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Charged Hexosaminides as New Substrates for β-*N*-Acetylhexosaminidase-Catalyzed Synthesis of Immunomodulatory Disaccharides

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Abstract: This work is a structure-activity relationship study that investigates the influence of the nature and amount of negative charge in carbohydrate substrates on the affinity of β-*N*-acetylhexosaminidases, and on the stimulation of natural killer cells. It describes synthetic procedures yielding novel glycosides that are useful in immunoactivation. Specifically, we present a thorough study on the ability of six C-6 modified β-*N*-acetylhexosaminides (aldehyde, uronate, 6-*O*-sulfate, 6-*O*-phosphate) to serve as substrates for cleavage and glycosylation by a library of β-*N*-acetylhexosaminidases from various sources. Four novel disaccharides with one or two (negatively) charged groups were prepared in synthetic reactions in good yields. Surprisingly, the 6-*O*-phosphorylated substrate, although cleaved by a number of enzymes from the series, worked neither as a donor nor as an acceptor in transglycosylation reactions. The results of wet experiments were supported by molecular modeling of substrates in the active site of two representative enzymes from the screening. All ten prepared compounds were examined in terms of their immunoactivity, namely as ligands of two activation receptors of natural killer (NK) cells, NKR-P1 and CD69, both with isolated proteins and whole cells. Sulfated disaccharides in particular acted as very efficient protectants of NK cells against activation-induced apoptosis, and as stimulants of the natural killing of resistant tumor cells, which makes them good candidates for potential clinical use in cancer treatment.

Keywords: β-*N*-acetylhexosaminidase; biotransformations; charged glycosides; glycosylation; molecular modeling; natural killer cells

Introduction

The employment of enzymes in carbohydrate synthesis has seen dynamic development in the past few decades. β-*N*-Acetylhexosaminidases[1] (EC 3.2.1.52, CAZy GH20; http://www.cazy.org) belong to the group of glycosidases with a well-documented broad synthetic potential;[2] at the same time, they are also extensively studied from the viewpoint of their structure and their involvement in human physiology and disease.[3] Previously, we have demonstrated that fungal β-*N*-acetylhexosaminidases possess a wide tolerance to substrates bearing various modifications, including *N*-acyls,[4] sulfates,[5] and an absent C-4 hy-

droxy.[6] Moreover, GlcNAc substrates with two unusual *C–N* linked aglycones, namely the azido group[7] and the aromatic triazole moiety,[8] were cleaved by some fungal β-*N*-acetylhexosaminidases; the azide even worked as an efficient glycosyl donor in transglycosylation reactions.[7]

As for the biological and especially the immunomodulatory activity of glycosides and glycoconjugates, our previous studies described the ability of *N*-acetyl-D-hexosamine structures to stimulate the immune response: they can activate natural killer (NK) cells by binding to their activation receptors, such as NKR-P1 and CD69 proteins.[9] A significantly enhanced binding affinity was observed in structures bearing negatively charged functional groups such as carboxyl or sulfate,[9a,10] but no systematic study on this phenomenon has been presented until now. The pilot study on C-6 oxidized *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-pyranosides[11,12] revealed the ability of β-*N*-acetylhexosaminidases, especially those of the *Penicillium* and *Talaromyces* genera, to cleave and transfer the glycosyls of sugar aldehydes. After *in situ* oxidation to a carboxyl group, the uronate-containing oligosaccharides β-D-Gal*p*NAcA-(1→4)-D-Glc*p*NAc and β-D-Gal*p*NAcA-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Man*p*NAc were prepared. These compounds are among the best known oligosaccharidic ligands of the CD69 receptor described so far. Another biological property studied in this type of compounds is their ability to protect NK cells against activation-induced apoptosis.[13]

In this work we report on the substrate specificity and synthetic potential of (mainly fungal) β-*N*-acetylhexosaminidases with C-6 modified *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-hexopyranosides (aldehyde **2**, uronate **3**, sulfates **4** and **6**, phosphates **5** and **8**; Figure 1). The results are supported by molecular modeling using two enzymes from the set. Four novel, negatively charged disaccharides were prepared in high yields. The immunomodulatory properties of all ten C-6-modified hexosaminides were tested using NK cell activation receptors NKR-P1A and CD69 and CD69^{high} lymphocytes. As a result, we could compare the influence of the number and character of the functionalities in the hexosaminides on the synthetic performance of β-*N*-acetylhexosaminidase and on the response in immunological tests.

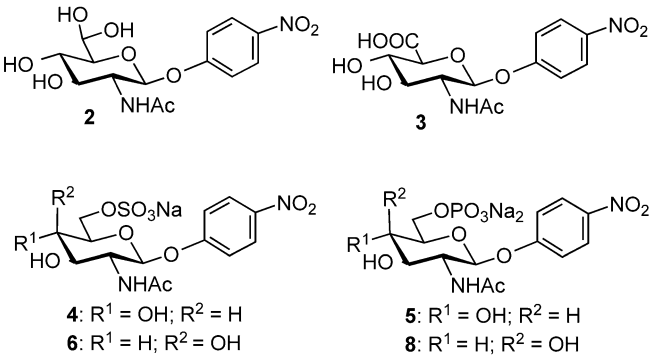
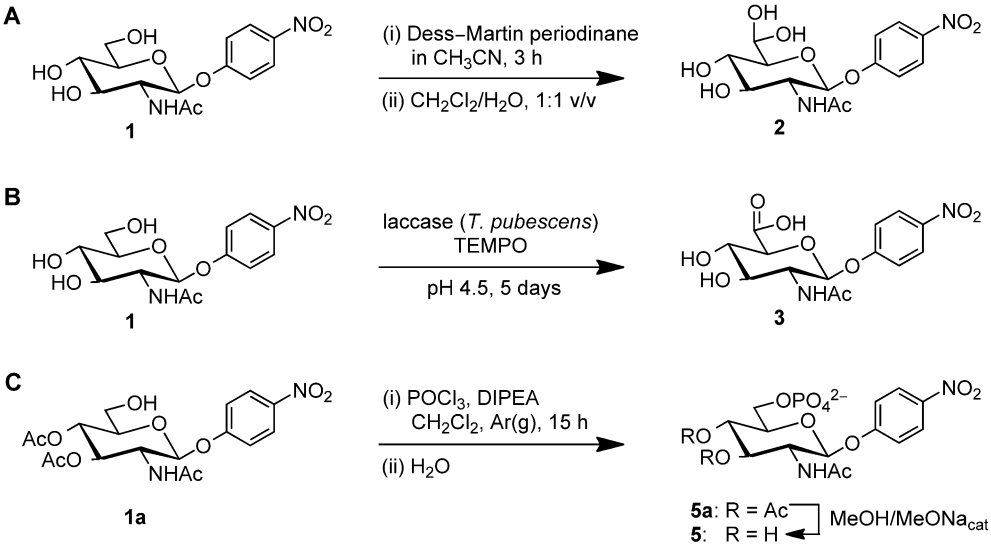


Figure 1. Substrates **2–6** and **8**.

Results and Discussion

Preparation of Substrates 2–6 and 8

The synthesis of aldehyde **2** (Scheme 1) was adapted from an elegant published procedure based on Dess–Martin oxidation.[14] NMR spectroscopy confirmed the formation of the required aldehyde together with



Scheme 1. Synthesis of β-*N*-acetylhexosaminidase substrates **2** (**A**), **3** (**B**) and **5** (**C**).

its hydrated form (geminal diol). Upon standing in anhydrous solution (DMSO), a gradual dehydration of the diol was observed, which is in agreement with the literature.[15,16] Uronate **3** was prepared in a high-yield, one-step reaction using the laccase from *Trametes pubescens* (EC 1.10.3.2) in tandem with TEMPO. The enzymatic oxidation proceeded *via* aldehyde **2**; however, it could not be efficiently stopped at that stage. The aldehyde traces (<5%) could be isolated by silica gel chromatography (EtOAc: MeOH:H_2O 6:8:0.25→6:8:1) but since the aldehyde yield was negligible anyway, a more convenient separation by gel permeation chromatography in water was opted for, which gave pure uronate **3**. Sulfates **4** (*gluco*-) and **6** (*galacto*-) were prepared in a 5-step synthesis starting from the respective unprotected *p*-nitrophenyl hexosaminide as already described in our previous work.[6] The preparation of phosphates **5** (*gluco*-) and **8** (*galacto*-) was the most challenging task and was complicated by the presence of the nitro moiety on the aromatic ring. Here, the optimal procedure was the phosphorylation of the respective partially protected *p*-nitrophenyl hexosaminide with $POCl_3$. Product purification was particularly challenging as this compound tended not to separate from the phosphate salts that originated in the reaction mixture due to its polarity.

