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Toward an Optimal Oligosaccharide Ligand for Rat Natural Killer Cell Activation Receptor NKR-P1[1]

Pavel Krist,* Eva Herkommerová-Rajnochová,* Jana Rauvolfová,* Tomáš Semeňuk,* Petra Vavrušková,* Jiří Pavlíček,† Karel Bezouška,† Ladislav Petruš,‡ and Vladimír Křen*,[2]

**Institute of Microbiology, Laboratory of Biotransformation, Academy of Sciences of Czech Republic, Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic; †Department of Biochemistry, Faculty of Science, Charles University Prague, Hlavova 8, CZ-128 40 Prague 2, Czech Republic; and ‡Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, SK-842 38 Bratislava, Slovakia*

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Aminosugars have a good affinity for the NKR-P1A protein, the major activating receptor at the surface of rat natural killer cells. We have systematically investigated the structural requirements of the recombinant soluble dimeric form of the receptor for its optimal carbohydrate ligands. While *N*-acetyl-D-mannosamine was the best neutral monosaccharide ligand, its participation in the context of an extended oligosaccharide sequence was equally important. The IC_{50} value for the GalNAcβ1 → ManNAc disaccharide was nearly 10^{-10} M with a further possible increase depending on the type of the glycosidic linkage and the aglycon nature. From the point of view of its availability, stability, and affinity for the receptor and a potential *in vivo* use, these studies are pivotal for the design of an oligosaccharide or glycomimetics suitable for further clustering into the multivalent glycodendrimers. © 2001 Academic Press

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Receptors for carbohydrates belong to several protein families. The recognition of the carbohydrate antigens by the immunoglobulin superfamily members (antibodies, T-cell receptors) has been studied, e.g., on anti-carbohydrate monoclonal antibodies. For many non-immunoglobulin carbohydrate-dependent interactions, however mostly the receptors related to the C-type animal lectins superfamily are responsible (1). Membrane proteins related to the C-type lectins are thought to constitute important activation (NKR-P1A) as well as inhibitory (Ly-49, CD94) receptors of natural killer cells (2); the intracellular signals transmitted by these molecules are known to regulate important effector functions of the NK cells, such as killing of malignantly transformed or virally infected cells, and production of various cytokines (3).

Our knowledge of the carbohydrate ligands for lectin immune receptors is seminal in their use for the manipulation of lymphocyte effector functions. In our previous work, we have shown that hexosamines (GlcNAc, GalNAc) in their free or clustered forms are important ligands for the major activating receptors of rat natural iller cells, NKR-P1A protein (4). Although the exact nature of the physiological ligand for this receptor remains unknown, we assume it to be related to high affinity sialylated or sulfated oligosaccharides found at the surface of certain NK cell sensitive (but not the NK cell resistant) tumor cells (5). Natural hexosamine-based oligosaccharides are also important as they are involved in the reactions of the surveillance and elimination of infected or malignantly transformed cells (6). Moreover, they have a prominent position in natural surface carbohydrates. In microorganisms (bacteria and fungi), aminosugars form essential components of cell walls (chitin, muramic acid). When released, they may trigger both local and systemic immune responses in the multicellular organisms (7). Recently, it has been found that various lipochitine molecules (NOD factors) (8) are involved, besides their role in nodulation processes of leguminous plants, also in embryogenesis of vertebrates (zebra-fish, frog) (9). *N*-Acetyl-D-mannosamine (ManNAc) is an important component of the linkage unit attaching teichoic acid to the pepti-

Abbreviations used: GlcN, 2-amino-2-deoxy-D-glucose GlcNAc; 2-acetamido-2-deoxy-D-glucose; GalN, 2-amino-2-deoxy-D-galactose; GalNAc, 2-acetamido-2-deoxy-D-galactose; ManN, 2-amino-2-deoxy-D-mannose; ManNAc, 2-acetamido-2-deoxy-D-mannose; TalNAc, 2-acetamido-2-deoxy-D-talose, Fuc, 6-deoxy-D-galactose; Man, D-mannose; Neu5Ac, 5-acetylneuraminic acid, KDO, 3-deoxy-D-*manno*-2-octulosonic acid; GlcUA, glucuronic acid; PAMAM, polyamidoamine (Starburst); BSA, bovine serum albumin; NK, natural killer; NKR-P1, natural killer rat cell protein-1; *p*NP, *p*-nitrophenyl.

[1] This paper is dedicated to 60th anniversary of Professor Joachim Thiem.

[2] To whom the correspondence should be addressed. Fax: +420-2-4752509. E-mail: kren@biomed.cas.cz.

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doglycan that is a major component of G^+ bacterial cell walls (10). In some pathogens (e.g., in *Streptococcus pneumoniae*), ManNAc participates in the forming the cell capsule responsible for their virulence (11).

There is a growing evidence that carbohydrates can be used as therapeutics, e.g. as vaccines (antimicrobial or anticancer) (12, 13) or for a direct treatment of some pathologic processes as envisaged for the Le^x antigen (14). Many natural carbohydrate ligands of the respective receptors are, however, not well suitable for *in vivo* applications because of their instability (e.g. half-life of the Le^x antigen in serum is ca 10 min (14)), lower affinity and a very high price. Therefore, glycomimetics overcoming these problems are being designed, carbohydrates are clustered to increase their effects, and the ligands are being optimized from all the above points (e.g., stability, activity, and availability).

Some high affinity carbohydrate ligands for the NKR-P1 receptor have been already identified (5) to be chondroitin and dermatan oligomers. Moreover, longer heparin-type oligosaccharide chains are efficient anticoagulants. In addition of that these compounds are considerably unstable *in vivo,* another major drawback for their application as immunostimulants is their extremely high price. Therefore, we have turned our attention to aminosugars as they were identified (as monosaccharides) to be the most effective ligands of NKR-P1 receptor.

A series of chitooligomers ranging from chitobiose ($n = 2$) up to chitoheptaose ($n = 7$) has been tested in this respect (15). With growing carbohydrate chain up to $n = 4$, the affinity to respective oligosaccharide was elevated in a ca half an order with every additional GlcNAc unit. Starting from chitopentaose, the affinity surprisingly dropped down, suggesting the tetrasaccharide chain to be optimal for the binding.

