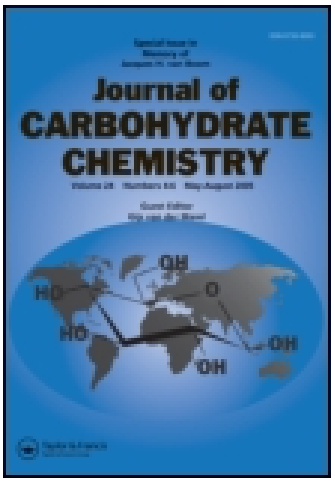

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ENZYMATIC SYNTHESIS OF *p*-NITROPHENYL β-CHITOBIOSIDE

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ABSTRACT

p-Nitrophenyl β-chitobioside (**2**) was prepared from *p*-nitrophenyl 2-acetamido-2--deoxy-β-D-glucopyranoside (**1**) using β-*N*-acetylhexosaminidase from *Aspergillus oryzae*. As minor by-product a (1→6)-linked regioisomer (**3a**) was formed. Addition of $(NH_4)_2SO_4$ and organic co-solvents (acetonitrile, dioxane) substantially increased the yield.

INTRODUCTION

We have recently demonstrated that hexosamines (GlcNAc, GalNAc) as monosaccharides or in multivalent conjugates are important ligands for the major activating receptor protein of rat natural killer cells, NKR-P1A.[1-3]

Testing a series of linear chitooligomers (n = 2-9) it was shown that oligosaccharide chain length is an important factor for the binding potency to the receptor.[4] Our present efforts aim at the synthesis of hexosamine oligosaccharide

building blocks that can eventually be turned into multivalent neoglycoconjugates, in order to combine effects of chain length and clustering.

p-Nitrophenyl glycosides are suitable building blocks for the preparation of cluster glycosides and glycodendrimers[5] and, moreover the aromatic moiety improves the binding to the NK receptors. Therefore, *p*-nitrophenyl β-chitobioside (**2**, *p*NP chitobioside) became our target as it can be expected to bind effectively to the NKR-P1 protein and can be clustered to multivalent conjugates.

The classical chemical preparation[6,7] of *p*NP chitobioside (**2**) is analogous to the synthesis of glycoside **1**, described in the literature.[8] Starting from very expensive chitobiose, it involves synthesis of the acetylated α-glycosyl chloride *via* peracetylated chitobiose, followed by phase transfer-catalyzed reaction with *p*-nitrophenol in acetone.[6,7]

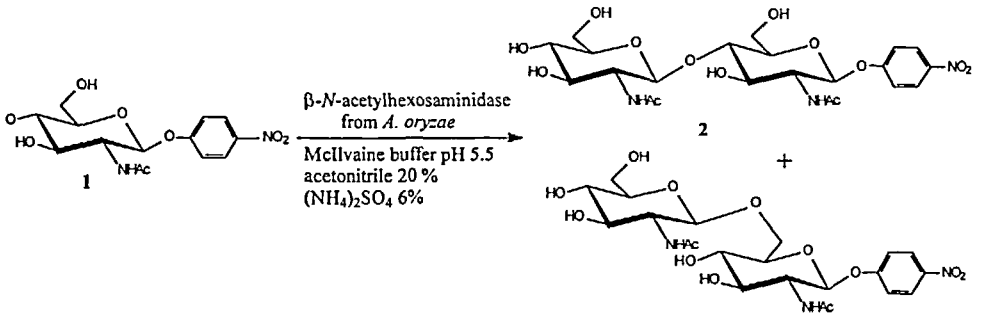
The bottleneck in this synthesis is the preparation of acetylated α-chitobiosyl chloride from chitobiose, employing acetic anhydride and HCl gas, which is accompanied by the cleavage of the glycosidic bond of the disaccharide. Overall yields related to chitobiose or peracetylated chitobiose did not exceed 10% in our hands, despite exhaustive reaction optimization (lit.[6] 18.5%, lit.[7] 6.5%). An alternative chemical synthesis of **2** based on attaching the GlcNAc unit to *p*NP GlcNAc (**1**) gave preferentially its 1→6 isomer as the 6-position is much more reactive than the 4-position.[9]