β-*N*-Acetylhexosaminidase-Catalyzed Hydrolysis of Compounds 2–6, 8 and Molecular Modeling

All the prepared substrates, i.e., aldehyde **2**, uronate **3**, sulfates **4** and **6** and phosphates **5** and **8**, were tested with a series of 24 fungal β-*N*-acetylhexosaminidases produced by mycelia stemming from the Culture Collection of Fungi (CCF), Charles University in Prague, CZ. In addition, four representatives from other sources were tested. As shown in Table 1, the best results were obtained with the fungal enzymes. Aldehyde **2** proved to be the best substrate, its hydrolysis rates being 10–30% of those measured with the standard substrate **1**. In the *gluco*-series, the ability of enzymes to cleave the substrates declined in the order aldehyde→sulfate→uronate→phosphate.

In contrast, the *galacto*-derivatives **6** (sulfate) and **8** (phosphate) were not cleaved by any enzymes from the set. This result corresponds to the previous conclusions observed with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-pyranosiduronic acid (not cleaved).[11] Apparently, the introduction of a charged group at the C-6 position of *p*-nitrophenyl hexosaminides is only tolerated by β-*N*-acetylhexosaminidases if the substrate is in the *gluco*-configuration (equatorial C-4 hydroxy group). This behavior is consistent irrespective of the nature of the charged group in the C-6 position (uronate, sulfate or phosphate).

Table 1. Hydrolysis of substrates **2–5** by β-*N*-acetylhexosaminidases.[a,b]

Enzyme source	**2**	**3**	**4**	**5**
Acremonium persicinum CCF 1850	–	–	–	–
A. awamori CCF 763[c]	++	–	–	–
A. flavofurcatis CCF 3061	+++	+	-	-
A. flavus CCF 642	+	–	–	–
A. niger CCIM K2	++	–	–	–
A. niveus CCF 3057	+	–	–	–
A. nomius CCF 3086	+++	+	–	–
A. oryzae CCF 147	++	+	–	–
A. oryzae CCF 1066	++	–	–	–
A. parasiticus CCF 1298	++	–	–	–
A. tamarii CCF 3085	++	–	–	–
A. versicolor CCF 2491	++	–	–	–
Fusarium oxysporum CCF 377	++	+	++	+
P. brasilianum CCF 2171[c]	++	+	+	+
P. chrysogenum CCF 1269	++	+	+	++
P. oxalicum CCF 1959	++	+	+	+
P. oxalicum CCF 2315	++	+	+	++
P. oxalicum CCF 2430	+	+	+	+
P. pittii CCF 2277	+++	++	+	–
T. flavus CCF 2573[c]	++	++	+++	++
T. flavus CCF 2686	+++	++	++	++
T. ohiensis CCF 2229	++	+	+	–
T. striatus CCF 2232	++	+	+++	–
Trichoderma harzianum CCF 2687	++	+	+	+
Canavalia ensiformis[d]	+++	–	–	–
Streptococcus pneumoniae	+	–	+	–
Bovine kidney	++	–	++	+
Human placenta (HEX A)	++	–	+	–

[a] Substrates **6** and **8** were not cleaved by any of the enzymes (hydrolysis rate <1%).

[b] (–) <2%, (+) 3–10%, (++) 11–20%, (+++) 21–30% hydrolysis rate relative to the hydrolysis of standard substrate *p*NP-GlcNAc (**1**).

[c] *A.* = *Aspergillus*, *P.* = *Penicillium*, *T.* = *Talaromyces*.

[d] *Canavalia ensiformis* = jack bean.

The screening results were compared to the conclusions of computational docking[17] and molecular dynamics simulations (MD) using two enzymes from the series: the β-*N*-acetylhexosaminidase from *Penicillium oxalicum*,[18] which has a broad substrate specificity[4] and cleaved all tested *gluco*-substrates well, and the enzyme from *Aspergillus oryzae*,[19] a more conservative hexosaminidase with a stricter substrate specificity.

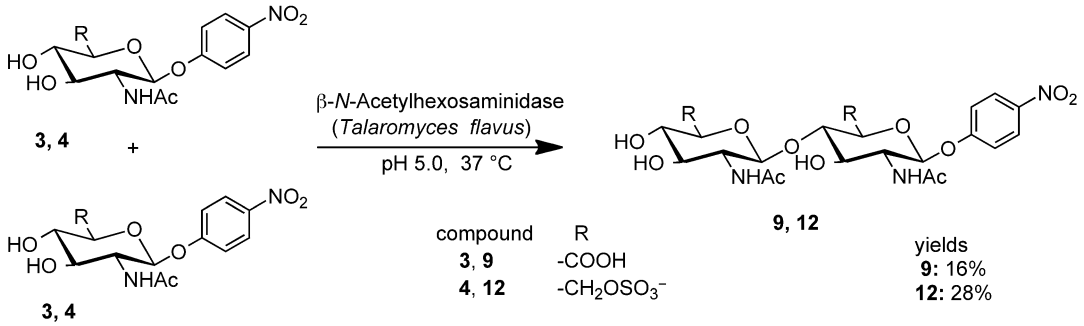
In both enzymes, ΔGs of the equilibrated enzyme-substrate complex with aldehyde **2** were comparable to the standard substrate **1** (for details see Table S1 in the Supporting Information). Uronate **3** provided weaker interaction with both enzymes due to the loss of hydrogen bonds fixing C-5 and C-6 carbons in the substrate pyranosyl ring. The strongly charged sulfate **4** and phosphate **5** in the *P. oxalicum* enzyme showed

just slightly lower ΔG values than the standard substrate **1** and they could well accommodate in the active site. Therefore, it could be expected that these substrates were cleaved by the enzyme, though possibly with lower affinity. In contrast, phosphate **5** in the *A. oryzae* enzyme initiated strong conformational changes within the active site. During MD the substrate C-4 hydroxy caused a strong shift and deformation of the loop Pro301–Gln312 above the active site (for reasoning and additional illustrating material see the Supporting Information), leading unambigously to the conclusion that the *A. oryzae* enzyme could not accommodate phosphate **5** in its native active site.

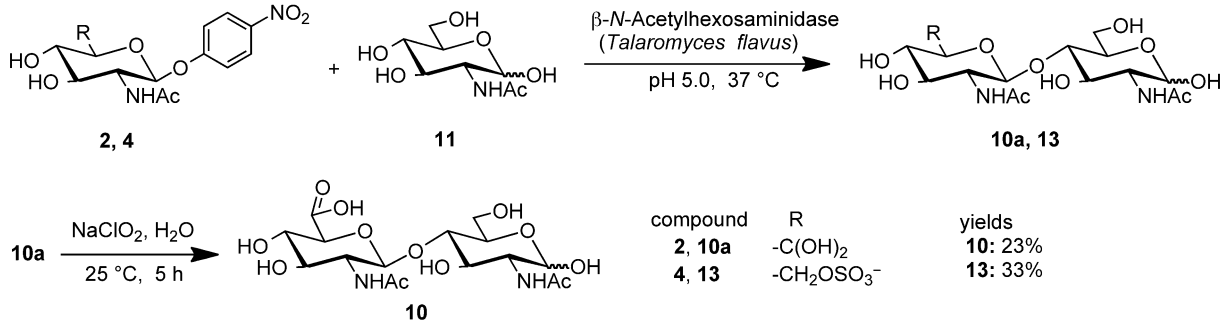
Syntheses Catalyzed by β-*N*-Acetylhexosaminidases

Substrates **2**–**5**, exhibiting good to excellent results in the screening for cleavage, were used in two types of synthetic transglycosylation reactions. In the first design, they served both as glycosyl donor and acceptor, resulting in a *p*NP-chitobiose type β(1→4)-bound disaccharide [β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→*O*)-*p*NP] carrying two charged groups (Scheme 2). In the other design, GlcNAc (**11**) as acceptor was glycosylated with a modified glycosyl, leading to a chitobiose-related disaccharide carrying one charged group at the non-reducing end (Scheme 3). All the reactions were first tested on an analytical scale in miligram amounts to optimize the amount of enzyme, reactant concentrations, reaction time, etc., and then repeated at a hundred mg scale to gain the disaccharidic products in a sufficient amount for biological studies. The catalyst used in all the preparative reactions presented was the β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686, which proved to be among the most efficient enzymes in the screening and, additionally, was known for its good synthetic capacity and tolerance to substrate modifications, as shown previously.[6,7,11]

The carboxyl group was introduced into the disaccharidic compounds in two ways – *via* aldehyde **2** in combination with *in situ* quantitative oxidation of the product by $NaClO_2$ or directly *via* uronate **3**. This was because, from our previous experience with *galacto*-derivatives,[11] we (correctly) supposed uronate **3** to be a much poorer substrate than aldehyde **2**, both in terms of the cleavage and transglycosylation. In contrast to the *galacto*-series where the uronate was not cleaved at all and the only way to the carboxy disaccharides was through the aldehyde, uronate **3** was a relatively good substrate for cleavage. However, the



Scheme 2. Autocondensation reactions with substrates **3** and **4** catalyzed by the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686.



