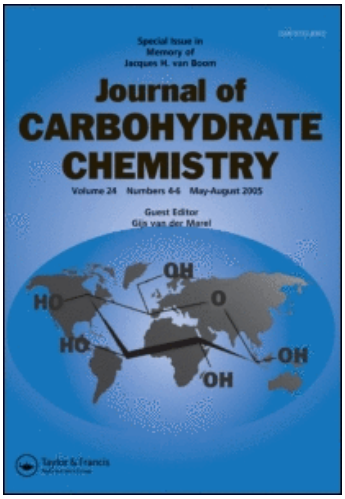

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PREPARATION OF ManNAc CONTAINING CHITOOLIGOMERS BY ISOMERISATION AND THEIR BINDING TO NKR-P1 PROTEIN

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ABSTRACT

4-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-mannopyranose (**2**) was prepared from diacetylchitobiose (**1**) by Lobry de Bruyn-Alberda van Ekenstein rearrangement under catalysis of $Ca(OH)_2$. The disaccharide **2** shows about ten times higher affinity than **1** towards NKR-P1 protein acting as a crucial activating receptor of rat natural killer cells (leukocytes). Chitotriose (**3**) can be epimerized analogously to give GlcNAcβ1-4GlcNAcβ1-4ManNAc (**4**) and its binding properties to NKR-P1 are also about ten times higher than those of **3**.

INTRODUCTION

In our recent work[1] modified chitooligomers were tested for their affinity towards NKR-P1 protein. Rat NKR-P1 antigen is a carbohydrate-binding protein from rat natural killer (NK) cells with remarkably high affinity for both calcium and certain monosaccharides such as GlcNAc.[2] Chitooligomers [-GlcNAcβ1-4-]$_n$ (n = 2-9) bind to this protein with high affinity, chitotetraose being superior.[3] Recent investigations revealed that NKR-P1 is an important activation antigen of NK cells as well as tumour infiltrating T lymphocytes, and that the recognition of carbohydrate ligands by this receptor is intimately linked to an increased ability to kill NK sensitive as well as certain NK resistant tumour cells.[4]

Various derivatives of chitooligomers were investigated as useful ligands for the mapping of the carbohydrate-binding site of NKR-P1. Until now, mostly modifications of chitooligomers at the non-reducing end were studied.[1,3] A terminal NHAc group was not essential for the binding to this lectin since its substitution with an OH group only slightly lowered the affinity for the receptor as shown, e.g., Glcβ1-4GlcNAc compared to GlcNAcβ1-4GlcNAc.[1] Replacement of a terminal β-D-GlcNAc with a β-D-Man residue also resulted in moderately lower binding affinity. However, the substitution by β(1→4)-linked Gal or a change of the glycosidic bond, e.g., to α(1→4)-linked Glc, resulted in a sharp decrease of the affinity.[3] Therefore, it seemed that for an efficient binding both the structure and linkage of the glycosidic residue at the non-reducing end and the length of the chitooligomer chain were important.

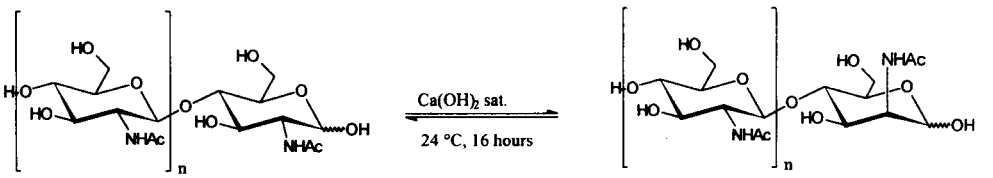
To investigate the influence of the structural changes at the reducing terminus, we started to study the epimerization of an equatorial to an axial 2-NHAc group catalysed by alkali metals.[5-9] Previously this reaction was successfully applied to the formation of ManNAc from GlcNAc using an optimised procedure with saturated aqueous $Ca(OH)_2$ which gave about 30 % yield of the ManNAc under thermodynamic equilibrium.[10]

Here, we report the preparation of the epimer at 2-position of chitobiose, 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-mannopyranose (**2**)

and that of the corresponding derivative **4** obtained from chitotriose **3** (as a mixture), and their binding activity towards the rat NKR-P1 protein.

RESULTS AND DISCUSSION

The rearrangement of reducing sugars in water catalyzed by alkali (Lobry de Bruyn-Alberda van Ekenstein transformation)[5-7] involves the ring opening, enolization of the aldehyde, isomerization at C-2, and subsequent ring closure. Ca^{2+} ions and ions of similar size such as La^{3+} stabilise well the transition state.[8]



1 n = 1
3 n = 2

2 n = 1
4 n = 2

With 2-acetamido-2-deoxy sugars, the number of products is smaller then with simple monosaccharides in which ketoses are also formed. As demonstrated by HPLC, reaction mixtures resulting from the treatment of chitobiose (**1**) and chitotriose (**3**) contained about 30% of the *manno*-analogues **2** and **4**, respectively. No other by-products were observed. Repeated HPLC was necessary for the final purification in preparative amounts.

