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# Synthesis of Sulfated Glucosaminides for Profiling Substrate Specificities of Sulfatases and Fungal β-*N*-Acetylhexosaminidases

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*Sulfated carbohydrates are components of many glycoconjugates, and are degraded by two major processes: cleavage of the sulfate ester by a sulfatase, or en bloc removal of a sulfated monosaccharide by a glycoside hydrolase. However, these processes have proved difficult to study owing to a lack of homogeneous, defined substrates. We describe here the synthesis of a series of* p*-nitrophenyl β-*D*-glucosaminides bearing sulfate esters at the 2-, 3-, 4- or 6-positions, by divergent routes starting with* p*-nitrophenyl 2-acetamido-2-deoxy-β-*D*-glucopyranoside. The sulfated* p*-nitrophenyl β-*D*-glucosaminides were used to study the substrate specificity of four sulfatases (from* Helix pomatia, Patella vulgata*, abalone, and* Pseudomonas aeruginosa*), and revealed significant differences in the preference of each of these enzymes for desulfation at different positions around the sugar ring. The 3-, 4- and 6-sulfated* p*-nitrophenyl 2-acetamido-2-deoxy-β-*D*-glucosaminides were screened against a panel of 24 fungal β-*N*-acetylhexosaminidases to assess their substrate specificity. While the 4- and 6-sulfates were substrates for many of the fungal enzymes investigated, only a single β-*N*-acetylhexosaminidase, that from* Penicillium chrysogenum*, could hydrolyze the 3-sulfated* p*-nitrophenyl glycoside. Together these results demonstrate the utility of sulfated* p*-nitrophenyl β-*D*-glucosaminides for the study of both sulfatases and glycoside hydrolases.*

## Introduction

Sulfated carbohydrates and glycoconjugates are critical mediators of functions as diverse as providing support to cells and the extracellular matrix,[1] spermatogenesis,[2] osmoprotection of halophilic bacteria,[3] and modulation of interspecies interactions.[4] For example, in response to alfalfa-derived flavonoid signals, the bacterial nitrogen-fixing symbiont *Sinorhizobium meliloti* releases Nod factors including a sulfated chitotetraoside, and this results in the formation of root nodules that are colonized by the bacterium.[5] The presence of a single sulfate group on the *S. meliloti* Nod factor determines the host specificity of the bacterium, and mutant bacteria unable to install the sulfate group lose the ability to induce nodulation in alfalfa but gain the ability to nodulate and colonize a new plant host, vetch.[6] Similarly, a critical role for sulfated carbohydrates has been determined for the process of rolling leukocyte adhesion, an early stage in the inflammatory response. The initial rolling event is mediated by binding of carbohydrate structures to an adhesion protein, L-selectin, which is common to all leukocytes. A preferred ligand for L-selectin is GlyCAM-1, which is expressed on high endothelial venules in response to the inflammatory insult.[7] The expression of a family of sulfated sialyl Lewis$^x$ structures (including 6-*O*-sulfated sialyl Lewis$^x$) on GlyCAM-1 is a prerequisite for L-selectin binding.[8]

Sulfated carbohydrates are degraded through two main processes. The first is the sulfatase-catalyzed removal of the sulfate group from the glycoconjugate, which may be followed by other enzymatic steps to complete the breakdown. For example, in higher organisms, most carbohydrate sulfatases are lysosomal residents and are responsible for the tightly orchestrated degradation of sulfated glycoproteins, glycosaminoglycans and glycolipids.[9] Most of these enzymes catalyze the hydrolysis of sulfate esters off the nonreducing terminus sugar, thus allowing the engagement of *exo*-acting glycoside hydrolases, which remove the desulfated terminal sugar residue. The second pathway for the degradation of sulfated glycoconjugates is through the cleavage of the sulfated carbohydrate by a glycosidase or lyase prior to removal of the sulfate ester. For example, the lysosomal glycoside hydrolase β-*N*-acetylhexosaminidase A can cleave GlcNAc-6-sulfate directly from the nonreducing terminus of a keratan sulfate chain.[10]

The characterization of the substrate specificities of both carbohydrate sulfatases and glycoside hydrolases that act on sulfated carbohydrates is problematic owing to the difficulty of acquiring suitable well-defined substrates for their study and, in the case of carbohydrate sulfatases, because of the lack of a group that can be detected by spectroscopic means following the cleavage reaction. Sulfated fluorogenic glycosides may be used in coupled assays to overcome this shortcoming.[11,12]

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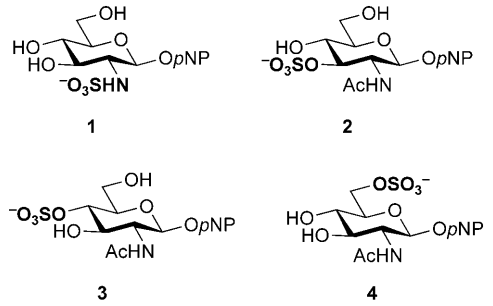
Thus, the sulfated glycoside acts as a substrate for the sulfatase of interest releasing the desulfated fluorogenic glycoside, which is then a substrate for a glycoside hydrolase, the action of which releases the fluorophore, thereby permitting the easy detection of sulfatase activity. A similar approach using sulfated chromogenic glycosides has been reported.[13,14] These approaches have allowed the monitoring of sulfatase activity in several systems, but the limited number of suitable substrates prevents its more widespread use.