Scheme 3. Glycosylation of GlcNAc (**11**) using substrates **2** and **4** catalyzed by the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686.

synthesis and subsequent complicated purification (preparative HPLC) resulted in quite a poor synthetic yield (16%). In contrast, aldehyde **2** gave *ca.* double the yield in the synthetic reaction. The *in situ* oxidation step of the product to a uronic acid with $NaClO_2$ was very straightforward, selective and efficient. Thus, we could conclude that it is easier to produce carboxy disaccharides using an aldehyde donor.

Sulfate **4** was the best glycosyl donor in the series, affording products **12** and **13** in very good yields by a simple purification with gel permeation chromatography (28 and 33%, respectively, after two hours of reaction time). The procedure described here gave substantially better results than those by Uzawa and coworkers,[20] especially as far as reaction time and amount of enzyme are concerned. Unfortunately, we were unable to prepare any transglycosylation products from phosphate **5**. Neither of the reaction designs described above gave any notable amounts of phosphorylated disaccharide products, although the catalytic function of the enzyme was apparently unchanged since β-D-Glc*p*NAc-(1→6)-β-D-Glc*p*NAc could be isolated from the reaction with acceptor **11**. No result was obtained, even when phosphate **5** was used as reaction acceptor with *p*NP-GlcNAc as donor. Instead of the expected phosphorylated product, we only isolated *p*NP-chitobiose. These results clearly show that phosphate **5**, although cleaved, does not work as a substrate in transglycosylation reactions.

Table 2. Affinity of carbohydrate ligands to NK cell activation receptors NKR-P1A and CD69, expressed on a logarithmic scale ($-\log IC_{50} \pm SD$). The values reflect the compound ability to inhibit binding between the respective protein and a standard high affinity ligand $GlcNAc_{23}BSA$.

Compound	NKR-P1A	CD69
GlcNAc (**11**)[a]	5.2±0.2	3.4±0.5
*p*NP-GlcNAc (**1**)	6.0±0.1	4.1±0.1
Aldehyde **2**	8.7±0.1	6.6±0.0
Uronate **3**	7.7±0.1	6.7±0.1
Sulfate **4**	9.8±0.1	8.6±0.2
Phosphate **5**	8.5±0.1	6.6±0.1
*p*NP-GalNAc	6.4±0.1	4.3±0.1
*p*NP-GalNAc aldehyde[b]	6.1	5.0
*p*NP-GalNAcA[b]	6.8	6.9
Sulfate **6**	9.7±0.1	8.6±0.0
Phosphate **8**	8.7±0.1	6.8±0.1
*p*NP-chitobiose	9.7±0.1	7.5±0.1
Disaccharide **9** (diuronate)	9.6±0.4	9.6±0.4
Disaccharide **12** (disulfate)	9.2±0.4	9.3±0.3
Chitobiose	8.5±0.1	6.3±0.2
Disaccharide **10** (uronate)	8.1±0.1	6.3±0.1
Disaccharide **13** (sulfate)	8.8±0.1	7.8±0.1

[a] GlcNAc (positive control) had IC_{50} values of 6.3×10^{-6} M (NKR-P1A) and 4.0×10^{-4} M (CD69).
[b] Data taken from ref.[11]

Prepared Compounds as Ligands of NK Cell Activation Receptors

All prepared compounds were tested in a competitive binding assay with two activation receptors of NK cells, rat NKR-P1 and human CD69 (see Experimental Section for details).[21] D-Mannose was used as the negative control, no inhibitory effects could be observed up to 10 mM concentrations (not shown). The results are summarized in Table 2 as $-\log IC_{50}$. In the monosaccharidic series, the introduction of an aldehyde or a charged group into the molecule produced a notable increase in binding, more pronounced with the NKR-P1 receptor. Sulfates **4** and **6** were the best ligands in the series, followed by phosphates, uronates and aldehydes. Generally, disaccharides carrying a *p*NP moiety were better ligands than the reducing disaccharides. In the disaccharidic series, outstanding results were observed with the CD69 receptor. Two charged groups in compounds **9** and **12** (carboxy and sulfate, respectively) produced an increase of two orders of magnitude compared to unmodified *p*NP-chitobiose. With $-\log IC_{50}$ values approaching 10 they ranked among the best ligands ever studied, comparable only to multivalent structures.[22] Apparently, the sulfate is the most efficient moiety for increasing ligand strength (although any charged group causes an increase in binding).

Immunoactivity of Prepared Compounds

In order to investigate the immunoactivity of the prepared compounds **2**–**6**, **8**, **9**, **10**, **12** and **13**, we first tested their effect on the activation of immune cells carrying cellular forms of the NKR-P1 and CD69 receptors, namely with highly NKR-P1 positive rat NK cells and $CD69^{high}$ human peripheral blood mononuclear cells (PBMC).[21] In both cases, the dicarboxylated disaccharide **9** and both sulfated disaccharides **12** and **13** displayed the highest effect. This correlated well with the competitive studies shown in Table 2. We also tested the ability of individual compounds to protect $CD69^{high}$ cells from apoptosis.[13] Again, compounds **9**, **12** and **13** were the best in this assay, followed by monosulfated *p*NP-GlcNAc and *p*NP-GalNAc **4** and **6**. Finally, the immunomodulatory properties of the compounds were tested using human PBMC and human tumor cells, both NK-sensitive (K562), and NK-resistant (RAJI). Here, compounds **9**, **12** and **13** strongly stimulated the natural killing of RAJI tumor cells. Such an outstanding ability to enhance natural killing of normally resistant tumor cells may result from a synergic activation of NK cells through NKR-P1 and their protection against apoptosis, as shown in separate experiments above.

Conclusions

This study presents a series of C-6-modified substrates derived from *p*NP-GlcNAc and *p*NP-GalNAc and studies their ability to act as substrates for a range of β-*N*-acetylhexosaminidases in cleavage and novel synthetic reactions. The compounds from the *gluco*-series were much better cleaved than those from the *galacto*-series; aldehyde and sulfate proved to be the best substrates. Interestingly, phosphate **5**, although cleaved by β-*N*-acetylhexosaminidases, did not work as a glycosyl donor or acceptor in transglycosylation reactions. Using the other substrates, four disaccharides in total were enzymatically synthesized. Furthermore, we compared the prepared compounds in terms of their immunomodulatory potential, namely their ability to serve as ligands of NKR-P1A and CD69 activation receptors and as protectants of $CD69^{high}$ lymphocytes against activation-induced apoptosis. Sulfates **4** and **6** were the best activating monosaccharides, especially for the NKR-P1 receptor. The positive effect of two charged groups in the (disaccharidic) molecule was clearly visible in their reaction with the CD69 receptor (compounds **9**, **12**). In the immunostudies with whole cells, compounds **9**, **12** and **13** strongly stimulated the natural killing of NK-resistant RAJI tumor cells.

In conclusion, sulfates were the most efficient compounds in the series, both as substrates (in terms of enzymatic cleavage and synthesis) and as the most active immunostimulants of natural killer cells. These compounds are especially valuable due to the favorable combination of high affinity and good stability, which distinguishes them from the previously tested natural sulfated oligosaccharides.[10,23] These features make these compounds promising lead structures for the further development of carbohydrate mimetics with potent immunomodulatory activities and the enzymatic synthetic methodology makes them available for further studies.

Experimental Section

General Materials and Methods

All reagents unless stated otherwise were used as purchased without further purification. TLC was performed on aluminum sheets precoated with Silica Gel 60 (F_{254} Merck, D); the spots were visualized by UV light (254 nm) and/or by spraying with 5% H_2SO_4 in ethanol and charred with a heat-gun. Flash chromatography was performed with Merck silica 60 (230–400 mesh) as the stationary phase.

NMR spectra of compounds **2** and **9** were measured in a Varian Mercury 300 MHz spectrometer (299.98 MHz for ^{1}H, and 75.44 MHz for ^{13}C), compound **4** in a Varian Unity Inova 400 MHz spectrometer (399.87 MHz for ^{1}H, and 100.55 MHz for ^{13}C), compounds **3**, **5**, **5a**, **6**, **8**, **12**, and **13** in a Bruker AVANCE III 400 MHz spectrometer (400.13 MHz for ^{1}H, and 100.61 MHz for ^{13}C), and compound **10** in a Bruker AVANCE III 600 MHz spectrometer (600.23 MHz for ^{1}H, and 150.93 MHz for ^{13}C) in DMSO-d_6 (99.9 atom% D, Sigma–Aldrich, Steinheim, D) or D_2O (100 atom% D, Sigma–Aldrich, Steinheim, D) at 303 K. The residual signal of the solvents was used as an internal standard (DMSO-d_6: $\delta_H = 2.500$, $\delta_C = 39.60$, D_2O $\delta_H = 4.508$). The carbon spectra in D_2O were referenced to the signal of acetone ($\delta_c = 30.50$). ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMQC, HMBC, and 1D-TOCSY spectra were measured using standard manufacturers' software. The proton spin systems of each sugar unit were assigned by COSY and 1D-TOCSY. The assignment was transferred to carbons by HSQC. The position of sugar moiety substitution was confirmed by long-range heteronuclear correlations in the HMBC spectrum and the configuration at C-1 was deduced from coupling constants $J_{H1,H2}$ observed in the ^{1}H NMR spectra. The position of the sulfate was indicated indirectly by the downfield shifts of the involved carbons (C-6). Chemical shifts are given on a δ scale [ppm], and coupling constants in Hz. Digital resolution enabled us to report the chemical shifts of protons to three, coupling constants to one and carbon chemical shifts to two decimal places. Some hydrogen or chemical shifts were read from HSQC or HMQC and are reported to two decimal places.