Chitooligomers and their derivatives can be suitable glycomimetics for the NK cell activation *in vivo* as they are sufficiently stable, easily available, biocompatible, as well as they can be conveniently modified and clustered. Chitooligomers themselves act as antibacterial agents (16). Also their immunostimulatory (17) and antitumor (against Sarcoma 180) (18) activities have been described.

We have performed series of preliminary experiments with chitooligomers and their simple derivatives modified either at the reducing (epimerization of GlcNAc into ManNAc) (19) or non-reducing (substitution of the NHAc group with OH) (20) moieties. We have studied also the effect of the clustering of GlcNAc to the affinity and found a dramatic increase—up to three orders—in the affinity of simple GlcNAc clusters (3–8 units) (21). These experiments have confirmed our assumption that chitooligomers are ideal lead structures for development of glycomimetics with a potential to activate NK cells *in vivo.*

To speed up the screening and optimization procedure, a combination of quite simple chemical methods with enzymatic ones was used (15). We have a strong background in enzymatic synthesis of aminosugars (22–24) based also on a large collection of β-*N*-acetylhexosaminidases (26, 27). Enzymatic methods are more straightforward than chemical ones, avoiding tedious protection/deprotection procedures. Quantities in the range of 10–100 mg necessary for characterisation, biological tests, and further modification can be quickly obtained by the approach what also speeds up the ligand optimization. We have also developed an enzymatic method for production of longer chitooligomers ($n = 6$–9) (28) not obtainable by hydrolysis of chitin as the lower ones.

The paper presents probing an optimal carbohydrate ligand for the NKR-P1 using commercial and newly prepared carbohydrates. The results demonstrate structural preferences leading to design of optimized ligand for NK cell stimulation.

MATERIALS AND METHODS

Materials. Monosaccharides, nitrophenyl glycosides and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside were from Sigma. Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside was synthesized according to a published procedure (29).

Construction of the expression plasmid. A soluble dimeric rat NKR-P1 protein was expressed using a prokaryotic expression vector pRSETB (Invitrogen, Groningen, The Netherlands). The DNA fragment containing the entire extracellular portion of the rat NKR-P1 starting with Val_{65} (4) was amplified by a polymerase chain reaction (Deep Vent DNA polymerase, New England BioLabs, Beverly, MA) from a plasmid pNKR-341P (4) using 5′-GATCTGTACG-ACGATGACGATAAGGTCTTAGTTCAAAAACCATCAGTG-3′ as a forward primer and an M13 universal primer hybridizing to the vector sequence as a reverse primer. This fragment was then linked with a pRSET expression vector sequence using an oligonucleotide 5′-GAAGGAGATATACATATGCGGGGT-3, as a forward primer, the M13 universal primer as a reverse primer, and the previously used forward primer as a linking primer. The amplified DNA was digested with NheI and with HindIII and ligated into the expression vector. The entire DNA sequence of an insert was checked by DNA sequencing using a Sanger dideoxy method performed using a SequiTherm Excel II DNA Sequencing kit (Epicentre Technologies, Madison, WI). The expression plasmid was designated pNKR348.

Protein expression and purification of NKR348. The expression vector was transformed into *Escherichia coli* strain BL-21pLysS (Invitrogen, Carlsbad, CA). The culture was grown at 37°C in LB medium with ampicillin and chloramphenicol to OD (at 550 nm) of 1.0, induced with 1 mM IPTG, and protein was produced for additional 4 h at 37°C. Inclusion bodies were prepared and stored at −80°C. Inclusion bodies were dissolved in 6 M guanidine–HCl and 50 mM DTT, and the solution was mixed with 8 M urea in 20 mM Tris–HCl, pH 8.0. The solution was centrifuged at 20,000 rpm in JA25.50 rotor (Beckman) for 30 min, and the clarified extract was applied onto a Q-Sepharose FF column (2.6 × 15 cm, Amersham–Pharmacia). The column was washed with 8 M urea in 20 mM Tris–HCl, pH 8.0, and eluted at 4 ml/min with a 60 min linear gradient of NaCl up to 1 M. NKR348 was in the flowthrough fraction, which was immediately applied onto a second purification column packed with Ni-NTA Superflow (1 × 15 cm, Qiagen, Hilden, Germany). The column was washed with 8 M urea in a 20 mM sodium

phosphate buffer, pH 7.8, and 500 mM NaCl, and eluted with a linear gradient into 8 M urea in a 20 mM phosphate buffer, pH 3.5, and 500 mM NaCl.

Refolding of NKR348. The purified NKR348 was immediately refolded by a dilution of 5 ml of the urea-containing protein solution (5 mg/ml) into 500 ml of the refolding buffer (50 mM Tris–HCl, pH 8.5 with 1 M L-arginine, 1 mM $CaCl_2$, 5 mM 2-cysteamine, 1 mM cystamine, 1 mM PMSF, 1 mM NaN_3, 1 μM leupeptin and 1 μM pepstatin). The protein solution was dialyzed against the intermediate buffer (50 mM Tris–HCl, pH 8.5, with 1 M urea, 1 M NaCl, 1 mM $CaCl_2$, 1 mM cysteamine, 0.2 mM cystamine, and 1 mM NaN_3), and the dimeric protein was recovered by ultrafilitrations through regenerated cellulose low binding membranes (Millipore, Bedford, MA) with a nominal cutoff limit of 100, 30, 10, and 3 kDa. The protein fraction recovered by 30 kDa membrane was extensively dialyzed against 10 mM Tris–HCl, pH 8.0, with 30 mM NaCl and 1 mM NaN_3, and the protein was further purified by anion exchange chromatography at a Milli Q HR 10/10 column (Amersham–Pharmacia) and by gel filtration on Superdex 75 PrepGrade (1.6 × 60 cm). The final preparation of the dimeric NKR348 was dialyzed extensively against a 10 mM Hepes buffer, pH 7.4, with 150 mM NaCl and 1 mM NaN_3, and stored at 10 mg/ml after concentration on a Centriprep 3 device (Millipore, Bedford, MA).

Preparation of NKR358. The histidine tag used during the purification procedure was removed by an enterokinase (New England BioLabs, Beverly, MA) digestion performed at 15°C. The cleaved protein was repurified by anion exchange chromatography and gel filtration as described above, and designated NKR358.