These limitations prompted us to search for an alternative approach enabling easier and cheaper preparation of **2**. Enzymatic methods[10] are often advantageous over chemical ones and when appropriately optimized and combined with suitable separation techniques they can be used for preparative purposes. β-*N*-Acetylhexosaminidase from *Aspergillus oryzae* (E.C. 3.2.1.52) proved to have excellent synthetic capabilities,[11-13] and it is available either commercially (Sigma) or by a simple preparation.[14] This enzyme prefers transglycosylation onto the 4-position of *gluco* configured sugars despite its lower chemical reactivity.

We report here a novel chemoenzymatic synthesis of *p*NP chitobioside (**2**) starting from *N*-acetyl-D-glucosamine using β-*N*-acetylhexosaminidase from *A. oryzae*.

RESULTS AND DISCUSSION

Starting from glycoside **1**, the desired product **2** was prepared in one step by enzymatic transglycosylation (Scheme). *p*NP GlcNAc (**1**) which can be easily prepared from *N*-acetyl-D-glucosamine in high yield[8] served both as glycosyl donor and acceptor.



As water activity can substantially influence the yields of enzyme-catalysed glycosylations[13] we have investigated the addition of high concentrations of inorganic salts ($(NH_4)_2SO_4$, LiCl) together with the addition of organic co-solvents. $(NH_4)_2SO_4$ is a commonly used 'salting-out' agent lowering the water activity, LiCl was chosen because of the large hydration sphere. In the preliminary experiments we have found that these two salts usually do not lower the activity of glycosidases. In the first step the optimum concentration of acetonitrile as co-solvent was studied (Figure 1). The co-solvent increased, *i.e.*, the solubility of glycoside **1**. This step was followed by optimization of the ammonium sulphate concentration (Figure 2). The activity of the enzyme was not impaired by ammonium sulphate addition. On the contrary, it was somehow increased,[13] and the enzyme was also more stable. Analogous additions of LiCl did not bring substantial increase of the yields as in the previous cases.[13] Addition of organic solvent increased the yields, partly by increasing of the *p*NP GlcNAc solubility and also by lowering water activity. The best reaction conditions comprised of 20 % acetonitrile, v/v, and 30 $g.L^{-1}$ of $(NH_4)_2SO_4$. During the scale up it was found that the

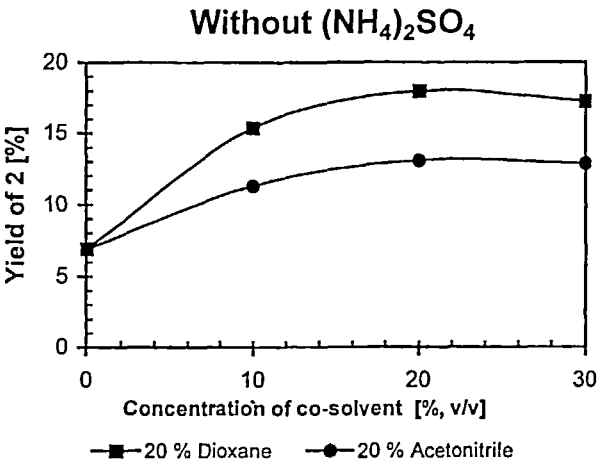


Figure 1 Yield of enzymatic synthesis of 2 - influence of co-solvents.