Mass spectrometry analysis and MS/MS experiments were performed using a LC^Q*DECA* ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with a static nanoelectrospray ion source. The spray voltage was maintained at 1.42 kV, the tube lens voltage was −10 V. The heated capillary was kept at 180 °C with a voltage of −37 V. Positive-ion full scans were acquired over the *m/z* range 150–2000. To confirm the structure of analyzed compound the MS/MS experiments were performed with normalized collision energy in range 20–35%, activation Q 0.25, activation time 30 ms and with isolation width *m/z* 3.0. MALDI-TOF mass spectra were measured on an ultraFLEX matrix-assisted laser desorption/ionization reflectron time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, D). The ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Positive spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of human angiotensin I *m/z* = 1296.69, MRFA peptide *m/z* = 524.26 and CCA matrix peak *m/z* = 379.09. A saturated solution of α-cyano-4-hydroxycinnamic acid or 2,5-dihydrobenzoic acid in 50% MeCN/0.3% acetic acid was used as the MALDI matrix. 1 μL of matrix solution was mixed with 1 μL of methanolic solution of the sample and 1 μL of the premix was loaded on the target, the droplet was allowed to dry at ambient temperature. The MALDI-TOF positive or negative spectra were collected in reflectron mode.

Enzymes

The laccase from *Trametes pubescens* (EC 1.10.3.2; 51 U/mg) was kindly donated by Prof. Dietmar Haltrich (BOKU University, Wien, Austria). The greenish lyophilisate had a laccase activity of 51 U/mg according to the standard activity determination, by monitoring the oxidation of ABTS [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] at 436 nm. The enzyme solution (10 μL) was added to a 1-mL

cuvette containing 20 mM acetate buffer pH 3.5 (890 μL) and ABTS (100 μL of a 10 mM solution of ABTS in H_2O). One enzyme unit is defined as the amount of laccase that oxidizes 1 μmol of ABTS under these conditions (ε_{ABTS} = 29.3 $mM^{-1} cm^{-1}$).

Commercial plant, animal and bacterial β-*N*-acetylhexosaminidases (EC 3.2.1.52) were purchased from Sigma. The fungal strains producing β-*N*-acetylhexosaminidases originated from the Culture Collection of Fungi (CCF), Department of Botany, Charles University in Prague (CZ). The strains were cultivated in submersed media as described previously.[4] Enzymes were obtained by $(NH_4)_2SO_4$ precipitation (80% saturation) of the cultivation media, and the precipitates were directly used in the enzymatic screening. The crude enzyme preparations were tested for contaminating sulfatase and phosphatase activities using the respective *p*-nitrophenyl substrate (*p*-nitrophenyl sulfate, *p*-nitrophenyl phosphate, Sigma) under the same conditions as the β-*N*-acetylhexosaminidase assay. For the synthesis of compounds **9**, **12** and **13**, a substantially purified β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686 was used. After 11 days of cultivation, the mycelium was filtered off and the enzyme was purified from the culture medium by cation exchange chromatography [Fractogel EMD SO_3^- (Merck, Germany), 10 mM sodium citrate/phosphate buffer pH 3.5, linear gradient 0–1 M NaCl] and gel permeation chromatography [Superdex 200 (Amersham Biosciences, SW), 50 mM sodium citrate/phosphate buffer pH 5 and 150 mM NaCl] using an Äkta Purifier protein chromatography system (Amersham Biosciences, SW). The β-*N*-acetylhexosaminidase activity was determined in an end-point assay as described previously,[5] with a 2 mM starting concentration of *p*NP-GlcNAc standard substrate **1** or substrates **2**–**6** and **8** and with 11–15 $mU mL^{-1}$ or 220–300 $mU mL^{-1}$ of β-*N*-acetylhexosaminidase, respectively.

Synthesis of Substrates for β-*N*-Acetylhexosaminidases

p-*Nitrophenyl 2-acetamido-2-deoxy*-β-D-gluco-*hexodialdo-1,5-pyranoside* (2): Dess–Martin periodinane (3.17 mL, 0.950 mmol, 0.3 M solution in CH_2Cl_2) was added to a suspension of *p*NP-GlcNAc (**1**; 300 mg, 0.876 mmol) in acetonitrile (3.7 mL). The mixture was stirred at room temperature for 3 h. The reaction was stopped by adding H_2O/CH_2Cl_2 (40 mL, 1:1 v/v) and the phases were separated. The aqueous phase was washed with CH_2Cl_2 several times, filtered, neutralized with aqueous 1 M NaOH and concentrated. The remaining water was removed by lyophilization and the residue was purified by silica gel chromatography (CH_2Cl_2:MeOH, 12:1) yielding the title compound **2** as a white amorphous solid; yield: 90 mg (0.264 mmol, 30%). According to NMR, the sample contains two components, the aldehyde and the geminal diol form of **2**, in a varying ratio of 34.5/65.5 (fresh solution) to 69/31 (1-day-old solution), respectively. ^{1}H NMR (DMSO; aldehyde): δ = 1.812 (s, 3H, Ac), 3.460 (dd, 1H, J = 10.0, 8.5 Hz, H-4), 3.577 (dd, 1, J = 10.0, 8.5 Hz, H-3), 2.738 (ddd, 1H, J = 10.0, 8.8, 8.2 Hz, H-2), 4.182 (dd, 1H, J = 10.0, 1.2 Hz, H-5), 5.368 (d, 1H, J = 8.2 Hz, H-1), 7.204 and 8.195 (AA′BB′, 4H, ΣJ = 9.3 Hz, *p*NP), 7.895 (d, 1H, J = 8.8, 2-NH), 9.633 (d, 1H, J = 1.2, H-6); ^{1}H NMR (DMSO; geminal diol): δ = 1.804 (s, 3H, Ac), 3.325 (m, 1H, H-5), 3.437 (m, 1H, H-4), 3.459 (m, 1H, H-5), 3.720 (ddd, 1H, J = 9.9, 9.0, 8.5 Hz, H-2), 5.002 (d, 1H, J = 2.5 Hz, H-5), 5.163 (d, 1H, J = 8.5 Hz, H-1), 7.171 and 8.195 (AA′BB′, 4H, ΣJ = 9.3 Hz, *p*NP), 7.803 (d, 1H, J = 9.0 Hz, NH). ^{13}C NMR (DMSO; aldehyde): δ = 23.05 (Ac), 54.92 (C-2), 70.41 (C-4), 73.38 (C-3), 78.94 (C-5), 98.01 (C-1), 116.69 (2×C-*ortho*), 125.85 (2×C-*meta*), 142.14 (C-*para*), 161.93 (C-*ipso*), 169.57 (C=O), 198.19 (C-6); ^{13}C NMR (DMSO; geminal diol): δ = 23.10 (Ac), 55.14 (C-2), 70.63 (C-4), 73.78 (C-3), 77.37 (C-5), 88.74 (C-6), 98.77 (C-1), 116.76 (2×C-*ortho*), 125.80 (2×C-*meta*), 141.94 (C-*para*), 162.35 (C-*ipso*), 169.49 (C=O); MS (ESI): m/z = 359.0 $[M+H]^+$, 381.1 $[M+Na]^+$, calcd. for $C_{14}H_{18}N_2O_9$: 358.1. MS confirmed that the aldehyde was present in the hydrated form.

p-*Nitrophenyl 2-acetamido-2-deoxy*-β-D-*glucopyranosiduronic acid* (3): *p*NP-GlcNAc (**1**; 300 mg, 0.876 mmol) and (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO; 37 mg, 0.237 mmol) were dissolved in 20 mM AcONa/AcOH buffer pH 4.5 (25 mL). Then, a solution of the laccase from *Trametes pubescens* (765 U) in the same buffer (1 mL) was carefully added to the suspension and the reaction was left under gentle shaking at ambient temperature. After several hours, the solution became clear. The reaction was monitored by TLC ($CHCl_3$: MeOH:EtOH:H_2O, 6:3.5:1:0.8). After 3.5 days, another portion of TEMPO (20 mg, 0.128 mmol) and enzyme (765 U) was added. After 5 days, the reaction was stopped by freezing and lyophilization. Purification by gel chromatography (Biogel P2, BioRad, USA, mobile phase water, flow rate 12.5 $mL h^{-1}$) afforded product **3** as a white solid; yield: 222 mg (0.623 mmol; 71%). Besides the major product **3** (R_f 0.3), traces of **2** (R_f 0.8; <5%) were observed in the reaction mixture; however, **2** could not be isolated using this purification method. ^{1}H NMR (D_2O): δ = 1.806 (3H, s, 2-Ac), 3.462 (1H, dd, J = 9.0, 9.6 Hz, H-4), 3.506 (1H, dd, J = 9.0, 10.1 Hz, H-3), 3.752 (1H, d, J = 9.6, H-5), 3.854 (1H, dd, J = 8.4, 10.1 Hz, H-2), 5.099 (1H, d, J = 8.4 Hz, H-1), 6.976 (2H, m, H-*ortho*), 8.026 (2H, m, H-*meta*); ^{13}C NMR (D_2O): δ = 22.34 (2-Ac), 55.43 (C-2), 72.15 (C-4), 73.43 (C-3), 76.38 (C-5), 98.86 (C-1), 116.88 (C-*ortho*), 126.35 (*meta*), 142.95 (C-*para*), 162.06 (C-*ipso*), 175.14 (C-6), 175.22 (2-CO); MS (ESI): found m/z = 357.0 $[M+H]^+$, 379.1 $[M+Na]^+$, calcd. for $C_{14}H_{16}N_2O_9$: 356.1.

