45, 172.73, 172.26, 158.96, 137.83, 129.31, 128.92, 128.82, 80.24, 79.67, 76.24, 72.50, 71.76, 67.74, 62.62, 56.06, 52.14, 38.49, 28.97, 22.92, 19.40; IR (KBr) 3295, 3091, 2957, 1749, 1703, 1654, 1546, 1296, 1271 cm^{-1}. Anal. Calcd. For $C_{24}H_{35}N_3O_{10}$: C, 54.85; H, 6.71; N, 8.00. Found: C, 54.59; H, 6.84; N, 7.56.

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(19) The coupling product (**1c**, 200.9 mg, 0.38 mmol) was dissolved in a mixture of DMF (2.0 mL) and 0.2 M phosphate buffer (pH 7.0, 2.0 mL). The pH was adjusted to 7.0 with 2 mol/L HCl, and papain (Sigma, P3375, 56.4 mg,

107 units) and L-cysteine (11.0 mg, 0.09 mmol) were added. Then the mixture was stirred at 35 °C with a controlled addition of 0.5 mol/L NaOH to maintain the pH at 7.0. After stirring for 7 h, the reaction mixture was brought to pH 8.0 with 0.5 mol/L NaOH and then filtered through a Celite pad and washed with H_2O. The filtrate was adjusted to be pH 4.7 by the addition of 2 mol/L HCl, concentrated in vacuo, and the residue was purified by silica gel column chromatography. Elution with EtOAc–MeOH–H_2O (7:2:1) afforded **1b** (119.2 mg, 67%). Analytical sample (colorless fine needles from acetone–H_2O); mp 174.0–176.0 °C(dec) [lit.[4] mp 172–174 °C]; $[\alpha]_D^{20}$ +8.1 (c 1.00, H_2O) [lit.[4] $[\alpha]_D^{25.5}$ +5.2 (c 1, H_2O)]; ^{1}H NMR (400 MHz, D_2O) δ 7.45 (m, 5 H), 5.17 (m, 2 H), 5.09 (d, 1 H, *J* = 9.8 Hz), 4.57 (dd, 1 H, *J* = 4.3, 7.8 Hz), 3.89 (dd, 1 H, *J* = 2.0, 12.2 Hz), 3.82 (dd, 1 H, *J* = 9.8, 10.3 Hz), 3.76 (dd, 1 H, *J* = 4.9, 12.2 Hz), 3.63 (dd, 1 H, *J* = 8.3, 10.3 Hz), 3.52 (m, 2 H), 2.89 (dd, 1 H, *J* = 4.4, 16.1 Hz), 2.79 (dd, 1 H, *J* = 7.8, 16.1 Hz), 1.96 (s, 3 H); ^{13}C NMR (100 MHz, D_2O) δ 175.39, 173.42, 172.19, 158.47, 136.89, 129.48, 129.13, 128.49, 79.09, 78.41, 74.96, 70.31, 68.02, 61.35, 55.17, 51.82, 38.31, 22.91; IR (KBr) 3292, 3073, 1716, 1689, 1655, 1542, 1374, 1256, 1065 cm^{-1}. Anal. Calcd. For $C_{20}H_{27}N_3O_{10}$: C, 51.17; H, 5.80; N, 8.95. Found: C, 51.00; H, 5.90; N, 8.75. The NMR spectrum of fully deprotected form(**1a**) was identical with that reported previously.[22]

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