Analysis of NKR358. The final protein preparation was analyzed by SDS polyacrylamide gel electrophoresis under reducing and nonreducing conditions, and the removal of histidine tag was checked by Western blotting using anti His_6 monoclonal antibody (Clontech, Santa Clara, CA), and by N-terminal sequencing (20 cycles of automated Edman degradations, Protein Sequencer LF3600, Beckman–Coulter, CA). The total molecular mass of the final protein was checked by MALDI and ion trap mass spectrometry (Bruker Biflex II and Finnigan LC-QDeca instruments, respectively). The unambiguous arrangement of the intra- and interchain disulfide bridges in NKR348 was confirmed on pepsin-digested protein using mass spectrometry for the evaluation of the bridging pattern (30).

Plate binding assays. NKR358 was radiolabeled with $Na^{125}I$ using Iodogen (Pierce, Rockford, IL). Binding and inhibition assays were performed as described previously (4), with minor modifications. Briefly, 96-well polyvinylchloride microplates (Titertek Immuno Assay-Plate, ICN Flow, Irvine, Scotland) were coated overnight at 4°C with 50 μl of $GlcNAc_{17}$BSA (Sigma) in TBS + C buffer (10 mM Tris–HCl 150 mM NaCl 1 mM $CaCl_2$ and 1 mM NaN_3). Plates were blocked with 1% BSA in TBS + C for 2 h at 4°C, incubated with the concentration of the radiolabeled protein corresponding to half of the saturating amount and various dilutions of the inhibitors (total reaction volume 100 μl), washed three times with TBS + C, and drained. 100 μl of scintillation solution was added, and the radioactivity in the individual wells was counted by β-counting (Microbeta, Wallac, Turku, Finland). All experiments were performed in duplicates, and the inhibition degree was calculated relative to the wells containing no inhibitor.

Preparation of 3-acetamido-2,6-anhydro-1,3-dideoxy-1-nitro-D-glycero-D-manno-heptitol. A freshly prepared MeONa solution (1 g sodium in 40 ml of dry methanol) was at room temperature added to a stirred solution of 2-acetamido-2-deoxy-D-galactose (5 g) in a mixture of dimethyl sulfoxide (20 ml) and nitromethane (8 ml). After 2 h 1-butanol (130 ml) was added and the mixture was left for 24 h at 0°C. The crystalline product was filtered and twice washed with a cold mixture of 1-butanol and methanol (1:1, 60 ml). The crystals were transferred to a stirred solution of water (100 ml) and crushed solid CO_2 (20 g). Amberlite IR 120 in the H^+ form (100–200 mesh, 30 g) was added and left to stir until the pH was neutral. The resin was filtered off, washed with water (3 × 30 ml) and the filtrate with the washings were heated at 100°C for 30 h. The reaction mixture was decolorized with activated charcoal (2 g) and added to a strongly basic anion exchanger in the OH^- form (Dowex 1 X-2 400, 5 g) and left under occasional stirring for 1 h. The anex was filtered out and washed with water (200 ml). A suspension of the washed anex in water (30 ml) was ca 1 h treated under stirring with crushed solid CO_2 (4 × 10 g) at 20°C. The anex was finally filtered out, washed with water (3 × 30 ml) and the combined filtrates evaporated under reduced pressure to a crude 3-acetamido-2,6-anhydro-1,3-dideoxy-1-nitro-D-*glycero*-D-*manno*-heptitol, yield 1.3 g (26%) (31, 32).

NMR spectrum was recorded on a Unity *Inova*—400 MHz spectrometer (399.90 MHz for ^1H, 100.55 MHz for ^{13}C) in D_2O at 30°C. The assignment was based on COSY, *J*-resolved, and HMQC experiments performed using the manufacturer's software. Acetone and residual signal of water (HDO δ_H 4.508 ppm, acetone δ_C 30.5 ppm) were used as an internal standard.

^1H NMR (399.90, D_2O, 303 K): 1.826 (3H, s, C$\mathbf{H_3}$CO), 3.46–3.54 (2H, m, H-6), 3.48 (1H, m, H-5), 3.580 (1H, dd, J = 3.1, 10.4 Hz, H-3), 3.712 (1H, t, J = 10.4 Hz, H-2), 3.775 (1H, d, J = 3.1 Hz, H-4), 3.919 (1H, ddd, J = 2.7, 8.9, 10.4 Hz, H-1), 4.429 (1H, dd, J = 8.9, 13.7 Hz, H-1′a), 4.531 (1H, dd, J = 2.7, 13.7 Hz, H-1′b).

^{13}C NMR (100.55, D_2O, 303 K): 22.36 (q, $\mathbf{C}H_3CO$), 49.26 (d, C-2), 61.42 (t, C-6), 68.35 (d, C-4), 71.78 (d, C-3), 75.70 (d, C-1), 76.92 (t, C-1′), 78.92 (d, C-5), 175.16 (s, $CH_3\mathbf{C}O$).

RESULTS AND DISCUSSION

New refolding protocol. Soluble dimeric rat NKR-P1 protein has been expressed in our laboratory using the prokaryotic expression vectors pMALc/pMALp (4). Since both original expression and refolding protocol were based on a tedious, multistep procedure, we have developed a protocol based on the well established prokaryotic expression in non-K strain of *Escherichia coli* (strain BL-21pLysS). Using the new protocol, 2–3 mg of soluble dimeric rat NKR-P1 protein devoid of any sequence tags could be obtained starting from 2 liters of the induced bacterial culture. NKR358 displayed electrophoretic properties on reducing and nonreducing SDS polyacrylamide gels similar to the previously characterized dimeric protein, NKR-341. The identity of both protein preparations was further confirmed by the MALDI mass spectrometry. However, the electrospray ionization mass spectrometry revealed subtle differences between the two preparations. The spectrum of NKR358 is consistent with that of a homogeneous molecule with the mass of 35,656 Da. This is compatible with a model in which all the disulfides would be in their oxidized forms, presumably forming the intra- and interchain disulfide bridges. On the other hand, the mass spectrum of NKR-341 revealed a heterogeneous mixture of species containing variously oxidized cystein −SH groups. The detailed investigations of the disulfide pairing in the dimeric molecule (data to be published elsewhere) corroborated these results by revealing multiple species of cystic peptides in NKR-341 that could not be seen in the newly refolded NKR358. Concomitant with these structural differences, NKR358 had a much better stability upon a prolonged storage even in the absence of

