Combined Application of Galactose Oxidase and β-*N*-Acetylhexosaminidase in the Synthesis of Complex Immunoactive *N*-Acetyl-D-galactosaminides

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Abstract: A high-yield preparatory procedure for the synthesis of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranoside (**2**) using the galactose oxidase from *Dactylium dendroides* in a batch reactor was developed. Enzymatic recognition of this aldehyde and the respective uronic acid **3** obtained by $NaClO_2$ oxidation was studied using a set of 36 fungal β-*N*-acetylhexosaminidases from *Acremonium*, *Aspergillus*, *Penicillium* and *Talaromyces* genera. The aldehyde **2** was readily hydrolysed by all tested β-*N*-acetylhexosaminidases but neither the uronic acid **3** nor its methyl ester **4** were accepted. Molecular modelling with docking into the active centre of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* revealed that the aldehyde **2** is processed as a C-6 *geminal* diol by the enzyme. The aldehyde **2** was tested for transglycosylation reactions using GlcNAc as an acceptor. The β-*N*-acetylhexosaminidase from *Talaromyces flavus* gave the best yields (37%) of the transglycosylation product 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose, which was oxidised *in situ* to yield the final product 2-acetamido-2-deoxy-β-D-galactopyranosyluronic acid-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**6**). Compounds **3** and **6** were shown to be high-affinity ligands for two natural killer cell activation receptors, NKR-P1A and CD69. For the latter receptor they turned out to be among the best ligands described so far. This increase was obviously due to the presence of a carboxy moiety.

Keywords: β-*N*-acetylhexosaminidase; galactose oxidase; glycosylation; modified substrate; molecular modelling; natural killer cell

Abbreviations: GalNAc: 2-acetamido-2-deoxy-D-galactopyranose; GalNAcA: 2-acetamido-2-deoxy-D-galactopyranosuronic acid; β-D-GalNAcA-(1→4)-D-GlcNAc: 2-acetamido-2-deoxy-β-D-galactopyranosyluronic acid-(1→4)-2-acetamido-2-deoxy-D-glucopyranose; GlcNAc: 2-acetamido-2-deoxy-D-glucopyranose; LacdiNAc: β-D-GalNAc-(1→4)-D-GlcNAc or 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose; ManNAc: 2-acetamido-2-deoxy-D-mannopyranose; NK cell: natural killer cell; *p*NP: *p*-nitrophenyl; *p*NP-β-GalNAc: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside; *p*NP-β-GalNAcA: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid; UDP-GalNAc: uridine diphosphate 2-acetamido-2-deoxy-α-D-galactopyranose.

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Introduction

Enzymatic methods have already become a viable alternative to organic chemistry in the synthesis of complex carbohydrate structures,[1] especially due to their selectivity and simplicity. These methods are, however, often limited by the strict specificity of the enzymes towards substrates and, especially in case of glycosidases, by unsatisfactory yields.[2] Ingenious combinations of various enzymes in a sequence or multi-enzyme one-pot methods bring additional advantages and open new methodological ways.[3]

Glycosidases (EC 3.2) are readily available in a large repertoire from different sources. They tolerate environmental stress and have a broad substrate specificity towards a variety of acceptors. Their application is somehow limited in respect of glycosyl donors contrary to glycosyl transferases where a large number of modified substrates – nucleotide sugars – were employed for synthetic purposes.[1b,4]

The first papers presenting the use of modified glycosyl donors with glycosidases were published in the late 1990 s,[5,6] the first use of a β-*N*-acetylhexosaminidase was demonstrated by Hušáková et al.[7] in a reaction with 6-*O*-acetylated glycosyl donors. A series of β-*N*-acetylhexosaminidases with a broad substrate specificity was identified in a recent study dealing with *p*-nitrophenyl 2-acylamido-2-deoxy-β-D-glucopyranosides.[8]

It is quite obvious that glycosidases do not tolerate major changes in the structure of their substrates (glycone part). However, a large group of well-accepted modifications in the pyranose ring are those at the C-6 position.[5,7,9] The introduction of a highly reactive functionality such as the aldehyde group brings about the possibility of further modifications, e.g., reduction, oxidation or conjugation (e.g., with amines or hydrazides). The latter has recently been accomplished by the use of biotinylated nucleotide sugars as substrates for glycosyl transferases.[4c] It was already demonstrated that some galactosidases can tolerate the aldehyde group[5,9] and are able to use such modified substrates for transglycosylation reactions.

Our research is oriented to biologically active glycosides and glycoconjugates,[10] which are able to stimulate the immune response of an organism by activation of NK cells through NKR-P1A protein and other cell surface activation receptors. These immunoactive structures are mostly composed of *N*-acetyl-D-hexosamines (GalNAc, GlcNAc, ManNAc) and they can be prepared in good yields using β-*N*-acetylhexosaminidases.[11,12] GalNAc itself and glycosides containing β-GalNAc moiety possess a high immunomodulatory activity. Moreover, the incorporation of groups able to carry a negative charge (COOH, SO_3H) into such structures strongly improves the binding affinity towards the activation receptor of NK cells.[10a,13] Therefore, we were interested in the synthesis of glycosides bearing 2-acetamido-2-deoxy-β-D-galactopyranosyluronic acid moiety together with other *N*-acetyl-D-hexosamines.

In this paper we demonstrate a high-yield preparative-scale procedure for *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranoside (**2**) and its selective oxidation into the respective uronic acid **3**. We studied the recognition of these glycosides by a set of 36 β-*N*-acetylhexosaminidases from our enzyme library[12] and the results were evaluated using molecular models of the modified substrates docked into the active centre of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066. A novel disaccharide was prepared by a transglycosylation reaction and a subsequent chemical oxidation. Finally, all prepared hexosamine structures were tested for their immunomodulatory activity using NK cell activation receptors NKR-P1A (rat) and CD 69 (human). Compounds **3** and **6** proved to be high-affinity ligands for these receptors, particularly for CD69.