In this work we report the synthesis of a series of *p*-nitrophenyl β-D-glucosaminides—**1**, **2**, **3** and **4**—sulfated at the 2-, 3-, 4- and 6-positions, respectively (Scheme 1), and their utility



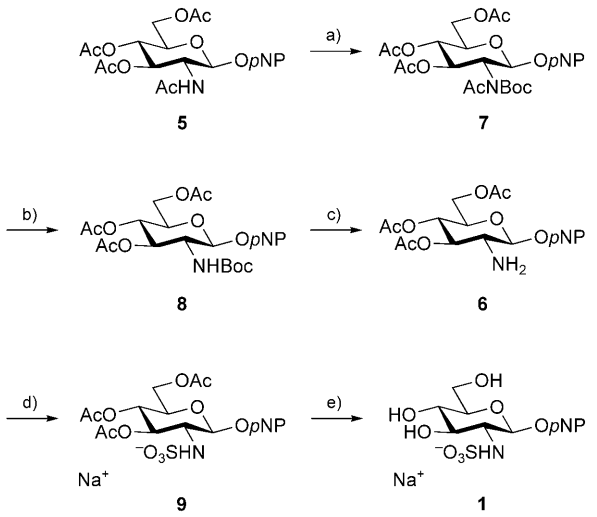

**Scheme 1.** Sulfated *p*-nitrophenyl 2-deoxy-β-D-glucosaminides.

in the study of substrate specificity of four sulfatases: three molluscan sulfatases from abalone, limpet and snail, and a bacterial sulfatase *Pa*AtsA from *Pseudomonas aeruginosa*. Additionally, we report the screening of **2**, **3** and **4** as substrates against a set of 24 fungal β-*N*-acetylhexosaminidases. These studies are the first to provide a comprehensive characterization of a range of sulfatases and β-*N*-acetylhexosaminidases against a complete set of regioselectively sulfated monosaccharides.

## Results and Discussion

### Synthesis of sulfated substrates

Each of the target substrates, the *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide sulfates **1**, **2**, **3** and **4**, were envisaged to be accessible from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide triacetate **5**. The first target, the 2-*N*-sulfate sodium salt **1**, was approached via the 2-amino derivative, *p*-nitrophenyl 3,4,6-tri-*O*-acetyl-β-D-glucosaminide **6** (Scheme 2). Treatment of **5** with $Boc_2O$ and catalytic DMAP gave the mixed imide **7** in quantitative yield. The *N*-acetyl group from the mixed imide **7** was cleaved by treatment with hydrazine hydrate (10 equiv) in MeOH. Whilst these conditions successfully removed the *N*-acetyl group of the mixed imide, concomitant de-*O*-acetylation was observed. This proved to be of little consequence as *O*-acetylation was easily achieved with acetic anhydride and pyridine to afford the acetylated carbamate **8**. The acid-labile *N*-Boc group of **8** was removed by treatment with neat trifluoroacetic acid to afford amine **6** in excellent yield (94% from **5**).

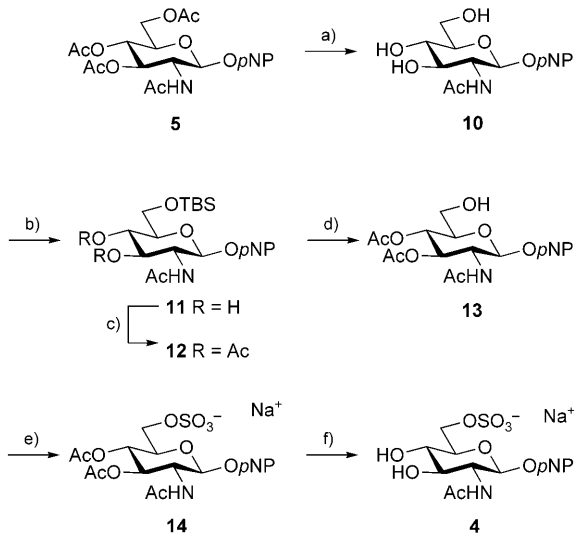



**Scheme 2.** a) $Boc_2O$, DMAP, THF, reflux, quant.; b) i: $N_2H_4{\cdot}H_2O$, MeOH, 40 °C; ii: $Ac_2O$, pyr., 94% from **5**; c) TFA, 30 min, 94%; d) $SO_3{\cdot}NMe_3$, pyr.; e) NaOMe, MeOH, 40% over two steps.

Numerous approaches have been used for the sulfation of amino sugars and related alcohols. While sulfation is relatively easy to achieve with complexes of sulfur trioxide and tertiary amines (for example, $SO_3{\cdot}$pyr or $SO_3{\cdot}Me_3N$) in pyridine, the purification of such products can be challenging, as traces of residual pyridine can catalyze the hydrolysis of the sulfate ester in the product; moreover, the resultant pyridinium salts are not particularly stable.[15] Thus, it is crucial to quantitatively remove residual pyridine and to rapidly convert the isolated pyridinium salt to a more stable alkali metal salt. Therefore, following sulfation of **6** with $SO_3{\cdot}Me_3N$ in anhydrous pyridine (Scheme 2), the majority of the solvent was evaporated. Residual pyridine was removed by size-exclusion chromatography (Sephadex LH-20) with MeOH/$H_2O$ (1:1) as eluent. Finally, the pyridinium counterion was exchanged for sodium by cation exchange (Dowex 400×8, $Na^{+}$ form) to afford the acetylated *N*-sulfate sodium salt **9**. Deacetylation of **9** with catalytic NaOMe in MeOH and purification by using the same procedure as described for the preparation of **9** (size-exclusion chromatography followed by cation exchange) gave the 2-sulfate **1** as the sodium salt.