p-*Nitrophenyl 2-acetamido-2-deoxy-6*-O-*sulfo*-β-D-*glucopyranoside, sodium salt* (4): Compound **4** was prepared as described previously,[5] and it was obtained in 52% yield over five steps starting from *p*NP-GlcNAc. ^{1}H NMR (D_2O): δ = 1.867 (3H, s, Ac), 3.459 (1H, dd, J = 9.6, 9.0 Hz, H-4), 3.556 (1H, dd, J = 10.2, 9.0 Hz, H-3), 3.780 (1H, ddd, J = 9.6, 5.7, 2.2 Hz, H-5), 3.894 (1H, dd, J = 10.2, 8.4 Hz, H-2), 4.085 (1H, dd, J = 11.4, 5.7 Hz, H-6u), 4.246 (1H, dd, J = 11.4, 2.2 Hz, H-6d), 5.139 (1H, d, J = 8.4 Hz, H-1), 6.989 (2H, AA′BB′, ΣJ = 9.3 Hz, 2×H-*ortho*), 8.019 (2H, AA′BB′, ΣJ = 9.3 Hz, 2×*H-meta*); ^{13}C NMR (D_2O): δ = 22.38 (Ac), 55.48 (C-2), 67.16 (C-6), 69.68 (C-4), 73.51 (C-3), 74.38 (C-5), 98.85 (C-1), 116.76 (2×C-*ortho*), 126.36 (2×C-*meta*), 142.87 (C-*para*), 161.88 (C-*ipso*), 175.19 (C=O). MS (ESI): found m/z = 422.7 $[M+H]^+$, 444.7 $[M+Na]^+$,calcd. for $C_{14}H_{18}N_2O_{11}S$: m/z = 422.0.

p-*Nitrophenyl 2-acetamido-2-deoxy-3,4-di*-O-*acetyl-6*-O-*phosphate-β-D-glucopyranoside* (5a): $POCl_3$ (0.5 mL, 5.462 mmol) was added dropwise under an argon atmosphere to a solution of dry *p*-nitrophenyl 2-acetamido-2-

deoxy-3,4-di-*O*-acetyl-β-D-glucopyranoside[6] (**1a**; 300 mg, 0.703 mmol) and dry ethyldiisopropylamine (DIPEA) in CH_2Cl_2 (11 mL, 1:10 v/v). After 15 h of stirring at ambient temperature, another portion of DIPEA (5 mL) was added, followed by the addition of water (1 mL). The reaction mixture was stirred for a further 1 h and, after the addition of EtOH (20 mL), it was evaporated to dryness. After purification by silica gel chromatography (mobile phase $CHCl_3$:MeOH, 10:3), compound **5a** was obtained as a white solid; yield: 300 mg (0.591 mmol, 84%). ^{1}H NMR (DMSO-d_6): δ = 1.769 (3H, s, 2-Ac), 1.941 (3H, s, 3-Ac), 1.990 (3H, s, 4-Ac), 3.740 (1H, ddD, J = 6.0, 11.6 Hz, $J_{H,P}$ = 8.4 Hz, H-6u), 3.843 (1H, ddD, J = 1.9, 11.6 Hz, $J_{H,P}$ = 6.1 Hz, H-6d), 4.047 (1H, dd, J = 8.4, 10.2 Hz, H-2), 4.106 (1H, ddd, J = 1.9, 6.0, 9.8 Hz, H-5), 4.860 (1H, dd, J = 9.6, 9.8 Hz, H-4), 5.213 (1H, dd, J = 9.6, 10.2 Hz, H-3), 5.493 (1H, d, J = 8.4, H-1), 7.247 (2H, m, H-*ortho*), 8.106 (1H, d, J = 9.0 Hz, 2-NH), 8.196 (2H, m, H-*meta*), 9.692 [2H, -OP(=O)(OH)$_2$]; ^{13}C NMR (DMSO-d_6): δ = 20.41 (3-Ac), 20.49 (4-Ac), 22.69 (2-Ac), 53.08 (C-2), 63.08 (C-6, $J_{C,P}$ = 4 Hz), 68.90 (C-4), 72.66 (C-3), 72.70 (C-5), 97.44 (C-1), 116.86 (C-*ortho*), 125.82 (C-*meta*), 142.21 (C-*para*), 161.69 (C-*ipso*), 169.28 (4-CO), 169.50 (2-CO), 169.72 (3-CO); ^{31}P NMR: δ = −1.581.

p-*Nitrophenyl 2-acetamido-2-deoxy-6-O-phospho-β-D-glucopyranoside, sodium salt (5):* A solution of sodium methoxide in methanol (25% w/w) was added dropwise to the suspension of **5a** (250 mg, 0.434 mmol) in dry methanol (5 mL), until the pH of the reaction mixture reached 13. After complete disappearance of the starting material as observed by TLC (acetonitrile:water, 4:1), the reaction mixture was directly loaded onto a Sephadex LH-20 (Sigma) column (mobile phase MeOH, flow rate 15 mL h^{-1}). Compound **5** was obtained as a white solid; yield: 100 mg (0.208 mmol, 48%). ^{1}H NMR (D_2O): δ = 1.803 (3H, s, 2-Ac), 3.503 (1H, dd, J = 9.1, 10.0 Hz, H-3), 3.525 (1H, ddd, J = 1.9, 3.6, 9.2 Hz, H-5), 3.577 (1H, dd, J = 9.1, 9.2 Hz, H-4), 3.824 (1H, ddD, J = 1.9, 12.2 Hz, $J_{H,P}$ = 5.9 Hz, H-6u), 3.852 (1H, dd, J = 8.4, 10.0 Hz, H-2), 3.892 (1H, ddD, J = 3.6, 12.2 Hz, $J_{H,P}$ = 7.7 Hz, H-6d), 5.115 (1H, d, J = 8.4, H-1), 7.006 (2H, m, H-*ortho*), 8.044 (2H, m, H-*meta*); ^{13}C NMR (D_2O): δ = 22.37 (2-Ac), 55.82 (C-2), 62.64 (C-6, $J_{C,P}$ = 4 Hz), 69.33 (C-4), 73.12 (C-3), 76.23 (C-5, $J_{C,P}$ = 7 Hz), 99.09 (C-1), 116.85 (C-*ortho*), 126.41 (C-*meta*), 142.96 (C-*para*), 162.02 (C-*ipso*), 175.15 (2-CO); ^{31}P NMR: δ = 4.759. MS (ESI): found m/z = 467.0 $[M+Na_2]^+$: calcd. for $C_{14}H_{19}N_2O_{11}P$: 422.1.

p-*Nitrophenyl 2-acetamido-2-deoxy-6-O-sulfo-β-D-galactopyranoside, sodium salt (6):* Compound **6** was synthesized analogously to compound **4**;[5] it was obtained in a 30% overall yield. ^{1}H NMR (DMSO-d_6): δ = 1.795 (3H, s, 2-Ac), 3.597 (1H, m, H-3), 3.732 (1H, ddd, ΣJ = 7.3 Hz, H-4), 3.819 (1H, dd, J = 12.0, 8.5 Hz, H-6u), 3.912 (2H, m, H-5, H-6d), 4.038 (1H, ddd, J = 10.6, 9.0, 8.4 Hz, H-2), 4.79 (1H, br s, 3-OH), 4.805 (1H, d, J = 4.5 Hz, 4-OH), 5.132 (1H, d, J = 8.4 Hz, H-1), 7.185 (2H, AA′BB′, ΣJ = 9.3 Hz, H-*ortho*), 7.743 (1H, d, J = 9.0 Hz, 2-NH), 8.188 (2H, AA′BB′, ΣJ = 9.3 Hz, H-*meta*); ^{13}C NMR (DMSO): δ = 23.08 (2-Ac), 51.65 (C-2), 65.03 (C-6), 67.54 (C-4), 70.88 (C-3), 73.47 (C-5), 98.91 (C-1), 116.70 (C-*ortho*), 125.75 (C-*meta*), 141.82 (C-*para*), 162.38 (C-*ipso*), 169.62 (CO); MS (ESI): found m/z = 422.7 $[M+H]^+$, 444.7 $[M+Na]^+$, calcd. for $C_{14}H_{18}N_2O_{11}S$: 422.0.