Results and Discussion

The C-6 oxidation of *p*NP-β-GalNAc (**1**) leading to the aldehyde **2** could theoretically be accomplished by various means, e.g., by laccase-TEMPO oxidation,[14] however, it is rather difficult to stop this reaction at the stage of the aldehyde and a mixture of the aldehyde and the uronic acid is usually formed. Therefore, we decided to use galactose oxidase, which is known to oxidise galactopyranosyl[5,9] and 2-amino-2-deoxy-D-galactopyranosyl structures selectively and quantitatively.[15] Galactose oxidase is highly specific for the carbohydrate C-6 position[16] and its preparative application to GalNAc structures has not been deeply exploited yet.[4,16] The problem of using galactose oxidase is the oxygen supply. Although a simple diffusive gas transport through the solution surface is mostly used in the literature,[9,17] the yields turned to be unsatisfactory in our hands. Bubbling by air or oxygen quickly deactivates the enzyme, probably due to denaturation in the gas/liquid interphase area. Therefore, we optimised a batch reactor with bubble-free oxygen supply as described by Bülter et al.[4c] by doubling the reaction volume and reducing the concentration of galactose oxidase twelve times compared to that used for the oxidation of UDP-GalNAc.[4c] This method enabled a quantitative oxidation on a 100-mg scale with a considerably lower consumption of enzymes and time compared to literature reports.[4c,9,17] An enormous problem consisted in the purification of the aldehyde as the compound is relatively unstable due to its high reactivity and a considerable decomposition occurs in the presence of organic solvents, under even slightly acidic or basic conditions or under heating.[18] A good solution was Dowex 50W-X8 in the Ca^{2+} cycle, which, however, required 100% conversion of the oxidation reaction because no starting material could be separated

by this method. *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid (**3**) was prepared chemically[17] as well as its methyl ester **4**.

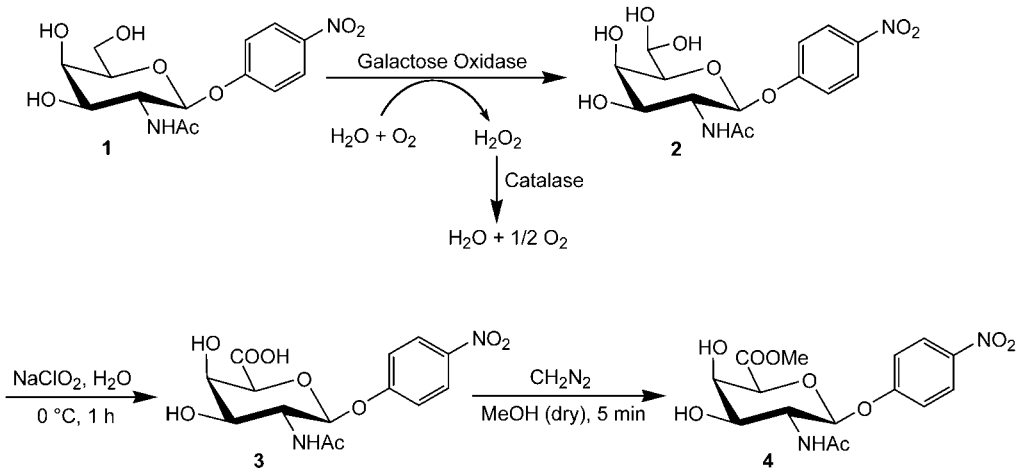
Compounds **2**, **3** and **4** were subjected to a screening for hydrolytic cleavage comprising 36 fungal β-*N*-acetylhexosaminidases from the genera of *Acremonium*, *Aspergillus*, *Penicillium* and *Talaromyces*. Compounds **3** and **4** proved to be poor substrates for all β-*N*-acetylhexosaminidases tested, with the cleavage rate lower than 1% compared to the standard substrate **1**. On the other hand, all tested enzymes cleaved substrate **2**, e.g., the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 hydrolysed it at a rate of 10% compared to **1** (Table 1).

Regarding the hydrolysis of the aldehyde **2**, we should consider the somehow neglected[5,9] question of whether β-*N*-acetylhexosaminidases recognise the aldehyde as such or in its hydrated form as a *geminal* diol (see Scheme 1), which is the prevailing form in the water solution.[18,19] The answer to this question as well as the confirmation of the other experimental results could be reached with the help of molecular modelling. The modified substrates **2**, **3** and **4** were docked into the active centre of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066. The stability of the enzyme-substrate complex reflects the drop of interaction energy (steric and electrostatic contributions, Table 2).

The steric energy contribution is in the same range for all examined compounds and is considerably lower than for the standard *p*NP-β-GalNAc (**1**, Figure 1). This is well understandable as glycosidases are known to be sensitive to any structural modifications of their substrates. On the other hand, major changes between the modified substrates are observed in the electrostatic part, in our case in the hydrogen bonding between substrate and enzyme (Figures 1 and 2). In case of the substrate **2**, its hydrated form exhibits stabilisation by hydrogen bonding with six amino acids whereas the aldehyde form lacks hydrogen bonding with two of them. This corroborates our hypothesis that substrate **2** is recognised by the enzyme as a *geminal* diol. In the cases of *p*NP-β-GalNAcA (**3**) and its methyl ester **4**, both substrates obviously bind weakly into the active site due to a loss in hydrogen bonding and, correspondingly, no hydrolysis was observed. Both substrates **3** and **4** have similarly low interaction energies, which demonstrates that the carboxylic group is not accepted due to its structural features and not because of the charge and possible anion solvation.

β-GalNAc structures bearing a carboxy group show strong immunoactivation properties.[10a,13] Therefore, we aimed to synthesise a disaccharide of β-D-GalNAcA-(1→4)-D-GlcNAc (**6**) – a LacdiNAc analogue. This could be accomplished either by enzymatic transfer of a 2-acetamido-2-deoxy-β-D-galactopyranosyluronic acid moiety onto GlcNAc or by transfer of a 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranosyl moiety followed by subsequent oxidation. The former method was impossible because *p*NP-β-GalNAcA (**3**) was not accepted by β-*N*-acetylhexosaminidases, so we opted for the aldehyde **2** as a donor with GlcNAc (**5**) as an acceptor under the catalysis by the β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686. The desired structure **6** bearing a carboxy group was obtained by *in situ* oxidation of the reaction mixture with $NaClO_2$. Interestingly, under the given conditions no oxidation at the free anomeric position occurred and the disaccharide **6** was isolated in 37% yield (Scheme 2).