The 6-sulfate **4** has previously been prepared by direct sulfation of the triol **10**,[13,16] or by sulfation of a selectively acetylated alcohol, followed by deprotection.[14] Here, we adopted the latter approach. Thus, **5** was deacetylated by using NaOMe in MeOH to afford **10** in quantitative yield (Scheme 3). Protection of the primary hydroxyl of **10** by silylation with *tert*-butylchlorodimethylsilane and imidazole in DMF afforded diol **11**. Acetylation of **11** under standard conditions gave **12**, which was subsequently treated with hydrogen fluoride–pyridine complex to give the 6-hydroxy derivative **13** in good yield (75%). Initially, we attempted sulfation of **13** using $SO_3{\cdot}Me_3N$ in pyridine;[14] in our hands $SO_3{\cdot}$pyr in pyridine/DMF gave better results. Purification was achieved by evaporation of the solvent and sequential size-exclusion and cation-exchange chromatographies of the residue to provide **14** as the sodium salt. Deacetylation

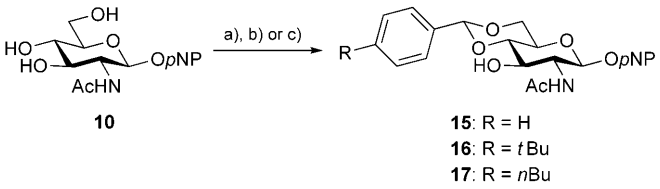
  




**Scheme 3.** a) NaOMe, MeOH, 40 °C, quant.; b) TBSCl, imidazole, DMF, 58%; c) $Ac_2O$, pyr., 86%; d) HF-pyr., THF, 0 °C→RT, 75%; e) $SO_3$·pyr., DMF, pyr., 50 °C; f) NaOMe, MeOH, 40% over two steps.

of **14** with NaOMe in MeOH furnished the 6-sulfate **4**, which was purified as before.

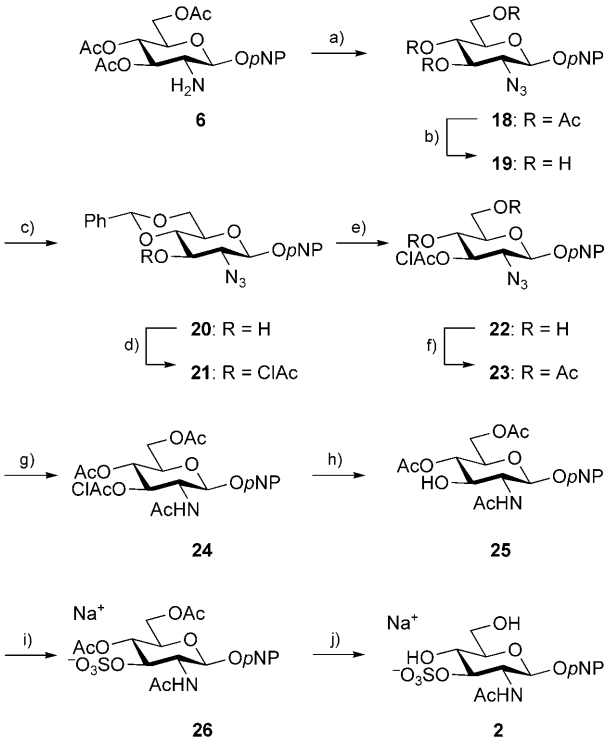
Our initial approach to the synthesis of the 3- and 4-sulfates **2**[17] and **3**[17] targeted the 4,6-benzylidene acetal **15** as a selectively protected intermediate (Scheme 4). However, attempts




**Scheme 4.** a) $PhCH(OMe)_2$, TsOH, MeCN; b) 4-*tert*-butyl benzaldehyde dimethyl acetal, TsOH, MeCN, 38%; c) 4-*n*-butyl benzaldehyde diethyl acetal, TsOH, MeCN.

to prepare **15** from **10** by treatment with benzaldehyde dimethyl acetal and catalytic TsOH in acetonitrile or chloroform foundered owing to the exceptionally poor solubility profile of **15** in these and a range of other organic solvents. The poor solubility of **15**, which we attribute to its highly symmetrical structure and the potential for hydrogen bonding to the 2-acetamido group, has been noted by others.[18] Attempts to introduce potential solubilizing groups onto the benzylidene acetal, through the preparation of the *tert*-butyl derivative **16** and the *n*-butyl derivative **17**, led to only marginal improvements in solubility, and so an alternative approach was required.