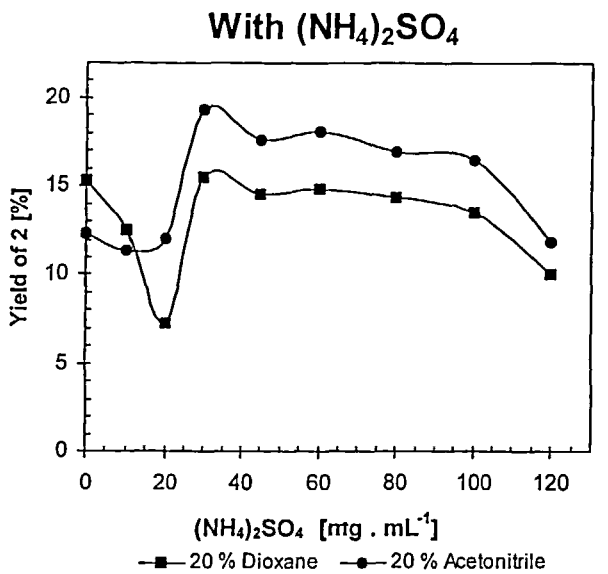


Figure 2 Yield of enzymatic synthesis of 2 - influence of ammonium sulphate concentration using an optimum co-solvent content of 20 %.

reaction slowed down after some time probably due to the enzyme inactivation, therefore, another portion of the enzyme was supplemented in the preparatory experiment. Analytical experiments were done with single dose of the enzyme only.

Despite the optimum ammonium sulphate concentration of 30 $g.L^{-1}$ found by analytical experiments, higher salt concentration (60 $g.L^{-1}$) in the preparatory experiment was used as it was observed that under these conditions the product **2** precipitates out that leads to further increase of its yield (shift of the thermodynamic equilibrium).

The enzymatic reaction was terminated by boiling. All nitrophenyl-containing compounds (free *p*-nitrophenol, unreacted **1**, *p*NP oligoglycosides formed) were separated from polar components (salts, sugars, proteins) by solid phase extraction on Amberlite XAD-4 resin column. After elution with MeOH the mixture was separated by gel chromatography on Sephadex LH-20 eluted with MeOH-H_2O (4:1, v/v). Product **2** was isolated in 22 % overall yield (based on consumed **1**; unreacted **1** could be recovered and reused). The structure of compound **2** was determined by NMR spectroscopy. Proton signals were assigned by COSY, TOCSY and HOM2DJ experiments. H-1 was differentiated from H-1' using its NOE to *ortho*-*p*NP protons and an HMBC crosspeak to carbon at 163.38 ppm (phenolic C of *p*NP). The proton assignment was transferred to ^{13}C NMR by HMQC. The site of attachment follows from the HMBC experiment (crosspeaks between H-1' and C-4 and between H-4 and C-1'). After submission of this paper, Nilsson et al.[15] described the preparation of a β(1→3)-linked *p*NP chitobiose analogue under very similar conditions and using the same enzyme from *A. oryzae*. However, they reported ^{13}C NMR spectra in DMSO only. We have, therefore, obtained the ^{13}C NMR of our compound in the same solvent and compared both spectra by calculation of a similarity index.[16] The high value of this criterion (S = 0.9958) indicates the identity of both compounds and, therefore, it appears that the structure of the *p*NP-chitobioside in the Nilsson paper[15] was incorrectly determined.

Besides the starting material and the disaccharide **2**, another minor product **3a** was isolated by Sephadex LH-20 chromatography, however, not in pure form. Therefore, it was peracetylated using acetic anhydride / pyridine under catalysis of 4-dimethylaminopyridine and the product further purified on silica gel ($CHCl_3$-MeOH

9:1, v/v) to afford a pure compound. It was identified by NMR as *p*-nitrophenyl 2 - acetamido - 6- *O*- (2 - acetamido - 2-deoxy-3,4,6-tri- *O*-acetyl- β-D-gluco-pyranosyl)-2-deoxy-3,4-di-*O*-acetyl-β-D-glucopyranoside (**3b**). Both H-6 protons of **3b** showed an upfield shift and C-6 was shifted downfield as compared to the spectrum of **2**. The formation of an analogous chitobiose regioisomer has been observed before during the enzymatic condensation of *N*-acetylglucosamine using the same enzyme.[11] This procedure allows the synthesis of *p*NP chitobioside on a preparatory scale.