p-*Nitrophenyl 2-acetamido-2-deoxy-6-O-phospho-β-D-galactopyranoside, cyclohexylamine salt (8):* The synthesis of compound **8** (*galacto-*) is analogous to that of **5** (*gluco-*). $POCl_3$ (0.4 mL, 4.370 mmol) was added dropwise under an argon atmosphere to the solution of dry *p*-nitrophenyl 2-acetamido-2-deoxy-3,4-di-*O*-acetyl-β-D-galactopyranoside[6] (**7**; 200 mg, 0.469 mmol) and dry DIPEA in CH_2Cl_2 (11 mL, 1:10 v/v). After 15 h of stirring at ambient temperature, another portion of DIPEA (5 mL) was added, followed by the addition of water (1 mL). The reaction mixture was stirred for a further 1 h and, after the addition of EtOH (20 mL), it was evaporated to dryness. The resulting yellow oil was dissolved in MeOH (20 mL) and DOWEX 50W resin (Sigma, 5 g) in H^+ form (prewashed with MeOH) was added. After 1 min of stirring, the reaction mixture was filtered and evaporated to dryness under vacuum. The yellowish solid was dissolved in dry methanol (5 mL) and a solution of sodium methoxide in methanol (25% w/w) was added dropwise, until the pH of the reaction mixture reached 13. After the starting material disappeared (TLC; acetonitrile:water, 4:1), the reaction mixture was directly loaded onto Sephadex LH-20 (Sigma) column (mobile phase MeOH, flow rate 15 mL h^{-1}). Fractions containing the product were collected and after evaporation mixed with DOWEX 50W resin in methanol. The resin was filtered off and after adding cyclohexylamine (5 mL), the mixture was dried under vacuum. The product was crystallized from water/*i*-PrOH to give **8** as white crystals; yield: 115 mg (0.272 mmol, 58%). ^{1}H NMR (D_2O): δ = 0.974 (1H, m, H-4′d), 1.097 (1H, m, H-3′d), 1.138 (1H, m, H-2′u), 1.437 (1H, m, H-4′u), 1.587 (1H, m, H-3′u), 1.778 (1H, m, H-2′u), 1.800 (3H, s, 2-Ac), 2.944 (1H, m, H-1′), 3.697 (1H, dd, J = 3.3, 10.9 Hz, H-3), 3.808 (2H, m, H-6), 3.865 (1H, m, H-5), 3.925 (1H, br d, J = 3.3 Hz, H-4), 4.025 (1H, dd, J = 8.4, 10.9 Hz, H-2), 5.061 (1H, d, J = 8.4, H-1), 7.023 (2H, m, H-*ortho*), 8.053 (2H, m, H-*meta*); ^{13}C NMR (D_2O): δ = 22.41 (2-Ac), 24.05 (C-3′), 24.55 (C-4′), 30.59 (C-2′), 50.66 (C-1′), 52.45 (C-2), 63.07 (C-6, $J_{C,P}$ = 4 Hz), 67.32 (C-4), 70.68 (C-3), 74.55 (C-5, $J_{C,P}$ = 7 Hz), 99.49 (C-1), 116.87 (C-*ortho*), 126.42 (C-*meta*), 142.91 (C-*para*), 162.16 (C-*ipso*), 175.34 (2-CO); ^{31}P NMR (D_2O): δ = 1.910. MS (ESI): m/z = 445.0 $[M+Na]^+$, 466.9 $[M+Na_2]^+$, calcd. for $C_{14}H_{19}N_2O_{11}P$: 422.1.

Molecular Modeling

Molecular dynamics simulation was performed using the previously reported dimeric models of the β-*N*-acetylhexosaminidases from *Aspergillus oryzae*[19] and *Penicillium oxalicum*.[18] All substrates were built with Yasara, and optimized by AM1 semi-empirical method.[24] Force field parameters were assigned using the AutoSMILES approach.[25] Initial ligand positions for docking experiments were determined by overlaying the substrate and enzyme with the crystal structure of bacterial chitobiase cocrystallized with the ligand (pdb code 1QBB) in Yasara.[26] In the second step the actual docking was performed using the version of Autodock 4.0[27] implemented in Yasara applying local Search Algorithm, grid space 0.375 nm, to determine the exact docked position of the standard substrate *p*NP-GlcNAc (**1**) as well as of individual C-6-modified substrates **2**–**5** in the active site. For molecular docking, the pyranosyl rings of the substrates were in a slightly distorted $^{1,4}B/^1S_3$ conformation to

resemble the 'reactive' conformations in the Michaelis structures reported by Vocadlo and co-workers.[28]

The molecular dynamics simulations of the substrate-enzyme complexes were run to further improve the docked positions, including the solvation effect, and to assess the stability of the docked position in the active site. All simulations were run for 3 ns in explicit TIP3[29] water with Yasara using the Yamber 2[30] force field; periodic boundary conditions with the simulation cell extended 0.8 nm on each side of the complex. Sodium ions were iteratively placed at the coordinates with the lowest electrostatic potential until the cell was neutral. Intramolecular forces were calculated every 1 fs, intermolecular every 2 fs; Lennard-Jones and electrostatic interactions were cut off at 0.76 nm, long-range electrostatic interactions were computed using the Particle-Mesh Ewald method,[31] with grid spacing 0.1 nm, fourth-order B-splines, and a tolerance of 10^{-4} for the direct space sum. The simulation was run in the following NPT ensemble: constant temperature (298 K), pressure and number of particles. The evaluation of ligand-enzyme complexes in time was analyzed on the basis of geometry and energy parameters. Root mean square deviation and binding energies were calculated with Yasara. Binding energies were calculated using a method applied earlier for substrate-galactosaminidase complexes.[32] It considers the internal energy obtained with the specified force field, including van der Waals solvation energy and a correction for the entropic cost of exposing the substrate/enzyme surface to water.[33,34] The solvation energy was calculated using the boundary fast method implemented in Yasara. Free energies of binding were calculated by AutoDock with Lamarckian Genetic Algorithm and grid space 0.285 nm[17] using substrate-enzyme complexes minimized in Yasara after 3 ns of MD.

Analytical Transglycosylation Reactions

For autocondensation reactions with substrates **3**–**5** (where the substrate served both as glycosyl donor and acceptor), the respective substrate (25–300 mM) was dissolved in sodium citrate/phosphate buffer and the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (2.5–30 U mL^{-1}) was added. For the glycosylation of GlcNAc with substrates **2**, **4** and **5**, the respective substrate (50–150 mM) and 2-acetamido-2-deoxy-D-glucopyranose (**11**; 200–600 mM) were dissolved in sodium citrate/phosphate buffer and the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (2.5–30 U mL^{-1}) was added. Phosphate **5** was also tested as a glycosyl acceptor: *p*NP-GlcNAc (50 mM) and **5** (200 mM) were dissolved in sodium citrate/phosphate buffer and the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (1.8 U mL^{-1}) was added. For the reactions with substrates **2--4**, 50 mM sodium citrate/phosphate buffer pH 5 was used. With phosphate substrate **5**, 200 mM sodium citrate/phosphate buffer pH 4 was used and after the substrate dissolved, the pH was adjusted to 5 with 200 mM citric acid. Here, a stronger buffering capacity was required to suppress the alkalization of the reaction medium by phosphate **5**. The reactions were incubated at 35 °C with shaking (850 rpm) for 24 h. Aliquots were taken at regular time intervals and analyzed by TLC (2-propanol:H_2O:NH_3 aq., 7: 2: 1).