Azido groups are unable to participate in hydrogen bonding interactions, and so replacement of the 2-acetamido group of the substrate was seen as a potential method for improving the solubility of key intermediates. Diazo transfer with $TfN_3$ is a widely used method for this transformation and is usually performed on deprotected sugars. We were therefore delighted that treatment of the protected amine **6** with $TfN_3$ and $NEt_3$ in the presence of catalytic $CuSO_4$[19] gave the azide **18** in excellent yield (93%; Scheme 5). Next, **18** was deacetylated with




**Scheme 5.** a) $TfN_3$, $CuSO_4$, $NEt_3$, $CH_2Cl_2$/MeOH (9:1), 93%; b) NaOMe, MeOH, 91%; c) $PhCH(OMe)_2$, TsOH, $CHCl_3$, 87%; d) $(ClAc)_2O$, 2,6-lutidine, DMAP, $CH_2Cl_2$, 98%; e) aq. TFA (70%), $CH_2Cl_2$, 0 °C→RT; f) $Ac_2O$, 2,6-lutidine, DMAP, $CH_2Cl_2$, 91% over two steps; g) $PPh_3$, AcCl, $CH_2Cl_2$, 64%; h) thiourea, MeOH, 61%; i) $SO_3$·pyr, DMF, pyr., 50 °C; j) NaOMe, MeOH, 44% over two steps.

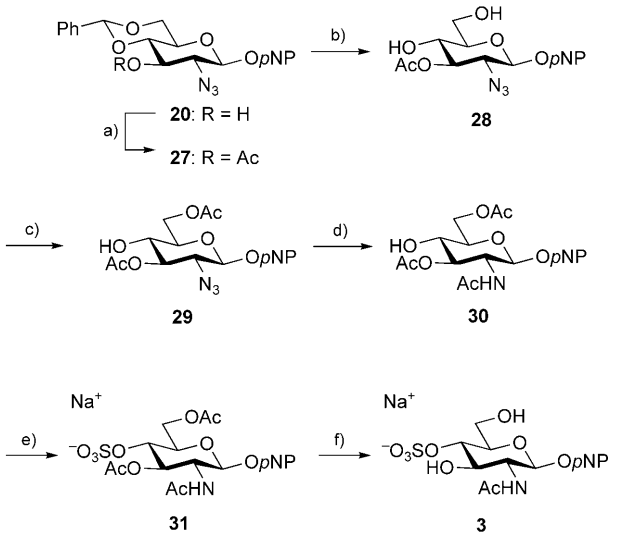
NaOMe in MeOH to afford the triol **19**. Reaction of **19** and benzaldehyde dimethyl acetal in the presence of catalytic TsOH in $CHCl_3$[20] gave the benzylidene derivative **20** in excellent yield (87%). In striking contrast to the GlcNAc derivatives **15**, **16** and **17**, the azide-containing benzylidene derivative **20** possessed a vastly improved solubility and was freely soluble in a wide range of organic solvents. As the only difference between **15** and **20** was the presence or absence of the 2-acetamido group, it can safely be concluded that the 2-acetamido group was the cause of the poor solubility of the GlcNAc derivatives seen earlier.

For the synthesis of the 3-sulfate **2**, the benzylidene derivative **20** was treated with chloroacetic anhydride, DMAP, and 2,6-lutidine to provide the chloroacetate **21** in quantitative yield (Scheme 5). Hydrolysis of the benzylidene acetal of **21** with aqueous trifluoroacetic acid (TFA; 70%) in $CH_2Cl_2$ gave the diol **22**. Acetylation of **22** with $Ac_2O$, 2,6-lutidine and DMAP afforded the protected derivative **23**. We next needed to convert the azide of **23** to an acetamide; however, while reduction of azides to amines can be readily achieved by nucleophilic reducing reagents ($LiAlH_4$, $NaBH_4$) or by catalytic hydrogenation, neither of these methods was compatible with the ester and nitro groups of **23**. Instead, we applied the direct

Staudinger reduction/acylation protocol of Vilarassa and co-workers, which allows the direct conversion of an azide to an amide without the intermediacy of an amine.[21] Depending on the order of addition of reagents, this process proceeds via either an iminophosphorane or a triazaphosphadiene/acyl chloride adduct.[22] Accordingly, **23** was subjected to $PPh_3$ and AcCl to afford the acetamide **24** in a 64% yield. Treatment of **24** with thiourea in MeOH afforded the 3-hydroxy derivative **25**, which was sulfated by using $SO_3$·pyr in DMF/pyridine to give the acetylated 3-sulfate **26**. Finally, the NaOMe-catalyzed deacetylation of **26** and purification as described above afforded the 3-sulfate **2**.

For the preparation of the final target, the 4-sulfate **3**, the benzylidene acetal **20** was acetylated with $Ac_2O$/pyridine to give the monoacetate **27** (Scheme 6). Hydrolysis of the benzylidene acetal of **27** with 70% aqueous TFA in $CH_2Cl_2$ afforded the diol **28**. Monoacetylation of **28** to give alcohol **29** was achieved with AcCl and 2,4,6-collidine at −40 °C in good yield (64%). Given the success of the modified Staudinger conditions employed for the reduction/acetylation of the chloroacetyl derivative **23**, the same protocol was applied to the alcohol **29**, which possesses a potentially reactive hydroxy group. Encouragingly, upon treatment of **29** with AcCl and $PPh_3$, the acetamide **30** was obtained in reasonable yield (54%). To complete the synthesis, acetamide **30** was sulfated with $SO_3$·pyr in DMF/pyridine, the solvent was evaporated, and the triacetate **31** was purified as before (size-exclusion chromatography followed by cation exchange to the sodium salt). Deacetylation of the triacetate **31** with catalytic NaOMe in MeOH and purification as described above gave the 4-sulfate **3**.