EXPERIMENTAL

General methods. Reactions were monitored by TLC on Silica gel F_{254} (Merck) with solvent system 1-propanol-water-28 % ammonia soln. (7:2:1, v/v). The spots were visualised by UV light and by charring with 10 % H_2SO_4 in EtOH. HPLC was measured under the following conditions: pump Waters 600, Waters PAD 996 detector, integration at 280 nm, mobile phase MeCN/H_2O 83:17 (v/v), flow rate 1 mL/min, column Lichrospher 100 NH_2 5μm, 250x4 mm (Watrex, Prague, CZ), column temperature 40 °C.

NMR spectra were measured on a Varian INOVA-400 spectrometer (399.90 MHz for ^1H, 100.56 MHz for ^{13}C) in CD_3OD (**2**) or $CDCl_3$ (**3b**) at 30 °C. Carbon signal multiplicity was determined by APT, data for **3b** were obtained from HMQC. 2D NMR experiments - COSY, TOCSY, HOM2DJ, HMQC and HMBC - were performed using manufacturer's software.

Positive-ion electrospray-ionization mass spectra were recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, D) of BE geometry. A sample dissolved in acetonitrile:water (2:1, v/v) was continuously infused through a stainless capillary held at 4.3 kV into the electrospray ion source *via* a linear syringe pump at a flow rate of 80 $\mu L.min^{-1}$. For a high resolution experiment the instrument was tuned to a resolution of about 6 000 (10% valley definition) and the measurement was carried out by the peak-matching method against a mixture of polypropylene glycols (average M_r = 725) as an internal standard.

CD spectra were recorded on Jobin Yvon Mark 6 spectrometer in the spectral region 200-400 nm.

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[8] (**1**) and β-*N*-acetyl-hexosaminidase from *A. oryzae*[14] were prepared as described previously. One unit (U) is defined as μmol of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside hydrolyzed at pH 5.5 and 37 °C.

Analytical experiments (reaction optimization) were performed as follows: To a solution of **1** (38 mg, 0.11 mmol) and respective amount of the salt ($(NH_4)_2SO_4$ or LiCl) in McIlvaine buffer (citric acid 50 mM / Na_2HPO_4 100 mM, mixed to afford pH 5.5, 800 μL) a respective amount of acetonitrile or dioxane was added and the solution was made up with the buffer to 1 mL. β-*N*-Acetylhexosaminidase from *A. oryzae* (2.3 U) was added, and the mixture was incubated under shaking at 37 °C. Samples (50 μL) were quickly diluted with 300 μL of MeOH (to inactivate the enzyme) and the products were analyzed by HPLC.

***p*-Nitrophenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)--2-deoxy-β-D-glucopyranoside** (**2**). To a solution of **1** (1.6 g, 4.67 mmol) and $(NH_4)_2SO_4$ (2.7 g, 20.4 mmol) in McIlvaine buffer (pH 5.5, 32 mL) acetonitrile (9.6 mL) was added at 50 °C. The solution was cooled to 37 °C, its final pH was adjusted to 5.5, and β-*N*-acetylhexosaminidase (36 U) was added. The mixture was incubated at 37 °C. The reaction course was monitored by TLC. After 2 h another portion of the enzyme (15 U) was added. After a total of 2.5 h the reaction was stopped by heating (100 °C, 10 min), and the rest of acetonitrile was removed at 40 °C under reduced pressure. The reaction mixture diluted with water (150 mL) was then applied to XAD-4 resin column (15 x 6.5 cm) and washed extensively with H_2O. Retained *p*NP derivatives were eluted with methanol. After evaporation of solvents, the residue was purified in three equivalent portions by gel filtration using Sephadex LH-20 (113 x 4.6 cm) eluted with methanol-water (4:1, v/v) as a mobile phase. The chromatography afforded **2** (215 mg, 22.2 % related to consumed donor) and 980 mg of substrate **1** was recovered. Crude **3a** was obtained as a minor by-product from the last separation step (Sephadex LH-20 chromatography) in the yield of 3.2 mg (3.8 %). After peracetylation (Ac_2O/py + 4-dimethylaminopyridine (0.2 % w/v), 48 h) and flash chromatography on silica gel