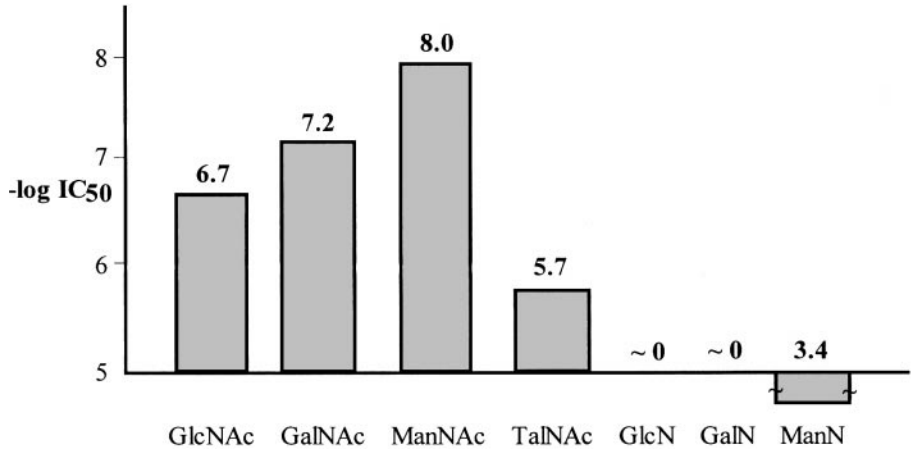

FIG. 1. Inhibition of binding of NKR-P1 to $GlcNAc_{23}BSA$ with simple aminosugars.

protective proteins such as BSA. Unlike NKR-341, NKR358 showed a minimal tendency to form disulfide-dependent aggregates.

Type of the sugar unit. From the previous experiments (4, 5) it has been known that monosaccharides bind to NKR-P1 with the following affinity: GalNAc > GlcNAc ⩾ Fuc > Man. We have, therefore, investigated basic structural preferences of the monosaccharidic ligands (Fig. 1). GalNAc proved to be about an order stronger ligand than GlcNAc. Surprisingly, ManNAc, which has never been tested before, was identified as a superior monosaccharide ligand for the receptor. We have been prompted to include this saccharide into the testing panel by our previous observation that disaccharide GlcNAcβ(1 → 4)ManNAc binds about 15× better than bare chitobiose (GlcNAcβ(1 → 4)GlcNAc) (18). Biochemical and structural aspects of these phenomena were studied in detail in another work (33).

The inhibition activity of *N*-acetyltalosamine is considerably weaker. This can be also caused by the fact that TalNAc in aqueous solutions mainly occurs (contrary to the other common aminosugars) in its furanose form (38). All good saccharide ligands identified up to now are pyranoses, and we assume that furanoses are not suitable for the binding.

Further, we have concentrated our attention to the basic D-glucose skeleton (Fig. 2). D-Glucose itself inhibits very weakly. It is obvious that substitution at the C-2 position is crucial for the binding. The removal of the OH group brings about no change of the inhibition effect as demonstrated with a 2-deoxy-D-glucose. However, the inversion of the inhibition effect as demonstrated with a 2-deoxy-D-glucose. However, the inversion of the OH group from its equatorial position (Glc) into the axial one as in D-mannose completely abolishes the binding. The replacement of the OH group with an NH_3^+ group (2-amino-2-deoxy-D-glucose is ionized in water below a neutral pH), also abolishes the binding possibly due to the positive charge, while the acetylation of this amino group in GlcNAc (eliminating the positive charge) results in a substantial increase of the binding (Fig. 1). On the other hand, 2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose were found to exert an inhibitory activity upon the human NK cells (39). They

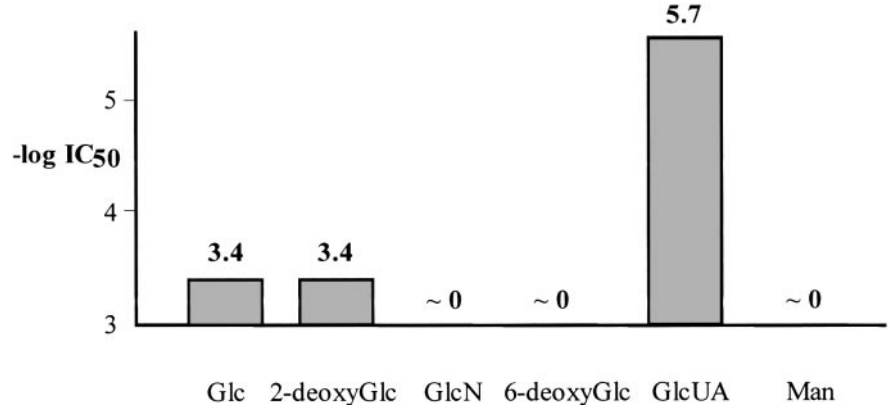

FIG. 2. Inhibition of binding of NKR-P1 to $GlcNAc_{23}BSA$ with derivatives of D-glucose.

inhibited to a similar extend the NK activity of both non-stimulated and interferon stimulated peripheral blood lymphocytes. The inhibitory effect of these two sugars was not reversed by a simultaneous exposure of the NK cells to these inhibitors and D-glucose. The authors (39) speculated that these two sugars either could block the receptors on the NK cells or impair the glycolytic pathway in the effector cells by the accumulation of phosphorylated derivatives of these sugars. Unfortunately, these authors did not test any acetamido sugar. It is necessary to add, however, that the receptors of the human NK cells differ from the rat cells (40).

Position C-6 is important as it is often sulfated in natural glycostructures or converted into carboxy group and thus negatively charged. Negatively charged carbohydrate structures (chondroitin sulfates, dermatan derivatives and heparans—all being sulfates and/or carrying carboxy groups) have been previously shown to strongly bind NKR-P1 (5) where they may interact with the Ca^{2+} ions (C-type lectin). Indeed, while 6-deoxy-D-glucose displayed no activity (elimination of H-bonding or ionic bonding) but D-glucuronic acid was a very good ligand with its IC_{50} being three orders of magnitude lower than that of D-glucose itself.