The affinity of compounds **1**–**4** towards two representative NK cell activation receptors, rat NKR-P1A, and human CD69 proteins, was tested. Due to their good availability and stability, monomeric soluble forms of these proteins, i.e., NKR-391 and CD69CWTY, which encompass the ligand-binding domains, were used for the tests.[20,21] To verify the identity and homogeneity of the protein preparations, the technique of Fourier transform-ion cyclotron resonance mass spectrometry[22] was employed in addition to the standard analyt-



Scheme 1. Chemoenzymatic synthesis of substrates **2** – **4**.

Table 1. Hydrolysis of substrate **2** by fungal β-*N*-acetylhexosaminidases.

Source of enzyme	**2**[a]
Acremonium persicinum CCF 1850	++
Aspergillus (A.) awamori CCF 763	++
A. caelatus CCF 3087	+
A. flavipes CCF 1895	+
A. flavipes CCF 3067	++
A. flavofurcatis CCF 3061	+
A. flavus CCF 3056	++
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. sojae CCF 3060	++
A. tamarii CCF 3085	++
A. terreus CCF 2539	++
A. terreus USA	+
Fusarium oxysporum CCF 377	+++
Hamigera avellanea CCF 2923	+
Chaetomium globosum CCF 430	+
Penicillium (P.) brasilianum CCF 2155	++
P. brasilianum CCF 2171	++
P. chrysogenum CCF 1269	+
P. funiculosum CCF 2985	+++
P. melinii CCF 2440	+
P. multicolor CCF 2244	+++
P. oxalicum CCF 1959	++
P. oxalicum CCF 2315	+++
P. oxalicum CCF 2430	++
P. pittii CCF 2277	+++
P. spinulosum CCF 2159	+++
Talaromyces (T.) flavus CCF 2573	+++
T. flavus CCF 2686	+++
T. ohiensis CCF 2229	++
T. striatus CCF 2232	+++
Trichoderma harzianum CCF 2687	++

[a] Hydrolysis of both the standard substrate **1** and the aldehyde **2** was spectrophotometrically determined as the liberated *p*-nitrophenol: the ratio of hydrolysis rates of **2** to **1** was 21–12% (+++), 11–7% (++), 6–1% (+).

Table 2. Interaction energies of substrates **1** – **4** with the β-*N*-acetylhexosaminidase from *A. oryzae*.

Substrate	Interaction energy [kJ/mol]		
	Total	Steric	Electrostatic
1	−242	−121	−121
2 (aldehyde)	−180	−89	−91
2 (diol)	−241	−86	−155
3	−136	−94	−42
4	−131	−79	−52

ical techniques described.[20,21] This method showed the proteins to be homogenous with $m/z = 11965.4$ and 11630.1 for the native (oxidised) forms of NKR-P1A and CD69, respectively. The biological activity of the soluble receptors after ^{125}I iodination was verified using simple sugars. D-Mannose served as a negative (non-inhibitory) control carbohydrate. GlcNAc (**5**), used as a reference compound, provided IC_{50} values of 10^{-5} M (NKR-P1A) and 10^{-3} M (CD69), which are in accordance with the previously reported values.[20,21]

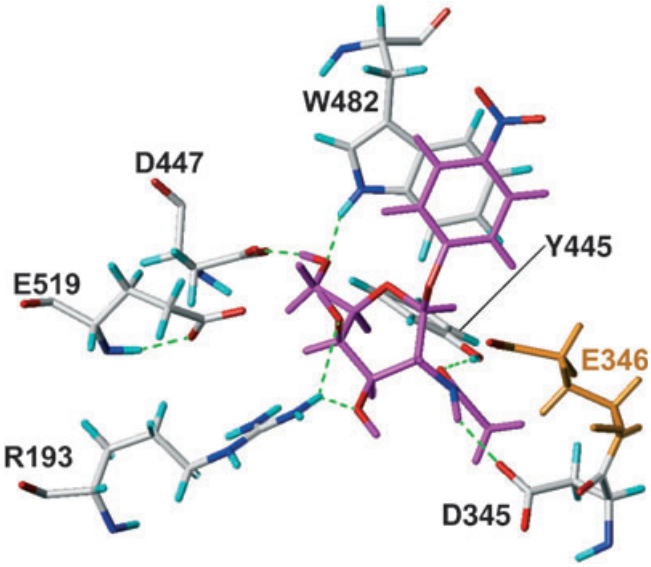


Figure 1. *p*NP-β-GalNAc (**1**) docked into the active site of the β-*N*-acetylhexosaminidase from *A. oryzae*. The catalytic glutamic acid 346 is shown in yellow, substrate **1** in violet. The ligand is fixed by hydrogen bonding and aromatic stacking of the phenol ring with Trp 482.

All inhibition assays were performed three times in duplicates and the results for NKR-P1 and CD69 are given in Figures 3 and 4, respectively, as $-\log IC_{50}$ with standard deviations. With the monomeric NKR-P1A, *p*NP-β-GalNAc (**1**; $IC_{50} = 1.3 \times 10^{-6}$ M) had a ten-fold higher inhibitory activity compared to the reference substance GlcNAc (**5**).[10a] While the oxidation of **1** into the aldehyde **2** caused only a negligible increase in IC_{50}, further oxidation to the uronic acid **3** resulted in an additional ten-fold increase in the inhibitory activity ($IC_{50} = 1.6 \times 10^{-7}$ M). A similar trend was observed in the disaccharide series: whilst a disaccharide of β-D-GalNAc-(1→4)-D-GlcNAc had an IC_{50} of 1.6×10^{-7} M, the analogous structure **6** bearing a GalNAcA moiety gave rise to a very potent disaccharide inhibitor with an IC_{50} of 10^{-8} M.