**Scheme 6.** a) $Ac_2O$, pyr., 86%; b) aq. TFA (70%), $CH_2Cl_2$, 76%; c) AcCl, 2,4,6-collidine, −40 °C, 64%; d) $PPh_3$, AcCl, $CH_2Cl_2$, 54%; e) i: $SO_3$·pyr, DMF, pyr., 50 °C; ii: Sephadex LH-20 and Dowex 400×8 $Na^+$ chromatography; f) NaOMe, MeOH, 39% over two steps.

### Examination of sulfated carbohydrates as sulfatase substrates

Sulfatases catalyze the hydrolysis of sulfate esters, resulting in the liberation of inorganic sulfate and the parent alcohol or amine. Detection of sulfatase activity can be achieved by monitoring the consumption of the sulfated substrate or the production of either of the products, inorganic sulfate or the desulfated alcohol (or amine). van Diggelen and co-workers used 4-methylumbelliferyl β-D-galactoside 6-sulfate as a substrate for a coupled assay to detect carbohydrate sulfatase activity; this enabled the study of MPS IVA cells, which lack *N*-acetylgalactosamine-6-sulfate sulfatase.[12] Incubation of this sulfated carbohydrate with the sulfatase resulted in desulfation and the formation of the 4-methylumbelliferyl β-D-galactoside, which was a substrate for endogenous galactosidase, resulting in the release of fluorogenic 4-methylumbelliferone. A similar assay was developed by Clinch et al. for the detection of bacterial carbohydrate sulfatases using chromogenic *p*-nitrophenol glycosides but using an exogenous glycoside hydrolase.[14]

The sulfatases from snail (*Helix pomatia*), limpet (*Patella vulgata*) and abalone (unspecified species) are commercially available, are widely used in analytical applications, and have been utilized for the preparation of monosulfated carbohydrates through the desulfation of readily accessible di- or trisulfated precursors.[23–25] For these reasons they were chosen for study. As the bacterium *Pseudomonas aeruginosa* has been observed to exhibit carbohydrate sulfatase activity,[26] arylsulfatase A from this organism (*Pa*AtsA) was also included.

As a coupling enzyme for these studies, we investigated the use of the β-*N*-acetylhexosaminidase *Ao*Hex (Sigma) from *Aspergillus oryzae*. *Ao*Hex belongs to glycoside hydrolase family 20,[27] and is a retaining enzyme that utilizes substrate-assisted catalysis by the 2-acetamido group on the substrate, which acts as a nucleophile to effect hydrolysis.[28] The product of desulfation of **1** is a 2-amino sugar that lacks the neighboring amide group and is not expected to be a substrate for this enzyme, and so it was excluded from these initial studies. No catalytic activity was seen towards the hydrolysis of the 3- and 4-sulfates, **2** and **3**, whereas the 6-sulfate was hydrolyzed to a limited degree. The kinetic parameters, $V_{max}$ and $K_m$, for *Ao*Hex catalysis were determined for the 6-sulfate **4** (Table 1). Compared to *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **10**, the 6-sulfate **4** was a 9400-fold poorer substrate in terms of the second order rate constant $V_{max}/K_m$. Thus, *Ao*Hex was deemed to have sufficient selectivity for use with **2**, **3** and **4** in the coupled assay.

**Table 1.** Kinetic constants for the *A. oryzae* β-*N*-acetylhexosaminidases with *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **10** and the 6-sulfate **4**.[a]

| Substrate | $V_{max}$ [mmol min$^{-1}$ mL$^{-1}$] | $K_m$ [mM] | $V_{max}/K_m$ |
|---|---|---|---|
| *p*-nitrophenyl *N*-acetyl-β-D-glucosamine **10** | 56 ± 1 | 0.70 ± 0.05 | 80 |
| 6-sulfate **4** | 0.102 ± 0.006 | 12 ± 2 | 0.0085 |

[a] Conditions: 50 mM $K_2HPO_4/KH_2PO_4$/0.05% BSA buffer, at pH 6.

Initially, the assay was optimized for the activity of the sulfatase and the coupling enzyme, *Ao*Hex. The best activity for the molluscan sulfatases using the artificial substrate *p*-nitrophenyl sulfate was found between pH 4 and 6, conditions under which the liberated *p*-nitrophenol ($pK_a = 7.2$) would be protonated and hence spectroscopically inactive. In order to improve the sensitivity of the assay, we therefore investigated the inclusion of α-cyclodextrin in the assay mixtures. An interesting feature of cyclodextrin complexes with various phenols is their ability to promote their ionization through lowering their $pK_a$ value.[29] Indeed, upon addition of 10 mM α-cyclodextrin to a solution of *p*-nitrophenol at pH 6, the extinction coefficient ($\varepsilon$) of *p*-nitrophenol increases fourfold, thus allowing more sensitive detection at this lower pH.

Armed with a method to enhance the spectroscopic properties of the liberated *p*-nitrophenol at low pH values, attention turned to the study of the sulfated derivatives as substrates by using the coupled assay. Unfortunately, the sulfatases from *P. vulgata* and abalone entrails were not stable at pH 6 for sufficient time to accurately determine linear rates under steady-state conditions in the coupled assay (6 h). Table 2 shows the relative rates of hydrolysis for **2**, **3** and **4** by the sulfatases from snail and *P. aeruginosa*, corrected to activity per unit of enzyme and for contaminating β-*N*-acetylhexosaminidase activity present in the sulfatase samples. As a point of reference, the activity seen towards the artificial, highly reactive sulfatase substrate, *p*-nitrophenyl sulfate is also included.