($CHCl_3$-MeOH 9:1, v/v) 2 mg of the pure compound **3b** were obtained mainly for the purpose of the structure determination (yield ca 2 %). Due to the paucity of the compound its CD spectrum was recorded as the optical rotation seemed to be unreliable. Data of **2**: $[\alpha]_D^{23}$ = -15.1° (*c* 0.96, H_2O-MeOH 1:1, v/v); ^{1}H NMR (CD_3OD) δ 1.987 (3 H, s, Ac), 2.044 (3 H, s, Ac), 3.336 (1 H, dd, $J_{3',4'}$=8.4 Hz, $J_{4',5'}$=9.7 Hz, H-4'), 3.395 (1 H, ddd, $J_{4',5'}$ = 9.7 Hz, $J_{5',6'a}$ = 2.2 Hz, $J_{5',6'b}$ = 6.4 Hz, H-5'), 3.478 (1 H, dd, $J_{2',3'}$ = 10.4 Hz, $J_{3',4'}$ = 8.4 Hz, H-3'), 3.609 (1 H, ddd, $J_{4,5}$ = 10.1 Hz, $J_{5,6a}$ = 1.9 Hz, $J_{5,6b}$ = 4.5 Hz, H-5), 3.673 (1 H, dd, $J_{5',6'b}$ = 6.4 Hz, $J_{6'a,6'b}$ = 11.9 Hz, H-6'b), 3.677 (1 H, dd, $J_{3,4}$ = 8.2 Hz, $J_{4,5}$ = 10.1 Hz, H-4), 3.700 (1 H, dd, $J_{6a,6b}$ = 12.3, $J_{5,6b}$ = 4.5 Hz, H-6b), 3.760 (1 H, dd, $J_{1',2'}$ = 8.4 Hz, $J_{2',3'}$ = 10.4 Hz, H-2'), 3.784 (1 H, dd, $J_{2,3}$ = 10.4 Hz, $J_{3,4}$ = 8.2 Hz, H-3), 3.882 (1 H, dd, $J_{6a,6b}$ = 12.3 Hz, $J_{5,6a}$ = 1.9 Hz, H-6a), 3.945 (1 H, dd, $J_{6'a,6'b}$ = 11.9 Hz, $J_{5',6'a}$ = 2.2 Hz, H-6'a), 4.047 (1 H, dd, $J_{1,2}$ = 8.4 Hz, $J_{2,3}$ = 10.4 Hz, H-2), 4.552 (1 H, d, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.228 (1 H, d, $J_{1,2}$ = 8.4 Hz, H-1), 7.189 & 8.224 (4 H, AA'BB', $\sum J$ = 9.3 Hz); ^{13}C NMR δ 23.20 q, 23.35 q (2 x NAc), 56.69 d (C-2), 57.67 d (C-2'), 61.84 t (C-6), 62.92 t (C-6'), 72.37 d (C-4'), 74.25 d (C-3), 76.18 d (C-3'), 77.18 d (C-5), 78.53 d (C-5'), 81.42 d (C-4), 100.21 d (C-1), 103.59 d (C-1'), 117.98 d (2 C, *ortho*-), 126.95 d (2 C, *meta*-), 144.38 s (*para*-), 163.91 s (*ipso*-), 174.06 s (C=O), 174.10 s (C=O); HR-ESI/MS: *m/z* 568.1750 $[M + Na]^+$ (568.2283 calcd. for $C_{22}H_{31}N_3O_{13}Na$).