With the monomeric CD69 protein, the increase in the inhibitory potency of the uronic acid derivatives was even more profound. Here, a gradual increase in the IC_{50} values was observed by *p*NP-β-GalNAc (**1**) and the aldehyde **2**, reaching IC_{50} values of 1.6×10^{-4} M and 10^{-5} M, respectively, compared to GlcNAc (**5**; Figure 4). However, the uronic acid **3** exhibited a significant increase with $IC_{50} = 10^{-7}$ M. Even more interestingly,

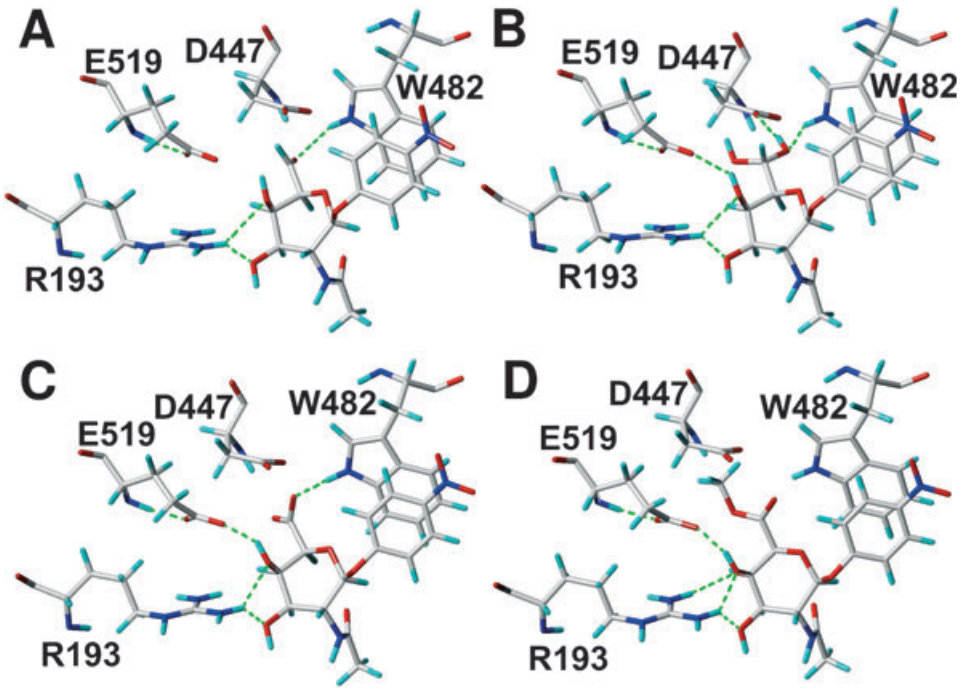
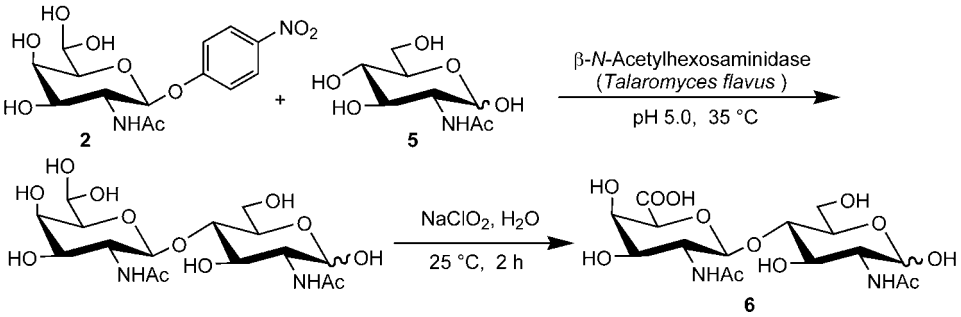


Figure 2. Substrates **2** – **4** docked into the active site of the β-*N*-acetylhexosaminidase from *A. oryzae*. **A**: aldehydic form of substrate **2**; **B**: *geminal* diol form of substrate **2**; **C**: *p*NP-β-GalNAcA (**3**); **D**: methyl ester **4**.



Scheme 2. Chemoenzymatic synthesis of disaccharide **6**.

the introduction of a carboxyl moiety into a disaccharide of β-D-GalNAc-(1→4)-D-GlcNAc led to a remarkable increase in IC_{50} of over four orders of magnitude, i.e., from 1.3×10^{-4} M (neutral disaccharide) to 1.5×10^{-8} M (the oxidised disaccharide **6**). Although the high affinities of certain negatively charged oligosaccharides for NKR-P1A have been reported,[13] this is to the best of our knowledge the first systematic study of the structure-activity relationship using a complete series of these compounds. Moreover, the very high inhibitory potency of the oxidised disaccharide **6** for CD69 (making it one of the best ligands known so far) represents an entirely novel observation, which could be effectively applied in the development of therapeutically useful glycomimetics.[10e]

Conclusion

In this paper we have demonstrated an optimised procedure for the preparation of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranoside (**2**). We found that this glycoside, contrary to its derivatives (*p*NP-β-GalNAcA, **3**), its methyl ester **4**, was readily accepted by fungal β-*N*-acetylhexosaminidases and we supported the experimental data by molecular modelling. A novel, highly immunoactive disaccharide **6** was prepared by a transglycosylation reaction and a subsequent chemical oxidation. This concept demonstrates the synthetic potential of the multi-enzyme approach and offers further possibilities for a selective and high yielding preparation of oligosaccharidic structures.