**Table 2.** Hydrolysis of carbohydrate sulfates by sulfatases [%].

| Substrate | *Pa*AtsA | Snail | Limpet[a] | Abalone[a] |
|---|---|---|---|---|
| *p*-nitrophenyl sulfate | 100 | 100 | 100 | 100 |
| 2-sulfate **1**[a] | < 0.01 | < 0.01 | < 0.01 | 0.3 |
| 3-sulfate **2** | 0.06 | 0.02[b] | 0.3 | 0.5 |
| 4-sulfate **3** | < 0.01 | 0.03[b] | < 0.01 | 0.4 |
| 6-sulfate **4** | < 0.01 | < 0.01 | < 0.01 | 0.2 |

[a] Turbidimetric assay. [b] Not corrected for contaminating β-*N*-acetylhexosaminidase activity.

Due to the instability of the *P. vulgata* and abalone enzymes under the assay conditions and the inability of *Ao*Hex to hydrolyze the 2-amino sugar formed from desulfation of **1**, we utilized a stopped turbidimetric assay that allows quantitation of the released sulfate through detection of the precipitated $BaSO_4$ formed upon addition of $BaCl_2$.[30] Table 2 shows the corrected carbohydrate sulfatase activity of the *P. vulgata* sulfatase towards **1**, **2**, **3**, and **4**, and of the three other sulfatases towards **1**.

Table 2 reveals that the 2-sulfate **1**, 4-sulfate **3** and 6-sulfate **4** were not substrates for the *P. aeruginosa* or *P. vulgata* sulfatases, whereas the 3-sulfate **2** was, albeit poor relative to *p*-nitrophenyl sulfate. Different results were found for the snail sulfatase, with no activity seen towards 2-sulfate **1** or 6-sulfate **4**, whereas the 3- and 4-sulfates **2** and **3** were hydrolyzed by this sulfatase. Finally, the sulfatase from abalone possessed the broadest specificity and was able to hydrolyze all of the carbohydrate sulfate esters, with the 3- and 4-sulfates being the best substrates (0.5 and 0.4% relative to *p*-nitrophenyl sulfate, respectively). More detailed kinetic analysis could not be performed owing to the limited amounts of the substrates available.

These results are reminiscent of those of Uzawa and coworkers who found that, of the same three molluscan sulfatases, only the abalone sulfatase could hydrolyze the 2-sulfate ester of *p*-nitrophenyl α-D-glucopyranoside.[25] Moreover, studies on sulfated D-*galacto*-sugars have revealed that the abalone sulfatase can hydrolyze a wider range of sulfated sugars than the other two molluscan sulfatases.[24] Finally, Uzawa observed that the sulfatase from *P. vulgata* was capable of hydrolyzing only the 3-sulfate ester of D-*galacto*-configured sugars.[24] We also observed that this sulfatase only acted upon the 3-sulfate **2**. It is noteworthy that the GlcNAc-3-sulfate residue is present within the high-affinity antithrombin III-binding pentasaccharide, and thus this enzyme might be of use in structural characterization of sulfated glycosaminoglycans.[31]

### Substrate profiling of a panel of 24 fungal β-*N*-acetylhexosaminidases

As discussed in the Introduction, certain glycosidases can tolerate the presence of sulfate esters on the substrate. Indeed, this substrate tolerance has allowed the exploitation of glycosidases as synthetic catalysts for the preparation of sulfated disaccharides by transglycosidation.[16,32] In order to gauge the possibility of using hexosaminidases as transglycosylation catalysts for the preparation of glycoconjugates utilizing the 2-, 3-, 4- and 6-sulfates **1**, **2**, **3** and **4** as glycosyl donors, we investigated the ability of a library of 24 fungal hexosaminidases to hydrolyze these compounds, relative to the desulfated substrate, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **10**. Solutions of each of the sulfated carbohydrates and the fungal β-*N*-acetylhexosaminidases were incubated at 35 °C and pH 5, and the extent of hydrolysis was quantitated by spectroscopic detection of liberated *p*-nitrophenol. Table 3 reveals that the ability of the *O*-sulfated substrates **2**–**4** to be hydrolyzed by the fungal β-*N*-acetylhexosaminidases varied widely and was dependent upon both the source of the enzyme and the position of sulfation. 2-Sulfate **1** was not cleaved by any of the hexosaminidases tested. This is in accordance with previous observations concerning the tolerance of these hexosaminidases to charged moieties at the 2-amino group and the requirements of substrate-assisted catalysis in the enzymatic mechanism.[33] The 6-sulfate **4** was a good substrate for the β-*N*-acetylhexosaminidases from *Fusarium*, *Penicillium*, *Talaromyces* and *Trichoderma*. Thus, the 6-sulfate **4** would be suitable for transglycosidations catalyzed by β-*N*-acetylhexosaminidases of *Talaromyces* genera, and likely much more so than that from *Aspergillus oryzae*, as published previously.[16] The 4-sulfate **3** was a substrate for the majority of β-*N*-acetylhexosaminidases, although hydrolysis of this compound was usually limited to < 10%. On the other hand, the 3-sulfate **2** was generally a poor substrate for all the enzymes investigated, except for that from *Penicillium chrysogenum*.