Subsequently, other monosaccharides having carboxy groups in the molecule, which are highly relevant for biological systems, e.g., 5-acetylneuraminic acid (Neu5Ac), 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and muramic acid were tested. Both Neu5Ac and KDO did not bind substantially with their respective $-\log IC_{50}$ values 4.3 and 4.2. A better affinity with $-\log IC_{50}$ 5.3 was observed for muramic acid. In addition to a carboxyl, its molecule contains also a free 2-amino group, which probably diminishes the binding in a similar way as in D-glucosamine.

The importance of the acetylation of the 2-amino group in the pyranose structure for the binding has evoked a question whether the acetylation is the most convenient acylation. Therefore, also the influence of the length of *N*-acyl was investigated. All the *N*-acyl derivatives examined, e.g., 2-deoxy-2-propionylamino-D-glucopyranose, 2-deoxy-2-butyrylamino-D-glucopyranose and 2-deoxy-2-isobutyrylamino-D-glucopyranose exhibited the $-\log IC_{50}$ value 6.4 which is a slightly worse value than that of acetyl derivative (GlcNAc $\sim$ 6.7); only 2-deoxy-2-palmitylamino-D-glucopyranose was slightly better with $-\log IC_{50} = 7.4$. Thus the choice of the acyl group has only a limited potential in the ligand optimization.

Other derivatizations done by us (41) were acylations at ligand C-6. Whereas 2-acetamido-6-*O*-acetyl-2-deoxy-D-glucopyranose ($-\log IC_{50} = 5.5$) was worse than GlcNAc, 2-acetamido-6-*O*-butyryl-2-deoxy-D-glucopyranose had exactly the same affinity as GlcNAc.

With regard to the optimal sugar unit of the NKR-P1 ligand, the subsequent general conclusions could be drawn:

1. 2-Acetamidosugars bind in the following order: ManNAc > GalNAc > GlcNAc $\gg$ TalNAc.

2. A 2-deoxy-2-acylamido group is crucial for the binding, but the length of the acyl group is not important.

3. In the C-6 position, the presence of a group with hydrogen bond-accepting properties is important (−OH, O-acyl, carboxyl). Its removal abolishes the binding.

4. While a negatively charged group in the molecule improves the binding, a positively chargable group (an unsubstituted amino group) has an opposite effect.

5. Hexopyranose structures seem to be optimal for the binding. The stereochemistry at the C-2 and C-4 positions is important, however, its changes influence the affinity within one order only. Furanose structures do not seem to be favorable for the binding.

Type of sugar linkage. In natural chitooligomers only $\beta(1-4)$ linkages occur. A comparison of chitobiose (GlcNAc$\beta(1 \rightarrow 4)$GlcNAc) and its regioisomer GlcNAc$\beta(1 \rightarrow 6)$GlcNAc revealed that natural $\beta(1 \rightarrow 4)$ compound is slightly better ($-\log IC_{50} = 7.0$) than the 1−6 isomer ($-\log IC_{50} = 6.7$).

Considerably more important is the type of the glycosidic bond (Fig. 3). In a series of *p*-nitrophenyl glycosides we can clearly see the basic structural preferences. Whereas both β-acetamidohexosaminides have about one order of magnitude better activities than the free sugar, the respective α-glycosides are considerably worse (by about three orders of magnitude). The increase of the affinity in the nitrophenyl β-glycosides can be ascribed to two possible effets. First, the free sugar exists in aqueous solution (H_2O or D_2O, 40°C), due to the mutarotation, as a mixtures of α- and β-anomers; the respective α:β values for GlcNAc, GalNAc and ManNAc are 0.68:0.32, 0.65:0.35 and 0.57:0.43 (38). When we consider that mainly β-anomer is responsible for the binding then we can assume that only less than a half of the compound participates in the process. When the anomeric position is fixed in the preferred β-position the specific binding should increase about two times. This hypothesis was checked with two simplest glycosides, e.g., methyl 2-acetamido-2-deoxy-α-D-glucopyranoside and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside. Indeed, the β-methyl glycoside was an approximately twice better inhibitor than GlcNAc itself, whereas the corresponding α-methyl glycoside was about five times worse than GlcNAc. Moreover, it seems that the neighbouring aromatic and electron rich (*p*-nitro group) moiety nonspecifically interacts with the receptor. Besides that, the *p*-nitrophenyl group is a very suitable linker for further clustering (after a reduction to the NH_2 group, the peptide or thioureido coupling via isothiocyanates can be easily performed). However, when the nitro group was in the *ortho* position the binding was strongly