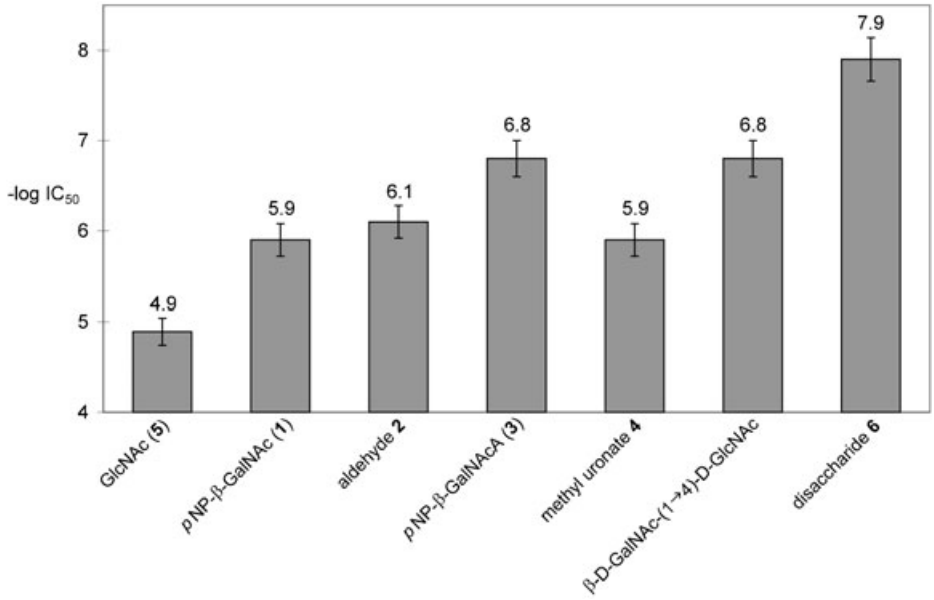


Figure 3. Affinity of new carbohydrate ligands towards rat NK cell activation receptor, NKR-P1A, expressed in the logarithmic scale ($-\log IC_{50}$). Data are average values from three independent experiments with standard deviations indicated by error bars.

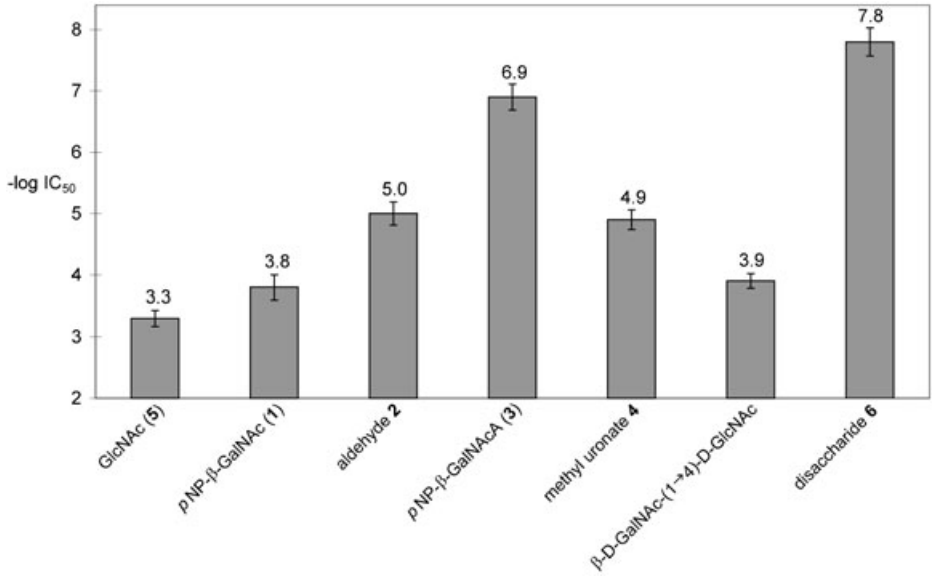


Figure 4. Affinity of new carbohydrate ligands towards human NK cell activation receptor, CD69, expressed in the logarithmic scale ($-\log IC_{50}$). Data are average values from three independent experiments with standard deviations indicated by error bars.

Experimental Section

Enzymes

Citrate/phosphate buffer (McIlvaine) pH 5.0 was prepared by mixing 0.1 M citric acid (24.3 mL) and 0.2 M Na_2HPO_4 (25.7 mL), then diluting with water to 100 mL and adjusting the pH to 5.0. The galactose oxidase from *Dactylium dendroides* (EC 1.1.3.9) was purchased from Worthington Biochemical Corporation (Canada) and used in the form of aliquots (100 μL, 0.5 U/μL) in 0.02 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.0. The catalase from bovine liver (EC 1.11.1.6) was purchased from Sigma. The fungal strains producing β-*N*-acetylhexosaminidases (EC 3.2.1.52) originated from the Culture Collection of Fungi (CCF), Department of Botany, Charles University Prague, or from the Culture Collection of the Institute of Microbiology (CCIM), Prague,[12] and were cultivated as described previously.[8]

Analytical Methods

TLC was performed on precoated Merck silica gel DC-Alufolien Kieselgel 60 F_{254} plates. The spots were visualised by charring with 5% H_2SO_4 in EtOH. Analytical HPLC was carried out on a Spectra Physics modular analytical system (San Jose, U. S.A) consisting of an SP 8800 ternary gradient pump, an SP 8880 autosampler and a Spectra Focus scanning UV/VIS detector. A Luna C8 (2) column, 250 × 4.6 mm with a guard column 4 × 3 mm (Phenomenex, USA) was used at ambient temperature with a mobile phase of MeCN: H_2O, 20: 80, and a flow rate of 0.5 mL/min. Retention times were: 14.28 min (**1**), 11.45 min (**2**) and 5.89 min (**3**). Compounds were detected at 200 nm. ^1H and ^{13}C NMR spectra were recorded on a Varian INOVA-400 spectrometer (399.89 MHz and 100.55 MHz, respectively) at 30 °C in the indicated solvents. Chemical shifts are expressed in the δ scale and were measured with digital resolution justifying the reported values to three or two decimal places, respectively. HMQC and HMBC readouts are accurate at one decimal place only. Residual solvent signals DMSO-d_6: $\delta_H = 2.50$, $\delta_C = 39.60$; CD_3OD: $\delta_H = 3.33$, $\delta_C = 49.30$) or internal acetone for D_2O solutions ($\delta_H = 2.030$, $\delta_C = 30.50$) were used for referencing. The reported assignments are based on gCOSY, HMQC, HMBC, and 1D-TOCSY experiments. Positive-ion MALDI mass spectra were measured on a Bruker BIFLEX II reflectron time-of-flight mass spectrometer (Bruker Daltonics, USA) equipped with a nitrogen laser (337 nm) and a gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Spectra were externally calibrated using the monoisotopic $[M+H]^+$ ion of angiotensin I (Sigma). A saturated solution of α-cyano-4-hydroxycinnamic acid (CCA) in 50% ACN/0.3% acetic acid was used as MALDI matrix. The matrix solution (1 μL) was premixed with a sample and loaded on the target and a droplet (1 μL) was allowed to dry at ambient temperature. The MALDI-TOF spectra were collected in reflectron mode. Positive ion electrospray (ESI) mass spectra were recorded on an LCQ^{DECA} ion trap mass spectrometer (Finnigan, San Jose, U. S.A) equipped with a nano-ESI ion source. Spray voltage was set at 2.0 kV. Samples dissolved in 50% MeOH were continuously infused into the ion source via a coated microcapillary. Full scan spectra were acquired over the *m/z* range of 50–500 Da. A 0.1% solution of Ultramark 1621 (PCR, Inc., Gainesville, FL, U. S. A.) in MeCN was used to calibrate the *m/z* scale of the instrument. Optical rotation was measured on a Perkin-Elmer 241 polarimeter at 589 nm.