The reaction mixture of chitotriose (**3**) and the epimerization product GlcNAcβ1-4GlcNAcβ1-4ManNAc (**4**) was not separable. The presence of **4** could be confirmed by NMR as indicated by the typical small coupling in the *manno*-configuration ($J_{1,2}$ = 1.4 Hz).[11] An integration in the ^{13}C NMR spectra of the anomeric carbons in the reducing residues (δ 93.32, 93.64, 95.84, 97.70 ppm) indicated the content of **4** to be 30.2 %.

The four oligosaccharides (**1-4**) were studied as inhibitors of binding of the recombinant soluble dimeric form of NKR-P1 protein to its high-affinity ligand.

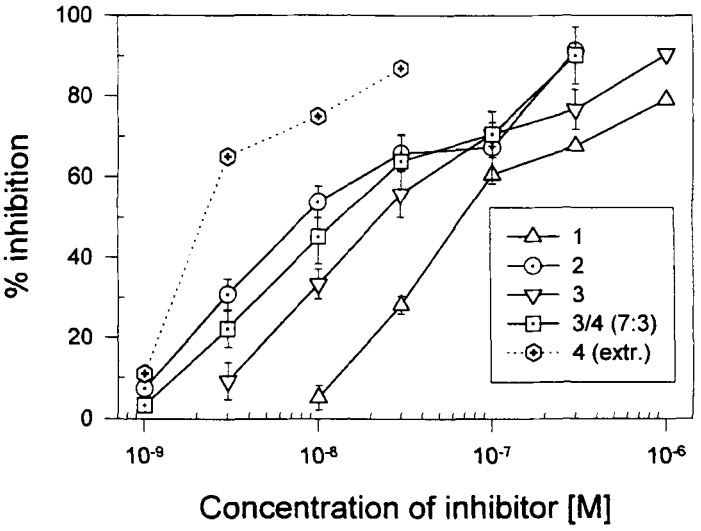


Figure 1. Investigation of the potencies of oligosaccharides **1-4** to inhibit the binding of the soluble dimeric form of rat NKR-P1A protein to its high affinity ligand. Details of the inhibition assays are given in the experimental section. Compound **4** was available only in mixture with compound **3**, and the inhibition data for pure **4** have been extrapolated from those for pure **3** and for a **3/4** mixture (69.8/30.2) using the formula % inhibition (**4**) = % inhibition (**3**) + {[% inhibition (**3/4**) - % inhibition (**3**)] x [100 / [molar % **4**]}.

Chitobiose (**1**) and chitotriose (**3**) were used as reference compounds since their affinities for NKR-P1 protein have been extensively studied in our previous work.[3] In accordance with these experiments, the two oligosaccharides gave IC_{50} of approximately 7×10^{-8} M and 2×10^{-8} M, respectively (Figure 1). However, the inhibitory potency of compound **2** was nearly ten times higher compared to chitobiose (**1**) (IC_{50} of 8×10^{-9} M).

A similar tendency was evident for the related pair of chitotriose **3** and its 2-epimer **4**. The mixture of **3** and **4** (7:3) was used directly for the inhibition tests with the NKR-P1 protein and an approximate effect of pure **4** was calculated from inhibition curves of pure **3** and that of a mixture of **3** and **4** with a known content of **4** (30.2 % - determined by NMR). This preparation was approximately twice as good as inhibitor of binding compared to pure chitotriose (**3**) (*i.e.* IC_{50} for a mixture of compounds **3** and **4**

in the approximate molar ratio 70 to 30 was around 1×10^{-8} M). When the inhibition data recorded for the above preparation were extrapolated to pure epimer **4** (Figure 1, dotted line), an IC_{50} of 2×10^{-9} M was calculated, which is again a ten times better inhibition potency than that recorded for the parent chitotriose **3**.

This study clearly indicates the impact of the epimerization of the 2-NHAc group in the chitooligomer chain for the oligosaccharide binding to this natural killer cell signalling protein. *N*-Acetylmannosamine is an important component of the linkage unit attaching teichoic acid to the peptidoglycans that are major components of Gram-positive bacterial cell walls.[12] In some pathogenic bacteria (*Streptococcus pneumoniae*) this component participates in forming the cell capsule responsible for evading the immune system and, therefore, for their virulence.[13] These facts could explain high affinity of the NK cell receptors to the ManNAc containing saccharides. Our findings also represent an important step in the development of potent immunostimulatory compounds for the use in anticancer and antimicrobial therapies involving activated lymphocytes.