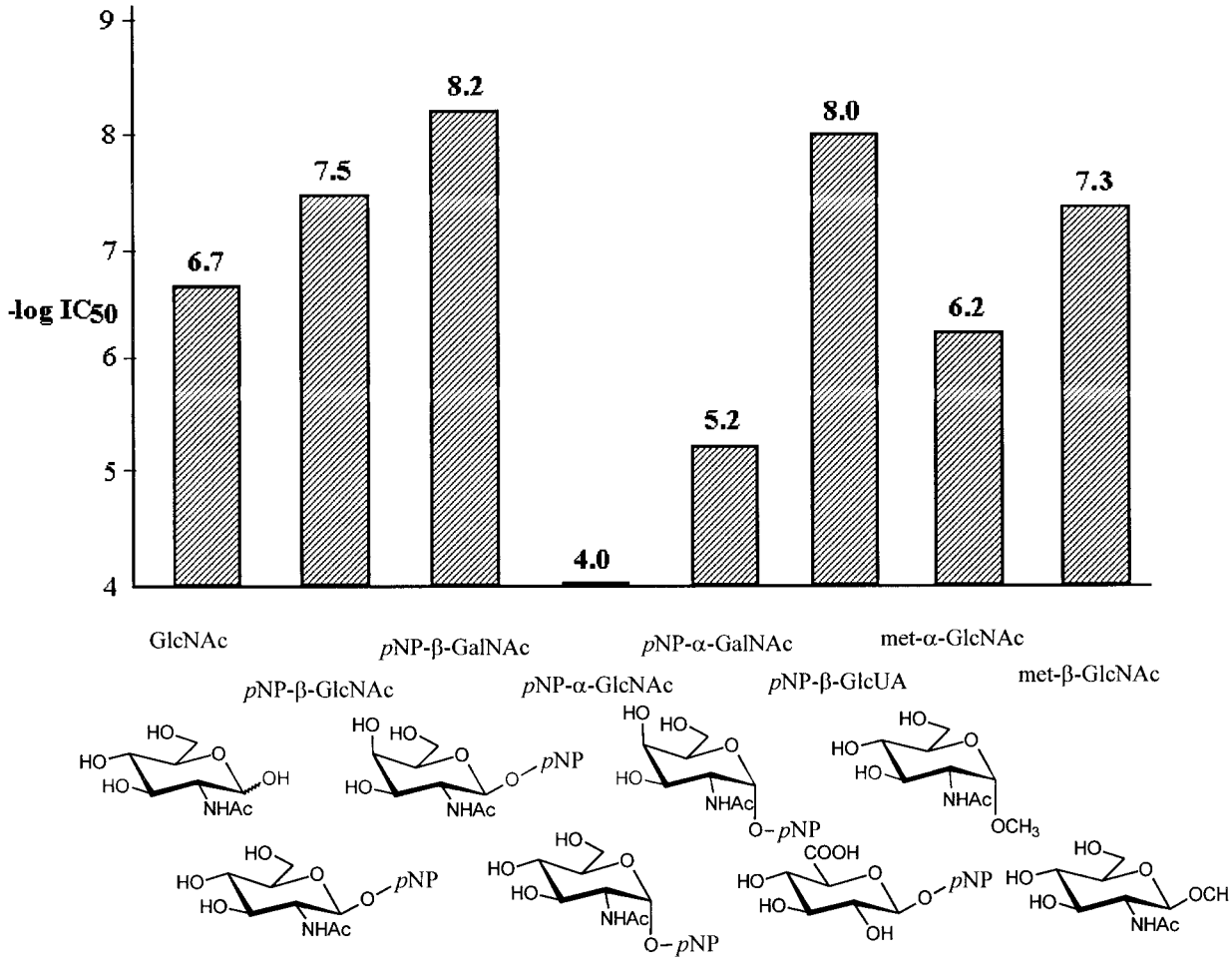

FIG. 3. Inhibition of binding of NKR-P1 to $GlcNAc_{23}BSA$ with different glycosidically linked carbohydrates. (*p*NP, *p*-nitrophenyl).

diminished; while *o*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucospyranoside exhibited the $-\log IC_{50}$ value of 5.7 (compared to 7.5 in respective *p*-nitro derivative), both *o*-nitrophenyl α-acetaminohexosaminides had no affinity at all. Apparently, the nitro group in the *ortho* position presumably has a negative sterical effect to the binding.

In this panel we can observe a high coherence with the preferences for the sugar type (GlcNAc vs GalNAc). We have also incorporated *p*-nitrophenyl β-D-glucuropyraoside uronic acid where the increase is the most notable—in more than 2 orders compared to the free sugar.

We have tested also both *p*-nitrophenyl α- and β-D-glucopyranosides and D-galactopyranosides and here virtually no affinity was observed. Interestingly, with *p*-nitrophenyl α- and β-D-mannopyranosides the $-\log IC_{50}$ values of 4.2 and 5.0, respectively, were obtained. A relatively high affinity of *p*-nitrophenyl α-L-fucopyranoside ($-\log IC_{50} = 6.0$) is also worth of mentioning.

For the preparation of glycodendrimers and neoglycoconjugates based on the aminosugars we were looking for suitable glycomimetics resistant to potential cleavage by the β-hexosaminidases when applied *in vivo*. Therefore, we have synthesized C-β-D-glycopyranosylnitromethanes ("nitromethyl β-C-glycosides") from GlcNAc (3-acetamido-2,6-anhydro-1,3-dideoxy-1-nitro-D-*glycero*-D-*gulo*-heptitol) and from GalNAc (3-acetamido-2,6-anhydro-1,3-dideoxy-1-nitro-D-*glycero*-D-*manno*-heptitol). Unfortunately, both derivatives had $-\log IC_{50}$ under 3 that render them not useful. We assume that this is an effect of the C-glycosidic linkage, despite it is in the preferred β-position.

Influence of the length of the glycosidic chain (chitooligomers) up to $n = 7$ was studied previously (15) and it was found that optimum number of glycosidic units is four; with a further extending of the chain the affinity drops (Table 1). We have extended our present study to chitooctamer and chitononamer not previously available that were prepared by a new enzymatic method (30) and the results obtained corroborated our previous results.

Then we have studied modification of optimal chitooligomers ($n = 3, 4$) at the nonreducing end. By the

TABLE 1

Inhibition of NKR-P1 Binding to $GlcNAc_{23}BSA$ with Linear Chitooligomers and $\beta(1 \rightarrow 6)$ Oligomers of GalNAc

Chitooligomer length (n)	$-\log IC_{50}$	$GalNAc\beta(1 \rightarrow 6)$ oligomer length (n)	$-\log IC_{50}$
1 (GlcNAc)	6.7	1 (GalNAc)	7.2
2	7.0	2	6.0
3	7.8	3	6.7
4	8.5	4	7.7
5	7.1	5	6.0
6	6.8	—	—
7	6.2	—	—
8	6.1	—	—
9	5.8	—	—

enzymatic reactions we have prepared chitooligomers substituted with $Glc\beta(1 \rightarrow 4)$, $Gal\beta(1 \rightarrow 4)$ (20) and $Glc\alpha(1 \rightarrow 4)$ (15). An oligosaccharide $Man\beta(1 \rightarrow 4)$-$GlcNAc\beta(1 \rightarrow 4)GlcNAc$ (42) was also obtained from the core structure of N-linked oligosaccharides. The results further support the conclusions drawn in the previous parts; α-glycosidic linkage is obviously detrimental to the activity as the α-D-glucosylated chitobiose has the worst activity from all trisaccharides tested, even worse than monosaccharide GlcNAc. On the other side β-Glc unit only slightly diminishes the activity of the trisaccharide, e.g., the change of the C-2‴ NHAc into OH has only small influence on the activity. Flipping of the C-2‴ OH from the equatorial (Glc) into the axial (Man) position has a bigger effect resulting in one order decrease of the binding. However, a much greater effect has the change of the C-4‴ OH from the equatorial (Glc) into the axial (Gal) position lowering the binding affinity in more than two orders. Analogous experiments were performed also in a panel longer in one GlcNAc unit (substituted chitotrioses) (16, 20) and the results are paralleling those shown in the Fig. 4.