Activity Assay for β-*N*-Acetylhexosaminidases[23]

The reaction mixture containing substrate **1** (2 mM, starting concentration) and β-*N*-acetylhexosaminidase (0.02–0.03 U/mL) in buffer (assay volume 50 μL) was incubated in microplates at 35 °C for 10 min. The assay was stopped by adding 0.1 M Na_2CO_3 (150 μL). Liberated *p*-nitrophenol was determined spectrophotometrically (414 nm) on a microplate reader Titertek Multiscan® MCC/ 340 (Flow Laboratories, McLean, USA). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute under the above conditions. The activity towards modified substrates **2**–**4** was determined analogously, with the amount of enzyme being 0.25–0.40 U/mL (for substrate **2**) and 0.45–0.70 U/mL (for substrates **3** and **4**). In the enzymatic screening, 36 β-*N*-acetylhexosaminidases were tested. They were classified according to the ratio of hydrolysis rates of the respective modified substrate and substrate **1** assayed under the same conditions and extrapolated to the same amount of enzyme.

Molecular Modelling

The primary sequence of the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 was aligned with the known X-ray structures of the β-*N*-acetylhexosaminidases from *Serratia marcescens* and *Streptomyces plicatus*, extracted from the Brookhaven Protein Database (PDB entries: 1QBA and 1HP4, respectively). The sequence data are available in DDBJ/EMBL/GeneBank databases (http://www.ncbi.nlm.nih.gov/) under the accession number AY091636. 3D models were generated by Modeller6 package.[24] For model refinement and minimisation, the SYBYL package with the TRIPOS force field (TRIPOS Associates Inc.) were used. The complete modelling including the alignment and energy minimisation was done exactly as described previously.[8] The docking of ligands was performed as described earlier.[8] The non-binding interaction energy between the model and the ligands within the optimised complex was calculated using the TRIPOS force field. This estimation of a real interaction energy neglects solvation and desolvation effects.

Tests of Affinity to NK Cell Activation Receptors, NKR-P1A and CD69[10a]

Compounds **1**–**6** were tested for their affinity towards two representative NK cell activation receptors, NKR-P1A and CD69 proteins. NKR-391 protein, the major activation receptor of rat NK cells, was expressed and purified as described previously.[10a,20] CD69CWTY protein that contained the soluble ligand-binding domain of the earliest activation receptor of lymphocytes and NK cells, CD69 antigen, was prepared as described earlier.[21] The proteins were purified from inclusion bodies that were dissolved in 6 M guanidine-HCl and 100 mM DTT, and refolded by dropwise addition into a hundred times larger volume of 50 mM Tris-HCl buffer pH 8.5 containing 0.4 M arginine, redox buffer (10 mM cysteamine and 1 mM cystamine), 1 mM PMSF, 1 μM leupeptin and 1 μM pepstatin. The folded protein was dialysed against low salt buffer, and purified by a combination of ion-exchange chromatography, reversed phase separation, and gel filtration as described previously.[21] The identity and homogeneity of the proteins were verified using SDS-PAGE under both reducing and non-reducing conditions, N-terminal sequencing (10 cycles of automated Edman degradation), and MALDI mass spectrometry, as described previously.[21] Moreover, Fourier transform-ion cyclotron resonance mass spectrometry was employed (APEX-Q, Bruker Daltonics, Bremen, Germany).[22] The proteins were radiolabelled with $Na^{125}I$ using Iodogen (Pierce, Rockville, IL, USA). Binding and inhibition assays were performed as described previously.[10a] Briefly, 96-well polyvinyl chloride microplates (Titertek Immuno Assay-Plate, ICN Flow, Irvine, Scotland) were coated overnight at 4 °C with $GlcNAc_{23}BSA$ (50 μL, 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 radiolabelled 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. Scintillation solution (100 μL) 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 with regard to the wells containing no inhibitor.

Oxidation by the Galactose Oxidase from *Dactylium dendroides* in a Batch Reactor with Bubble-Free Aeration[4c]

Oxidation of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**1**) was performed in a batch reactor with bubble-free aeration.[4c] The mixture of glycoside **1** (140 mg, 0.409 mmol), the galactose oxidase from *Dactylium dendroides* (100 U) and the catalase from bovine liver (40,500 U) in 0.02 M Na_2HPO_4/NaH_2PO_4 buffer pH 6.0 (100 mL) was pumped through a thin-walled silicon tube [Tygon® 3350 (P-lab, CZ), wall thickness 0.8 mm, i.d. 4 mm, length 600 mm] placed in an oxygen-storage flask with an oxygen pressure of 50 kPa. Both the inlet and the outlet of the tube were air-tightly connected to a batch reactor (200 mL) where the reaction mixture was slowly stirred. The reaction was monitored by TLC (propan-1-ol:H_2O:NH_3 aq., 7:2:1) and HPLC and stopped after 5 h by freezing. Lyophilisation gave the crude product **2**, which was further purified.

Analytical Transglycosylation Reactions Catalysed by β-*N*-Acetylhexosaminidases

Substrate **2** (4 mg, 0.012 mmol) and 2-acetamido-2-deoxy-D-glucopyranose (**5**; 26 mg, 0.118 mmol) were dissolved in citrate/phosphate buffer pH 5.0 (392 μL). The reaction was started by the addition of β-*N*-acetylhexosaminidase (0.75 U) and was incubated at 37 °C with shaking for 24 h. Aliquots were analysed by TLC (propan-1-ol : H_2O : NH_3 aq., 7:2:1).