Data of **3b**: CD spectrum: *c* 1.32.10^{-3} M, trifluoroacetic acid; $[\Delta\varepsilon]_{210}^{20}$ = -1.25, $[\Delta\varepsilon]_{235}^{20}$ = 0, $[\Delta\varepsilon]_{245}^{20}$ = +0.15, $[\Delta\varepsilon]_{260}^{20}$ = 0, $[\Delta\varepsilon]_{300}^{20}$ = -0.40; ^{1}H NMR ($CDCl_3$) δ 1.859, 1.961, 2.022, 2.044, 2.063, 2.071, 2.096 (all s, each 3 H, 7 x Ac), 3.561 (1 H, dd, $J_{5,6b}$ = 5.7 Hz, $J_{6a,6b}$ = 11.2 Hz, H-6b), 3.596 (1 H, ddd, $J_{4',5'}$ = 10.0 Hz, $J_{5',6'a}$ = 4.8 Hz, $J_{5',6'b}$ = 2.4 Hz, H-5'), 3.887 (1 H, ddd, $J_{1',2'}$ = 8.5 Hz, $J_{2',3'}$ = 10.6 Hz, $J_{2',NH'}$ = 8.4 Hz, H-2'), 3.907 (1 H, ddd, $J_{4,5}$ = 10.0 Hz, $J_{5,6b}$ = 5.7 Hz, $J_{5,6a}$ = 2.0 Hz, H-5), 4.008 (1 H, dd, $J_{5,6a}$ = 2.0 Hz, $J_{6a,6b}$ = 11.2 Hz, H-6a), 4.088 (1 H, ddd, $J_{1,2}$ = 8.2 Hz, $J_{2,3}$ = 10.5 Hz, $J_{2,NH}$ = 8.4 Hz, H-2), 4.111 (1 H, dd, $J_{5,6'b}$ = 2.4 Hz, $J_{6'a,6'b}$ = 12.3 Hz, H-6'b), 4.241 (1 H, dd, $J_{5',6'a}$ = 4.8 Hz, $J_{6'a,6'b}$ = 12.3 Hz, H-6'a), 4.611 (1 H, d, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.048 (1 H, dd, $J_{3',4'}$ = 9.3 Hz, $J_{4',5'}$ = 10.0 Hz, H-4'), 5.087 (1 H, dd, $J_{3,4}$ = 9.0 Hz, $J_{4,5}$ = 10.0 Hz, H-4), 5.223 (1 H, dd, $J_{2',3'}$ = 10.6 Hz, $J_{3',4'}$ = 9.3 Hz, H-3'), 5.445 (1 H, dd, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 9.0 Hz, H-3), 5.450

(1 H, d, $J_{1,2}$ = 8.2 Hz, H-1), 5.558 (1 H, d, $J_{2,NH}$ = 8.4 Hz, N-H), 5.655 (1 H, d, $J_{2',NH'}$ = 8.5 Hz, N'-H), 7.102 and 8.238 (4 H, AA'BB', $\sum J$ = 9.2 Hz); ^{13}C NMR ($CDCl_3$) δ 20.6 q (5 C), 23.2 q, 23.3 q, 54.8 d (C-2'), 54.9 d (C-2), 62.0 t (C-6'), 67.6 t (C-6), 69.7 d (2 C, C-4, C-4'), 72.1 d (C-5), 72.2 d (C-3), 72.9 d (C-3'), 74.0 d (C-5'), 97.9 d (C-1), 100.8 d (C-1'), 117.0 d (2 C), 126.3 d (2 C). HR-ESI/MS: *m/z* 778.2285 $[M + Na]^+$ (778.2283 calcd. for $C_{32}H_{41}N_3O_{18}Na$).

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