Synthesis of Oligosaccharides by β-*N*-Acetylhexosaminidases

p-*Nitrophenyl 2-acetamido-2-deoxy*-β-D-gluco-*pyranosyluronic acid*-(1→4)-2-acetamido-2-deoxy-β-D-gluco-*pyranosiduronic acid* (9): Substrate **3** (100 mg, 0.281 mmol) was dissolved in sodium citrate/phosphate buffer pH 5 (936 μL). The β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686 (30 U) was added and the mixture was shaken at 35 °C. After 3.5 h, the reaction was stopped by heating to 100 °C for 2 min. The reaction mixture was cooled to room temperature and centrifuged (13,000 rpm, 10 min), concentrated under vacuum and loaded onto a Biogel P2 (BioRad, USA) column (water, flow rate 12.5 mL h^{-1}). Fractions containing product **9** were collected, lyophilized and further purified by reversed-phase HPLC. HPLC was carried out in a modular system consisting of DeltaChrom SDS 020 and 030 pumps (Watrex, CZ), a Spectra 100 Variable UV/VIS CE detector (Thermo Separation Products, USA), Basic Marathon Plus autosampler (Watrex, CZ) and dyn./stat. mixing chamber (SunChrom GmbH, D). Clarity AS software (Chromservis, CZ) was used for evaluation. A Luna (C8) column, 250×4.6 mm, with a 4×3 mm guard column (Phenomenex, USA), was used at ambient temperature for analytical scale analysis (mobile phase MeCN:H_2O 20:80, flow rate 0.8 mL min^{-1}). For the purification of product **9** a Biospher SI C8 5 μm semipreparative column 250×8 mm (Watrex, CZ) was used (mobile phase MeCN:H_2O 20:80, flow rate 3.2 mL min^{-1}). The retention time of compound **9** was 5.89 min (analytical column) and 3.97 min (preparative column). Compounds were detected at 210 nm. Disaccharide **9** was obtained as a white solid; yield: 7.7 mg (0.022 mmol, 16%). ^{1}H NMR (D_2O): δ = 1.719 (3H, s, 2′-Ac), 1.742 (3H, s, 2-Ac), 3.338 (2H, m, H-3′,H-4′), 3.492 (1H, dd, J = 8.4, 10.3 Hz, H-2′), 3.595 (1H, dd, J = 8.5, 10.0 Hz, H-3), 3.679 (1H, dd, J = 8.5, 9.2 Hz, H-4), 3.766 (1H, d, J = 9.7 Hz, H-5), 3.815 (1H, dd, J = 8.2, 10.0 Hz, H-2), 3.977 (1H, d, J = 9.2 Hz, H-5), 4.377 (1H, d, J = 8.4 Hz, H-1′), 5.102 (1H, d, J = 8.2 Hz, H-1), 6.857 (2H, m, H-*ortho*), 7.911 (2H, m, H-*meta*); ^{13}C NMR (D_2O, HSQC and HMBC readouts): δ = 22.4 (2′-Ac), 22.7 (2-Ac), 54.7 (C-2), 55.4 (C-2′), 71.7 (C-3, C-3' or C-4′), 73.2 (C-3' or C-4′), 74.0 (C-5), 74.5 (C-5′), 80.1 (C-4), 98.8 (C-1), 101.6 (C-1′), 116.8 (C-*ortho*), 126.5 (C-*meta*), 143.1 (C-*para*), 161.6 (C-*ipso*), 170.9 (C-6), 172.4 (C-6′), 175.0 (2′-CO), 175.1 (2-CO); MS (MALDI-TOF): m/z = 574.2 $[M+H]^+$, 596.2 $[M+Na]^+$: calcd. for $C_{22}H_{27}N_3O_{15}$: 573.1.

***2-Acetamido-2-deoxy*-β-D-gluco-*pyranosyluronic acid*-(1→4)-2-acetamido-2-deoxy-D-gluco-*pyranose* (10):** Compound **2** (51 mg, 0.150 mmol) and 2-acetamido-2-deoxy-D-glucopyranose (**11**; 232 mg, 1.050 mmol) were suspended in 50 mM sodium citrate/phosphate buffer pH 5 (2.96 mL), the β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686 [$(NH_4)_2SO_4$ precipitate; 6 U] was added and the reaction was incubated at 37 °C with shaking (850 rpm). The reaction progress was monitored by TLC (2-propanol: H_2O:NH_4OH aq., 7:2:1). After 4 h, the reaction was stopped by boiling for 2 min. The reaction mixture was cooled to room temperature and $NaClO_2$ (38 mg, 0.337 mmol) was added. After 5 h, the oxidation was complete, the reaction mixture was centrifuged (13,500 rpm, 10 min), concentrated under vacuum and loaded onto Bio Gel P2 (BioRad, USA)

column (mobile phase water, flow rate 9.5 mL h^{-1}). Disaccharide **10** was obtained as a white solid; yield: 15 mg (0.034 mmol; yield 23% referred to donor **2**). According to NMR, **10** was a mixture of two anomers ($\alpha/\beta = 1.6$). ^{1}H NMR (D_2O; α-anomer): $\delta = 1.855$ (3H, s, 2-Ac), 1.888 (3H, s, 2′-Ac), 3.389 (1H, m, H-4′), 3.416 (1H, ddd, $J = 10.0$, 8.5 Hz, H-4), 3.421 (1H, m, H-3′), 3.483 (1H, m, H-6u), 3.587 (1H, m, H-5′), 3.599 (1H, m, H-6d), 3.607 (1H, m, H-2′), 3.667 (1H, dd, $J = 10.8$, 3.5 Hz, H-2), 3.704 (1H, ddd, $J = 10.0$, 5.0, 2.0 Hz, H-5), 3.732 (1H, dd, $J = 10.8$, 8.5 Hz, H-3), 4.415 (1H, d, $J = 8.5$ Hz, H-1′), 5.018 (1H, d, $J = 3.5$ Hz, H-1); ^{1}H NMR (D_2O; β-anomer): $\delta = 1.855$ (3H, s, 2-Ac), 1.888 (3H, s, 2′-Ac), 3.342 (1H, ddd, $J = 9.8$, 5.5, 2.0 Hz, H-5), 3.382 (1H, m, H-4′), 3.405 (1H, m, H-4), 3.414 (1H, m, H-3′), 3.462 (1H, m, H-6u), 3.502 (1H, m, H-2), 3.538 (1H, m, H-3), 3.578 (1H, m, H-5′), 3.594 (1H, m, H-2′), 3.640 (1H, m, H-6d), 4.412 (1H, d, $J = 8.5$ Hz, H-1′), 4.515 (1H, d, $J = 8.4$ Hz, H-1); ^{13}C NMR (D_2O; α-anomer): $\delta = 22.16$ (2-Ac), 22.39 (2′-Ac), 53.92 (C-2), 55.72 (C-2′), 60.39 (C-6), 69.47 (C-3), 70.18 (C-5), 72.18 (C-4′), 73.63 (C-3′), 75.68 (C-5′), 80.45 (C-4), 90.62 (C-1), 101.49 (C-1′), 174.71 (CO), 174.85 (CO'), 175.46 (C-6′; ^{13}C NMR (D_2O; β-anomer): $\delta =$ 22.16 (2-Ac), 22.39 (2′-Ac), 55.70 (C-2′), 56.35 (C-2), 60.53 (C-6), 72.18 (C-4′), 72.76 (C-3), 73.61 (C-3′), 74.81 (C-5), 75.72 (C-5′), 80.04 (C-4), 95.12 (C-1), 101.46 (C-1′), 174.78 (CO'), 175.01 (CO), 175.46 (C-6′). MS (MALDI-TOF): $m/z = 439.06$ $[M+H]^+$, 461.18 $[M+Na]^+$, 477.15 $[M+K]^+$, calcd. for $C_{16}H_{26}N_2O_{12}$: 438.15.

p-*Nitrophenyl* 2-acetamido-2-deoxy-6-O-*sulfo*-β-D-gluco-*pyranosyl*-(1→4)-2-acetamido-2-deoxy-6-O-*sulfo*-β-D-gluco-*pyranoside* (12): Substrate **4** (60 mg, 0.135 mmol) was dissolved in sodium citrate/phosphate buffer pH 5 (1350 μL). The β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686 (0.2 U) was added and the mixture was shaken at 35 °C. After 4 h, the reaction was stopped by heating to 100 °C for 2 min. The reaction mixture was cooled to room temperature and centrifuged (13,000 rpm, 10 min), concentrated under vacuum and loaded onto a Biogel P2 (BioRad, USA) column (water, flow rate 11.5 mL h^{-1}). Disaccharide **12** was obtained as a white solid; yield: 14 mg (0.019 mmol, 28%). ^{1}H NMR (DMSO-d_6): $\delta = 1.820$ (3H, s, 2-Ac), 1.895 (3H, s, 2′-Ac), 3.130 (2H, m, H-3′, H-4′), 3.386 (1H, m, H-5′), 3.511 (1H, dd, $\Sigma J = 18.2$ Hz, H-4), 3.608 (1H, m, H-2′), 3.640 (1H, dd, $\Sigma J = 18.3$ Hz, H-3), 3.770 (1H, ddd, $J = 10.2$, 8.7, 8.4 Hz, H-2), 3.812 (1H, dd, $J = 11.3$, 6.1 Hz, H-6′u), 3.837 (1H, m, H-5), 3.896 (1H, dd, $J = 11.1$, 1.9 Hz, H-6u), 4.064 (1H, dd, $J = 11.1$, 3.9 Hz, H-6d), 4.077 (1H, dd, $J = 11.3$, 2.0 Hz, H-6′d), 4.436 (1H, d, $J = 8.6$ Hz, H-1′), 5.279 (1H, d, $J = 8.4$ Hz, H-1), 7.217 (2H, AA′BB′, $\Sigma J = 9.3$ Hz, H-*ortho*), 7.665 (1H, d, $J = 9.5$ Hz, 2′-NH), 8.023 (1H, d, $J =$ 8.7 Hz, 2-NH), 8.173 (2H, AA′BB′, $\Sigma J = 9.3$ Hz, H-*meta*); ^{13}C NMR (DMSO-d_6): $\delta = 22.96$ (2′-Ac), 22.99 (2-Ac), 54.68 (C-2′), 54.74 (C-2), 63.70 (C-6), 65.52 (C-6′), 70.23 (C-4′), 71.76 (C-3), 72.53 (C-5), 74.69 (C-3′), 75.15 (C-5′), 80.55 (C-4), 97.72 (C-1), 101.95 (C-1′), 116.72 (C-*ortho*), 125.72 (C-*meta*), 141.97 (C-*para*), 162.02 (C-*ipso*), 169.29 (CO'), 169.67 (CO); MS (ESI): $m/z = 705.7$ $[M+H]^+$, 727.7 $[M+Na]^+$, calcd. for $C_{22}H_{31}N_3O_{19}S_2$: 705.1.