Synthesis of Substrates

***p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranoside (2):** Compound **2** was prepared by oxidation catalysed by the galactose oxidase from *Dactylium dendroides* in a batch reactor with bubble-free aeration from the starting material **1**. After lyophilisation, the reaction mixture was dissolved in water (30 mL), centrifuged (Beckman Model JS-21 centrifuge, Beckman Coulter, Inc., U. S. A.; 15,000 rpm, 15 min) and the supernatant was purified by column chromatography on Dowex 50W-X8 (Sigma) in Ca^{2+} cycle with water as a mobile phase and a flow rate of 60 mL/h. The pure compound **2** was obtained as a white solid; yield: 124 mg (0.364 mmol; 89%); $[\alpha]_D^{20}$: −138.7° (c 0.21, water). According to NMR, compound **2** is a mixture of the geminal diol and the free aldehyde (diol/aldehyde = 4.26).

Free aldehyde **2**: ^{1}H NMR (DMSO-d_6): δ = 1.808 (s, 3H, 2-Ac), 3.722 (ddd, *J* = 3.4, 6.1, 10.4 Hz, 1H, H-3), 4.064 (ddd, *J* = 8.5, 8.9, 10.4 Hz, 1H, H-2), 4.163 (ddd, *J* = 1.4, 3.4, 4.4 Hz, 1H, H-4), 4.419 (dd, *J* = 0.7, 1.4 Hz, 1H, H-5), 5.040 (d, *J* = 6.1 Hz, 1H, 3-OH), 5.147 (dd, *J* = 0.7, 4.4 Hz, 1H, 4-OH), 5.318 (d, *J* = 8.5 Hz, 1H, H-1), 7.244 (m, 2H, H-*ortho*), 7.825 (d, *J* = 8.9 Hz, 1H, 2-NH), 8.208 (m, 2H, H-*meta*), 9.500 (s, 1H, H-6); ^{13}C NMR (DMSO-d_6): δ = 23.0 (2-Ac), 51.5 (C-2), 68.1 (C-4), 70.1 (C-3), 79.5 (C-5), 98.2 (C-1), 116.7 (C-*ortho*), 125.6 (C-*meta*), 141.9 (C-*para*), 162.1 (C-*ipso*), 169.7 (2-CO), 199.7 (C-6).

Geminal diol **2**: ^{1}H NMR (DMSO-d_6): δ = 1.802 (s, 3H, 2-Ac), 3.274 (dd, *J* = 0.2, 6.6 Hz, 1H, H-5), 3.568 (ddd, *J* = 3.1, 6.1, 10.4 Hz, 1H, H-3), 3.912 (ddd, *J* = 0.2, 3.1, 4.2 Hz, 1H, H-4), 4.033 (ddd, *J* = 8.5, 9.0, 10.4 Hz, 1H, H-2), 4.705 (d, *J* = 4.2 Hz, 1H, 4-OH), 4.801 (d, *J* = 6.1 Hz, 1H, 3-OH), 4.899 (ddd, *J* = 6.3, 6.6, 7.3 Hz, 1H, H-6), 5.073 (d, *J* = 8.5 Hz, 1H, H-1), 5.841 (d, *J* = 7.3 Hz, 1H, 6-OH), 5.962 (d, *J* = 6.3 Hz, 1H, 6-OH), 7.200 (m, 2H, H-*ortho*), 7.655 (d, *J* = 9.0 Hz, 1H, 2-NH), 8.178 (m, 2H, H-*meta*); ^{13}C NMR (DMSO-d_6): δ = 23.0 (2-Ac), 51.7 (C-2), 67.4 (C-4), 71.1 (C-3), 77.8 (C-5), 88.2 (C-6), 99.1(C-1), 116.7 (C-*ortho*), 125.6 (C-*meta*), 141.7 (C-*para*), 162.5 (C-*ipso*), 169.6 (2-CO); MS (MALDI-TOF): *m/z* = 363 $[M+Na]^+$, 341 $[M+H]^+$, 323.

***p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid (3):** The starting material **2** (112 mg, 0.329 mmol) was suspended in water (41 mL) under stirring at 0 °C and $NaClO_2$ (82 mg, 0.907 mmol) was added in several portions during 1 h. The reaction was monitored by TLC (AcOEt:MeOH:H_2O, 7:3:0.5) and HPLC. It was stopped after 7 h by evaporation under vacuum to dryness. The crude compound **3** was purified by column chromatography on Merck silica gel 60 (40–63 μm) (AcOEt:MeOH:H_2O, 7:3:1) affording compound **3** as a yellowish honey; yield: 97 mg (0.272 mmol, 83%); $[\alpha]_D^{20}$: −11.9° (*c* 0.30, water). ^{1}H NMR (D_2O): δ = 1.788 (s, 3H, 2-Ac), 3.700 (dd, *J* = 3.4, 10.9 Hz, 1H, H-3), 4.009 (dd, *J* = 8.4, 10.9 Hz, 1H, H-2), 4.042 (d, *J* = 1.3 Hz, 1H, H-5), 4.096 (dd, *J* = 1.3, 3.4 Hz, 1H, H-4), 4.996 (d, *J* = 8.4 Hz, 1H, H-1), 6.976 (m, 2H, H-*ortho*), 8.008 (m, 2H, H-*meta*); ^{13}C NMR (D_2O): δ = 22.3 (2-Ac), 52.1 (C-2), 69.5 (C-4), 70.9 (C-3), 75.7 (C-5), 99.2 (C-1), 116.9 (2 × C-*ortho*), 126.3 (2 × C-*meta*), 142.8 (C-*para*), 162.4 (C-*ipso*), 174.6 (C-6), 175.4 (2-CO); MS (ESI): *m/z* = 401 $[M+2\ Na]^+$, 379 $[M+Na]^+$, 357 $[M+H]^+$.