EXPERIMENTAL

General methods. ^{1}H and ^{13}C NMR spectra were measured on a Varian VXR-400 spectrometer (399.95 MHz for ^{1}H, 100.58 MHz for ^{13}C) in D_2O at 25 °C. The sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid was used as an internal reference for both nuclei. Chemical shifts are given in the δ scale; digital resolution was 0.0002 and 0.006 ppm, respectively, *J* values are given in Hz COSY, delay-COSY, and HOM2DJ experiments were performed using the manufacturer's software. Carbon multiplicities were determined by APT (Attached Proton Test). High-resolution mass spectrometry in electrospray ionization (HR-MS ESI) were carried out on a Finnigan MAT 95 double-focusing instrument (Finnigan MAT, Bremen, Germany) of BE geometry by the peak-matching method against a mixture of polypropylene glycols (average M_r = 425, Aldrich) as an internal standard. Optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22 °C. TLC was carried out using silica gel

60 GF_{254} (Merck) with the solvent system 1-propanol - water - conc. ammonia (7:2:1), twice developed. The spots were visualised by charring with 5% H_2SO_4 in EtOH. Analytical HPLC was carried out on an SP 8800 high performance liquid chromatograph (Spectra Physics, CA, U.S.A.) equipped with UV detector operated at 210 nm; Lichrospher 100 NH_2 column (250 x 4 mm, 5 μm) (Watrex, Czech Rep.); the mobile phase was acetonitrile - water (79 : 21, v/v) at a flow rate 0.6 mL/min, ambient temperature; retention times were 19.20 and 21.15 for **1** and **2**, respectively. Preparative HPLC was performed on a Spectra Physics modular preparative system (San Jose, CA, USA) comprised of an SP8810 Ti pump, a Rheodyne injection port with a 100 μL sample loop, a Spectra 100 variable wavelength UV/VIS detector at 210 nm and a ChromaJet SP 4400 integrator. The column (250 x 8 mm) was packed with 5 μm Lichrospher 100 NH_2 and eluted with acetonitrile - water (79 : 21, v/v) at a flow rate of 2 mL/min. and ambient temperature. Chromatography was repeated twice to give a preparative yield of 10 % of **2**. Retention times: (**1**) 22.15 min., (**2**) 24.1 min.

Quantitative inhibition assays with the recombinant dimeric soluble form of NKR-P1, NKR-341 protein, were performed as described previously.[2,3] Briefly, tested oligosaccharides and ^{125}I-NKR-341 protein were incubated in duplicates in microtiter wells coated with $GlcNAc_{16}BSA$ (Sigma) 2 h at 4 °C. Wells were washed four times, dried, counted in a gamma counter, and % of inhibition calculated after subtracting of background counts.

4-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-mannopyranose (2). Chitobiose (**1**) (80 mg, 0.19 mmol) obtained by acidic cleavage of chitin[14,15] was dissolved in saturated aqueous $Ca(OH)_2$ solution (2 mL) and left overnight at laboratory temperature. After ca. 20 hours the solution was saturated by CO_2 and precipitated $CaCO_3$ was filtered off. The remaining solution was freeze-dried, and the mixture of **2** and **1** was separated by preparative HPLC under the conditions given in the general methods. Analytical yield of **2**: 25 % (as determined by HPLC); preparative yield was 7.9 mg (10 %). $[\alpha]_D^{23}$ = + 10.4 (*c* 0.13, H_2O). ^{1}H NMR (D_2O) δ 2.048, 2.068, 2.072 and 2.088 (each 3H, each s, 4 x NHAc), 3.946 (1 H, dd, $J_{2,3}$ = 4.7 Hz, $J_{3,4}$ = 9.2 Hz , H-3β), 4.147 (1 H, dd, $J_{2,3}$ = 4.8 Hz, $J_{3,4}$ = 9.1 Hz, H-3α), 4.336

(1 H, dd, $J_{1,2}$=1.7 Hz, $J_{2,3}$ = 4.8 Hz, H-2α), 4.485 (1 H, dd, $J_{2,3}$ = 4.7 Hz, $J_{1,2}$ = 1.6 Hz, H-2β), 4.559 (1 H, d, $J_{1',2'}$ = 8.4 Hz, H-1'α), 4.583 (1 H, d, $J_{1',2'}$ = 8.5 Hz, H-1β'), 5.019 (1 H, d, $J_{1,2}$ = 1.6 Hz, H-1β), 5.122 (1 H, $J_{1,2}$ = 1.7 Hz, H-1α). ^{13}C NMR (D_2O) δ 24.81 q, 24.95 q, 25.00 q ($CO\underline{C}H_3$), 55.55 d, 56.27 d, 58.45 d (C-2'β), 58.55 d (C-2'α), 62.82 t, 63.03 t, 63.41 t (C-6'), 70.47 d, 72.59 d (C-4'), 73.25 d, 73.66 d, 76.34 d (C-3'), 77.79 d, 78.75 d (C-5'), 79.48 d, 79.91 d, 95.60 d, 95.82 d, 104.26 d (C-1'), 177.44 s, 177.30 s, 177.58 s ($\underline{C}$=O). HRMS (ESI) *m/z* 447.1599 ([M + Na], $C_{16}H_{28}O_{11}N_2Na$, calc. 447.1591).

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