***2-Acetamido-2-deoxy-6-O-sulfo*-β-D-gluco-*pyranosyl*-(1→4)-2-acetamido-2-deoxy-D-gluco-*pyranose* (13):** Compound **4** (40 mg, 0.090 mmol) and 2-acetamido-2-deoxy-D-*gluco*-pyranose (**11**; 78 mg, 0.353 mmol) were suspended in 50 mM sodium citrate/phosphate buffer pH 5 (1200 μL), the β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686 (1 U) was added and the reaction was incubated at 35 °C with shaking (850 rpm). After 6.5 h, the reaction was stopped by boiling for 2 min. The reaction mixture was cooled to room temperature and centrifuged (13,000 rpm, 10 min), concentrated under vacuum and loaded onto a Biogel P2 (BioRad, USA) column (water, flow rate 11.5 mL h^{-1}). Disaccharide **13** was obtained as a white solid; yield: 16 mg (0.030 mmol, 33%). According to NMR, **13** was a mixture of two anomers ($\alpha/\beta = 1.8$). ^{1}H NMR (DMSO-d_6; α-anomer): δ 1.820, 1.822 (2×3H, 2×s, 2-Ac, 2′-Ac), 3.105 (1H, dd, $\Sigma J = 16.5$ Hz, H-4′), 3.285 (1H, dd, $\Sigma J = 17.8$ Hz, H-4), 3.32 (1H, m, H-3′), 3.35 (1H, m, H-5′), 3.458 (1H, m, H-2′), 3.47 (1H, m, H-6u), 3.56 (1H, m, H-6d), 3.572 (2H, m, H-2, H-5), 3.663 (1H, dd, $\Sigma J = 18.2$ Hz, H-3), 3.781 (1H, dd, $J = 12.2$, 6.6 Hz, H-6′u), 4.070 (1H, m, H-6′d), 4.367 (1H, d, $J = 8.2$ Hz, H-1′), 4.932 (1H, d, $J = 3.2$ Hz, H-1), 7.729 (1H, br d, 2′-NH), 7.782 (1H, d, $J = 7.5$ Hz, 2-NH); ^{1}H NMR (DMSO-d_6; β-anomer): $\delta = 1.820$, 1.822 (2×3H, 2×s, 2-Ac, 2′-Ac), 3.105 (1H, dd, $\Sigma J = 16.5$ Hz, H-4′), 3.162 (1H, m, H-5), 3.261 (1H, dd, $\Sigma J = 17.8$ Hz, H-4), 3.32 (1H, m, H-3′), 3.35 (1H, m, H-5′), 3.36 (1H, m, H-2), 3.374 (1H, m, H-6u), 3.441 (1H, m, H-2′), 3.508 (1H, dd, $\Sigma J = 18.0$ Hz, H-3), 3.614 (1H, m, H-6d), 3.781 (1H, dd, $J = 12.2$, 6.6 Hz, H-6′u), 4.070 (1H, m, H-6′d), 4.387 (1H, d, $J = 8.0$ Hz, H-1′), 4.462 (1H, d, $J = 8.3$ Hz, H-1), 7.729 (1H, br d, 2′-NH), 7.812 (1H, d, $J = 8.4$ Hz, 2-NH); ^{13}C NMR (DMSO-d_6; α-anomer): $\delta =$ 22.67, 23.03 (2-Ac, 2′-Ac), 53.88 (C-2), 55.47 (C-2′), 60.05 (C-6), 65.55 (C-6′), 68.64 (C-3), 69.88 (C-5), 70.30 (C-4′), 73.62 (C-3′), 74.98 (C-5′), 81.70 (C-4), 90.12 (C-1), 102.05 (C-1′), 169.11 (CO'), 169.31 (CO); ^{13}C NMR (DMSO-d_6; β-anomer): $\delta = 22.67$, 23.03 (2-Ac, 2′-Ac), 55.47 (C-2′), 56.65 (C-2), 60.24 (C-6), 65.61 (C-6′), 70.35 (C-4′), 72.23 (C-3), 73.71 (C-3′), 74.85 (C-5), 74.98 (C-5′), 81.28 (C-4), 95.45 (C-1), 101.83 (C-1′), 169.15 (CO'), 169.75 (CO); MS (ESI): $m/z = 504.7$ $[M+H]^+$, 526.7 $[M+Na]^+$, calcd. for $C_{16}H_{28}N_2O_{14}S$: 504.1.

Competitive Inhibition Assay with NKR-P1 and CD69 Activation Receptors of NK Cells

The inhibition assays were performed as described previously,[35] with the following modification: soluble NKR-P1 and CD69 protein receptors were labeled with fluorescent labels (fluorescein and rhodamine, respectively). The concentrations of bound protein receptors in the microtiter wells were determined by fluorescence measurement ($\lambda_{ex}/\lambda_{em} = 496/519$ nm and $\lambda_{ex}/\lambda_{em} = 546/577$ nm for NKR-P1 and CD69, respectively) using a Safire 2 spectrophotometer (Tecan, AT). The results are given as a negative logarithm of the ligand concentration required to cause 50% inhibition of the receptor binding to the standard high-affinity ligand $GlcNAc_{23}BSA$ ($-\log IC_{50}$). Proteins were labeled by covalently attaching fluorescent labels using *N*-hydroxysuccinimide fluorescein and *N*-hydroxysuccinimide rhodamine (both by Pierce Biotechnology, USA) for rat NKR-P1 A and human CD69 receptors, respectively.

The 96-well round-bottomed plate was coated with $GlcNAc_{23}BSA$ ligand, blocked with 2% BSA and after incubation at 4 °C for 2 h, the plate was washed three times with PBS. The labeled proteins and serial dilutions of studied

compounds (**1**–**6**, **8**–**13** and respective reference compounds, see Table 2) were put into each well, incubated at 4 °C for 1 h, then the plate was washed three times with PBS and incubated at 4 °C overnight with 0.1 M sodium acetate buffer supplemented with 0.1% octyl β-D-*gluco*-pyranoside and 0.1% Triton X-100. Then, the solution was transferred to 96-well flat-bottomed UV-transparent plates and the results were obtained by fluorescence measurement. Complete inhibition curves were constructed and the IC_{50} values were calculated from at least three independent experiments.

Immunological Tests

Rat NK cells were purified from mononuclear spleen cells using Ficoll-Paque centrifugation (Sigma) followed by the immunodepletion of T cells, B cells, and monocytes, and subsequent IL-2 activation in order to enhance the surface expression of the NKR-P1A receptor.[36] Peripheral blood mononuclear cells were obtained from standard blood fractions enriched in leukocytes (buffy coats from the local Blood Transfusion service) after dilution with RPMI1640 medium (Sigma), and Ficoll-Paque centrifugation. Cells were incubated overnight in complete RPMI1640 medium in plastic cell culture dishes to allow the adherent cells to attach. Collected non-adherent fraction of PBMC (N-PBMC) contained mostly lymphocytes (T, B, and NK cells). Lymphocytes from donors expressing CD69 in less than 5% of cells were designated $CD69^{low}$. Lymphocytes from donors with more than 20% CD69-positive cells were further activated by incubation at a density of 2×10^6 cells mL^{-1} in complete RPMI1640 medium for 4 h with PMA (50 ng mL^{-1}) and ionomycin (500 ng mL^{-1}). This procedure increased the surface expression of CD69 to 75–85%, as analyzed by flow cytometry using monoclonal antibody against CD69 labeled with phycoerythrin. Such lymphocytes were designated as $CD69^{high}$. Cellular activation assays and tests of natural killing were essentially performed as described previously.[9b] For apoptosis assays, the cells were resuspended at a density of 2×10^6 mL^{-1} in complete RPMI1640 medium, aliquoted into round-bottomed 96-well plates, and the tested compounds in various concentrations were added into duplicate wells. The compounds were added 12 and 6 h before determining the percentage of apoptotic cells using Annexin V-FITC/Hoechst 33258 staining and flow cytometry. The percentage of apoptotic cells (Annexin V^+/Hoechst 33258^-) observed in the presence of PBS alone and in the presence of 5×10^{-6} M arsenite were used as the negative and positive control, respectively.

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