Methyl (*p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosid)uronate (4): Compound **3** (45 mg, 0.126 mmol) was dissolved in dry MeOH (25 mL) and stirred with Dowex 50W-X2 in H^+ cycle (0.5 mL of the suspension) for 15 min. Dowex was then filtered off and the reaction mixture was concentrated to a volume of 10 mL. 1 M CH_2N_2 in dry MeOH (approximately 2 mL) was added dropwise until the reaction reached equilibrium (did not colour off any more). TLC (AcOEt:MeOH:H_2O, 7: 3: 1) confirmed complete conversion. Immediately, the reaction mixture was evaporated under vacuum to dryness and the crude compound **4** was purified by column chromatography on silica gel (gradient of CH_2Cl_2:MeOH 12:1 to 6:1). Pure compound **4** was obtained as a white solid; yield: 19 mg (0.051 mmol, 41%); $[\alpha]_D^{20}$: −36.5° (*c* 0.20, water). ^{1}H NMR (CD_3OD): δ = 1.993 (s, 3H, Ac), 3.808 (s, 3H, OMe), 3.905 (dd, *J* = 10.8, 3.3 Hz, 1H, H-3), 4.248 (dd, *J* = 10.8, 8.5 Hz, 1H, H-2), 4.269 (dd, *J* = 3.3, 1.4 Hz, 1H, H-4), 4.574 (d, *J* = 1.4 Hz, 1H, H-5), 5.312 (d, *J* = 8.5 Hz, 1H, H-1), 7.225 and 8.222 (AA′BB′, Σ*J* = 9.3 Hz, 4H, *p*NP); ^{13}C NMR (CD_3OD), HMQC and HMBC readouts: δ = 23.1 (Ac), 52.8 (OMe), 53.7 (C-2), 70.7 (C-4), 71.9 (C-3), 75.8 (C-5), 99.9 (C-

1), 117.8 (2 × C-*ortho*), 126.7 (2 × C-*meta*), 144.2 (C-*para*), 163.7 (C-*ipso*), 170.2 (C-6), 174.3 (2-CO); MS (ESI): $m/z =$ 393 $[M+Na]^+$.

2-Acetamido-2-deoxy-β-D-galactopyranosyluronic acid-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (6): Substrate **2** (36 mg, 0.106 mmol) and 2-acetamido-2-deoxy-D-glucopyranose (**5**; 234 mg, 1.058 mmol) were dissolved in citrate/phosphate buffer pH 5.0 (3528 μL). The β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686 (6.5 U) was added and the mixture was shaken at 37 °C. After 4.5 h the reaction was stopped by heating to 100 °C for 2 min. The reaction mixture was cooled to room temperature and $NaClO_2$ (30 mg, 0.332 mmol) was added in three portions. After the oxidation was complete (TLC; CH_2Cl_2:MeOH:EtOH:H_2O, 6:3.5:1:1.2) the reaction mixture was centrifuged (14,500 rpm, 10 min), concentrated under vacuum and loaded onto a Bio Gel P2 (BioRad, U. S. A.) column (mobile phase water, flow rate 15.4 mL/h). The disaccharide **6** was obtained as a white solid; yield: 17.1 mg (0.039 mmol, 37% referred to donor **2**); $[\alpha]_D^{20}$: +7.2° (*c* 0.25, water). According to NMR, compound **6** was a mixture of two anomers (α/β = 1.42).

α-Anomer of **6**: ^{1}H NMR (D_2O): δ = 1.817 (s, 3H, 2-Ac), 1.845 (s, 3H, 2′-Ac), 3.366 (dd, J = 8.3, 10.0 Hz, 1H, H-4), 3.437 (dd, J = 4.9, 12.1 Hz, 1H, H-6u), 3.557 (dd, J = 2.2, 12.1 Hz, 1H, H-6d), 3.583 (dd, J = 10.9, 3.5 Hz, 1H, H-3′), 3.623 (dd, J = 3.5, 10.9 Hz, 1H, H-2), 3.673 (ddd, J = 10.0, 2.2, 4.9 Hz, 1H, H-5), 3.728 (dd, J = 8.4, 10.9 Hz, 1H, H-2′), 3.733 (dd, J = 10.9, 8.3 Hz, 1H, H-3), 3.861 (d, J = 1.3 Hz, 1H, H-5′), 4.024 (dd, J = 3.5, 1.3 Hz, 1H, H-4′), 4.285 (d, J = 8.4 Hz, 1H, H-1′), 4.985 (d, J = 3.5 Hz, 1H, H-1); ^{13}C NMR (D_2O): δ = 22.12 (2-Ac), 22.42 (2′-Ac), 52.43 (C-2′), 53.87 (C-2), 60.53 (C-6), 69.33 (C-4′), 69.53 (C-3), 70.11 (C-5), 71.06 (C-3′), 75.47 (C-5′), 80.63 (C-4), 90.54 (C-1), 101.72 (C-1′), 174.32 (C-6′), 174.68 (2-CO), 174.99 (2′-CO).

β-Anomer of **6**: ^{1}H NMR (D_2O): δ = 1.845 (s, 6H, 2-Ac, 2′-Ac), 3.311 (ddd, J = 9.7, 1.9, 5.1 Hz, 1H, H-5), 3.361 (dd, J = 7.5, 9.7 Hz, 1H, H-4), 3.418 (dd, J = 5.1, 12.1 Hz, 1H, H-6u), 3.471 (dd, J = 8.1, 10.5 Hz, 1H, H-2), 3.534 (dd, J = 10.5, 7.5 Hz, 1H, H-3), 3.574 (dd, J = 10.9, 3.6 Hz, 1H, H-3′), 3.597 (ddd, J = 1.9, 12.1 Hz, 1H, H-α), 3.716 (dd, J = 8.4, 10.9 Hz, 1H, H-2′), 3.853 (d, J = 1.3 Hz, 1H, H-5′), 4.021 (dd, J = 3.6, 1.3 Hz, 1H, H-4′), 4.280 (d, J = 8.4 Hz, 1H, H-1′), 4.482 (d, J = 8.1 Hz, 1H, H-1); ^{13}C NMR (D_2O): δ = 22.42 (2-Ac, 2′-Ac), 52.43 (C-2′), 56.24 (C-2), 60.41 (C-6), 69.33 (C-4′), 71.06 (C-3′), 72.86 (C-3), 74.79 (C-5), 75.47 (C-5′), 80.18 (C-4), 95.13 (C-1), 101.72 (C-1′), 174.32 (C-6′), 174.99 (2-CO, 2′-CO); MS (ESI): m/z = 483 $[M+2\,Na]^+$, 461 $[M+Na]^+$.

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