Although not all the above modifications improved the binding affinity valuable structural data were obtained. Our main task has been, however, to design and to prepare ligands with the activity higher than the natural chitooligomers. Another aim was to have shorter oligosaccharides than, e.g., chitotriose or chitoteraose (up to now the best ones), with a sufficient or even better activity to simplify further clustering chemistry and to limit a potential decomposition *in vivo*. Also the price of the material is important in the light of potential applications (the prices of chitooligomers grow exponentially with the number of units).

GalNAc has a better affinity than GlcNAc. A natural oligomer of GalNAc analogous to chitooligosaccharides

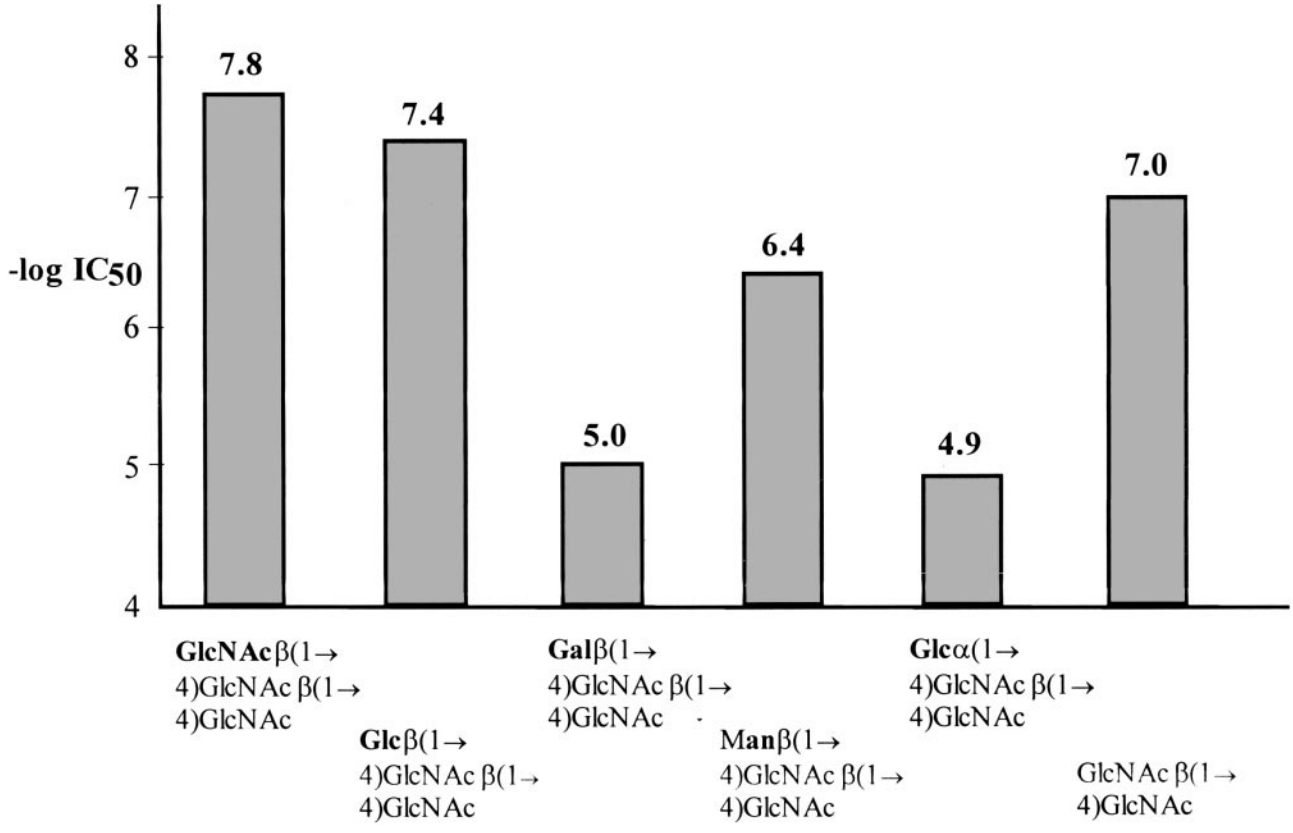

FIG. 4. Inhibition of binding of NKR-P1 to $GlcNAc_{23}BSA$ with chitobiose substituted at the nonreducing end.