**Table 3.** Hydrolysis of the sulfated derivatives **2**–**4** by various fungal β-*N*-acetylhexosaminidases relative to *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **10**.[a]

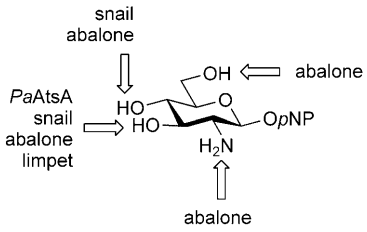
| Enzyme source | 3-Sulfate **2**[b] | 4-Sulfate **3**[b] | 6-Sulfate **4**[b] |
|---|---|---|---|
| *Acremonium persicinum* CCF 1850 | − | + | − |
| *Aspergillus awamori* CCF 763 | − | − | + |
| *A. flavofurcatis* CCF 3061 | − | ++ | − |
| *A. flavus* CCF 642 | − | − | − |
| *A. niger* CCIM K2 | − | − | − |
| *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 | − | + | +++ |
| *Penicillium brasilianum* CCF 2171 | − | + | ++ |
| *P. chrysogenum* CCF 1269 | +++ | +++ | + |
| *P. oxalicum* CCF 1959 | − | ++ | ++ |
| *P. oxalicum* CCF 2315 | − | + | ++ |
| *P. oxalicum* CCF 2430 | − | + | ++ |
| *P. pittii* CCF 2277 | − | ++ | ++ |
| *Talaromyces flavus* CCF 2573 | − | + | ++++ |
| *T. flavus* CCF 2686 | − | + | +++ |
| *T. ohiensis* CCF 2229 | − | ++ | + |
| *T. striatus* CCF 2232 | − | ++ | ++++ |
| *Trichoderma harzianum* CCF 2687 | − | ++ | ++ |

[a] 2-Sulfate **1** was not cleaved by any of the enzymes tested. [b] >20% (++++), 11–20% (+++), 6–10% (++), 1–5% (+) or <1% (−).

## Conclusions

Two different assays were developed to monitor the hydrolysis of four carbohydrate sulfates by sulfatases: 1) a coupled assay using *Ao*Hex as the coupling enzyme and 2) a stopped turbidimetric assay, which quantitated liberated inorganic sulfate through the formation of a $BaSO_4$ precipitate. These assays were used to investigate four sulfatases: three commercial molluscan arylsulfatases from snail (*Helix pomatia*), limpet (*Patella vulgata*) and abalone; and recombinantly expressed AtsA from *Pseudomonas aeruginosa*. All four sulfatases could cleave the 3-sulfate **2**, both the snail and abalone sulfatases could cleave the 4-sulfate **3**, whereas only the abalone sulfatase could cleave the 2- and 6-sulfates **1** and **4** (Scheme 7). While the carbohydrate sulfatase activity seen for all of these sulfatases was low relative to the activity seen towards the artificial substrate *p*-nitrophenyl sulfate, this is not unexpected, as the leaving group ability of the carbohydrate in the carbohydrate sulfate is poor relative to *p*-nitrophenol ($pK_a = 7.2$) in *p*-nitrophenyl sulfate. Nonetheless, distinct carbohydrate sulfatase activity was observed and could be quantified.

The sulfated *p*-nitrophenyl glucosaminides **1**–**4** were also tested for their ability to be hydrolyzed by a family of fungal β-*N*-acetylhexosaminidases. The 6-sulfate **4** was a good substrate for β-*N*-acetylhexosaminidases from a range of species in the genera *Fusarium*, *Penicillium*, *Talaromyces* and *Trichoderma*. The 4-sulfate **3** was also accepted as a substrate by the same




**Scheme 7.** Regioselectivity of sulfate hydrolysis by the sulfatases studied here.

β-*N*-acetylhexosaminidases. With these positive results, future work will investigate the ability of the 4- and 6-sulfates **3** and **4** to act as glycosyl donors in transglycosidation reactions for the preparation of more complex glycoconjugates. On the other hand, the 3-sulfate **2** was generally a poor substrate for all β-*N*-acetylhexosaminidases, with the exception of the β-*N*-acetylhexosaminidase from *Penicillium chrysogenum*.

## Experimental Section

**General methods**: All chemicals were obtained from Sigma–Aldrich, except for *N*-acetylglucosamine, which was obtained from CMS Chemicals UK, and used without further purification. Anhydrous dichloromethane and THF were deoxygenated and dried prior to use according to Pangborn's method by using a commercially available system (Seca Solvent Systems).[34] Acetonitrile was dried over $CaH_2$ and distilled prior to use. Pyridine was dried over KOH and distilled prior to use, or stored over 3 Å molecular sieves. 2,4,6-Collidine was distilled and stored over KOH. DMF and MeOH were dried over 3 Å molecular sieves. TLC was performed with aluminium sheets precoated with Silica Gel 60 ($F_{254}$ Merck), and the plates were visualized by irradiation with UV light (254 nm) and/or charring with a mixture of 5% sulfuric acid in MeOH. Flash chromatography was performed according to the method of Still et al. by using Silica Gel 60 (Merck).[35] NMR spectra were obtained on Varian Unity 400 or 500 instruments. Melting points were obtained by using an electrothermal melting-point apparatus or a Reichert–Jung hot stage and are uncorrected unless otherwise stated. Optical rotations were recorded on a Jasco DIP-1000 polarimeter and are reported in $10^{-1}$ deg cm$^2$ g$^{-1}$. IR spectra were obtained on a Perkin–Elmer Spectrum One FTIR spectrometer with a zinc selenide/diamond Universal ATR sampling accessory as a thin film. Elemental analyses were performed by C.M.A.S (Belmont, Victoria). Low-resolution mass spectrometry was performed by Sioe See Volaric (Melbourne University, Australia). High-resolution mass spectrometry was performed by Chris Barlow or Adrian Lam on a Finnigan hybrid LTQ-FT mass spectrometer (Thermo Electron Corp.) or by Dr. Sally Duck at the Chemistry Department, Monash University.