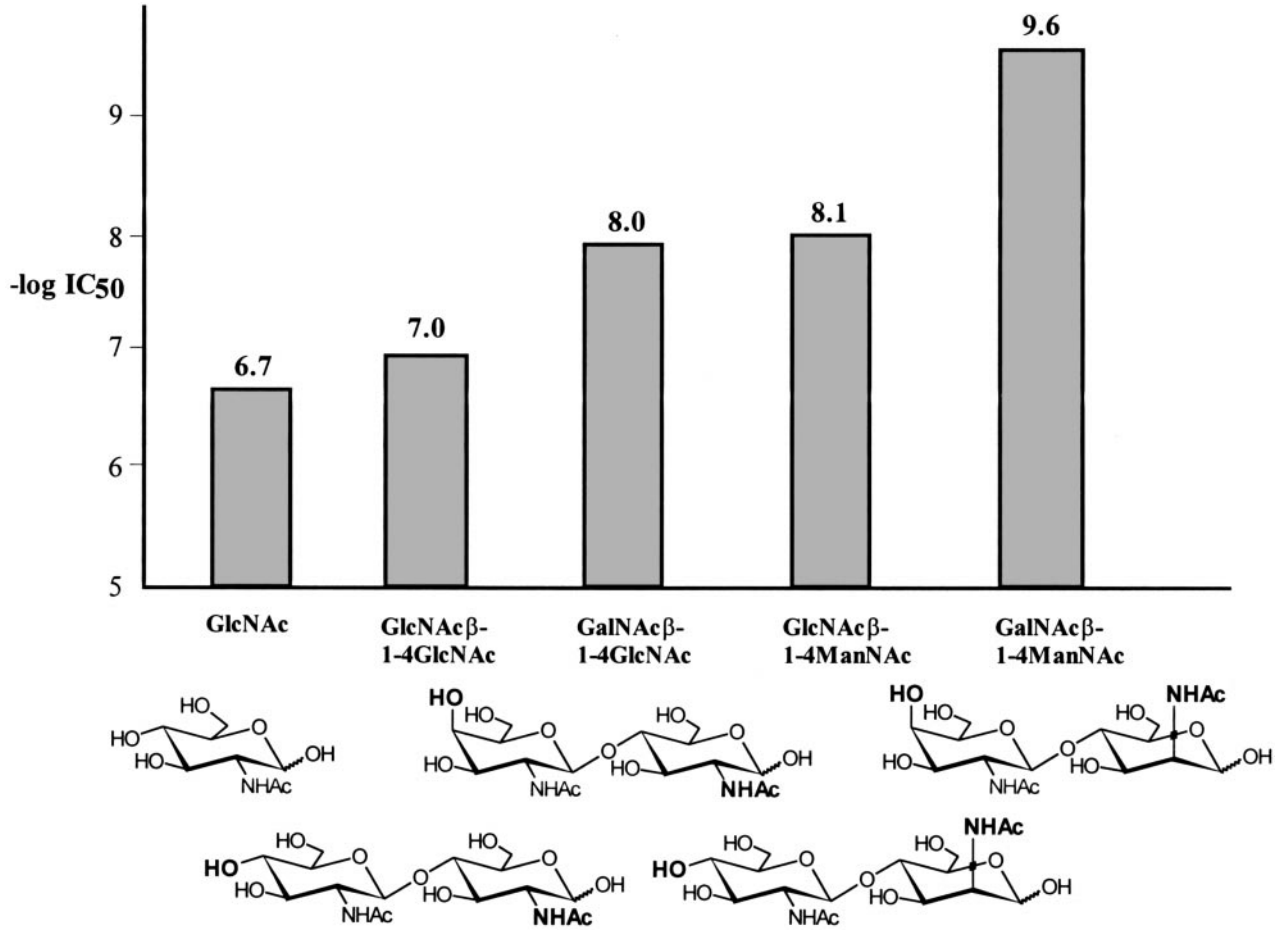

FIG. 5. Inhibition of binding of NKR-P1 to $GlcNAc_{23}BSA$ with $\beta(1 \rightarrow 4)$ diacetamidohexopyranobioses.

does not exist. Also its sterical form would be, due to axial position of the C-4 OH in GalNAc, much more different from those of chitooligomers (a linear $\beta(1 \rightarrow 4)$ equatorial-equatorial geometry due to the glycosidic linkages). Oligomers of GalNAc$\beta(1 \rightarrow 6)$ can be prepared quite easily by the condensation of GalNAc in liquid HF (34) which affords series of homooligosaccharides ($n = 2–5$) that can be separated by a gel filtration (BioGel P4). The results (Table 1) are in the accordance with the above conclusions: Extending the glycosidic chain increases the binding up to tetraose, pentaose has a lower affinity. Although GalNAc itself has a higher affinity than GlcNAc, the $\beta(1 \rightarrow 6)$ glycosidic linkage diminishes the activity, so that the respective oligomers are always worse ligands than analogous linear chitooligomers.

We have, therefore, concentrated to the disaccharides with the optimal $\beta(1 \rightarrow 4)$ linkage. Lead structure for the modifications was chitobiose composed of two GlcNAc units, which is, nevertheless, the acetamidohexopyranose with the lowest affinity to NKR-P1 (Fig. 1). When the equatorial C′-4 OH (GlcNAc) at the nonreducing end was flipped to the axial position (GalNAc) (35) an expected affinity increase by one order of magnitude as observed (Fig. 5). In the case of higher $\beta(1 \rightarrow 4)$ saccharides, this change without distortion of overall structure due to axial nature C-4 OH in GalNAc can be done only at their nonreducing end. On the other side, a flip of the C-2 NHAc from the equatorial (GlcNAc) into the axial position (ManNAc) at the reducing end of the disaccharide has been done by us previously (18). Here, we have expected a positive effect of the ManNAc that was up to now identified as the monosaccharide with the highest affinity to NKR-P1. Also this manipulation brought about an increase in more than one order. An rather logical step was then the design of the combination of these two changes—disaccharide GalNAc$\beta(1 \rightarrow 4)$ManNAc that was prepared by Lobry de Bruyn–Alberta van Ekenstein epimerization (36) of GalNAc$\beta(1 \rightarrow 4)$GlcNAc. This disaccharide, which had IC_{50} nearly 10^{-10} M, seems to represent an optimal compromise from the point of view of the availability, stability, and the affinity for the receptor. It is necessary to state that another potential modification, e.g., ManNAc$\beta(1 \rightarrow 4)$ at the nonreducing end could be a potentially very strong ligand,

however, a synthesis of respective disaccharide has not been accomplished yet due to technical problems. The synthesis of the β-ManNAc structures is one of the most challenging problem in carbohydrate chemistry (37) and, moreover, the C-4 position in GlcNAc is the least reactive group in this sugar.

For a potential *in vivo* application multivalent glycomimetics should be used. We already proved that glycoclusters based on single β-GlcNAc (clustered on PAMAM cores) (21) improve the binding to the NKR-P1 in many orders compared to oligosaccharides. Cluster with 3 carbohydrate units has $-\log IC_{50} = 9.0$, tetracluster 9.8, and octacluster 10.5. The binding of these simple polyvalent glycoclusters was so strong that complexes formed defined precipitates (21).

Such a dramatic binding affinity was, however, achieved only with a dendrimer based at a non-optimized molecule of GlcNAc. As a first step to a preparation of the dendrimers based on the optimized structures we have prepared a building block, which could be easily clustered either to the synthetic cores or to proteins. *p*-Nitrophenyl β-chitobioside, prepared enzymatically (25) has a suitable linker—*p*-nitrophenyl group—that can be easily transformed *via* reduction and thiophosgene reaction into a phenylisothiokyanato group, which could be coupled to primary NH_2 groups at the core structures. This molecule combines the effect of a longer chitooligomer chain with the β-linkage to the phenyl moiety which together causes an increase of $-\log IC_{50}$ to the value of 9.6. This is more than 3 orders higher than for GlcNAc so that we could expect from clustering of the corresponding nitrophenyl glycoside of such a disaccharide ligand, or even of the "optimal" disaccharide GalNAcβ(1 → 4)ManNAc, a substantial increase of the affinity.

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