***p*-Nitrophenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-*N*-(*tert*-butyloxycarbonyl)-2-deoxy-β-D-glucopyranoside (7)**: A mixture of the *p*-nitrophenyl triacetate **5** (4.52 g, 9.65 mmol), $Boc_2O$ (2.9 mL, 13 mmol) and DMAP (236 mg, 1.93 mmol) was refluxed for 30 min. The resulting solution was concentrated and chromatographed (EtOAc/petroleum spirits 3:7) to afford the mixed imide **7** as a colorless foam (5.47 g, quant.). $[\alpha]_D^{23} = -16.7^\circ$ ($c = 1.22$, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 1.51$, 1.59 (2s, 9H; $C(CH_3)_3$), 2.03, 2.07, 2.11 (3s, 9H; 3OAc), 2.34, 2.45 (2s, 3H; NAc), 3.94–3.99 (brm, 1H; H5), 4.15, 4.29 (2brd, 2H; H6,6′), 4.55 (brt, 1H; H2), 5.18 (brt, 1H; H3),

5.76–5.89 (m, 1H; H4), 6.12 (d, 1H; H1), 7.00–7.50 (br m, 2H; *p*NP), 8.19 (d, 2H; *p*NP); HRMS (ESI$^+$) *m/z* 591.1791 [*M*+Na]$^+$, requires 591.1807 ($C_{25}H_{32}N_2NaO_{13}$); elemental analysis calcd (%) for $C_{25}H_{32}N_2O_{13}$: C 52.81, H 5.67, N 4.93; found: C 52.90, H 5.71, N 4.79.

**p-Nitrophenyl 3,4,6-tri-*O*-acetyl-2-*tert*-butyloxycarbonylamino-2-deoxy-β-D-glucopyranoside (8)**

*1)* p-*Nitrophenyl 2-*tert-*butyloxycarbonylamino-2-deoxy-β-D-glucopyranoside*: A solution of the mixed imide **7** (5.46 g, 9.61 mmol) and hydrazine hydrate (4.7 mL) in MeOH (150 mL) was stirred at 40 °C for 5 h. The resulting suspension was concentrated to afford the deprotected carbamate as a bright yellow amorphous solid that was used without further purification (quant.) or recrystallized to yield a colorless amorphous solid. M.p. 205.5–206 °C (MeOH/$H_2O$); $[\alpha]_D^{24} = -25.9°$ ($c = 0.930$, MeOH); $^1$H NMR (400 MHz, $CD_3OD$): $\delta =$ 1.44 (s, 9H; $C(CH_3)_3$), 3.40 (dd, $J_{1,2} = 8.4$, $J_{2,3} = 9.4$ Hz, 1H; H2), 3.46–3.52 (m, 2H; H3,5), 3.63 (dd, $J_{3,4} = 9.9$, $J_{4,5} = 9.2$ Hz, 1H; H4), 3.70 (dd, $J_{5,6} = 1.9$, $J_{6,6'} = 11.9$ Hz, 1H; H6), 3.91 (dd, $J_{5,6'} = 5.6$ Hz, 1H; H6′), 5.11 (d, 1H; H1), 7.16–7.18, 8.19–8.22 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $CD_3OD$): $\delta = 28.93$ (3C, $C(CH_3)_3$), 58.34, 62.60, 71.92, 75.75, 78.60 (5C, C2,3,4,5,6), 80.35 (1C, $C(CH_3)_3$), 100.96 (1C, C1), 117.77, 126.86, 144.03, 158.55 (6C, *p*NP), 164.06 (1C, C=O); HRMS (ESI$^+$) *m/z* 423.1365 [*M*+Na]$^+$, requires 423.1379 ($C_{17}H_{24}N_2NaO_9$); elemental analysis calcd (%) for $C_{17}H_{24}N_2O_9$: C 51.00, H 6.04, N 6.99; found: C 50.93, H 6.10, N 6.94.

*2)* p-*Nitrophenyl 3,4,6-tri-*O*-acetyl-2-*tert*-butyloxycarbonylamino-2-deoxy-β-D-glucopyranoside (**8**)*: A suspension of the crude deprotected carbamate (5.05 g, 9.60 mmol), $Ac_2O$ (6 mL) and pyridine (12 mL) was stirred for 16 h, after which the resulting suspension was diluted with EtOAc (250 mL), washed with HCl (3×100 mL, 1 M) and sat. aq. $NaHCO_3$ (2×100 mL), dried ($MgSO_4$), and concentrated to afford the acetylated carbamate **8** as a colorless amorphous solid (4.74 g, 94% from **5**). M.p. 210–213 °C (corrected), $[\alpha]_D^{23} = -22.8°$ ($c = 0.54$, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 1.42$ (s, 9H; $C(CH_3)_3$), 2.06, 2.08, 2.09 (3s, 9H; $3CH_3$), 3.81 (ddd, $J_{2,3} =$ 9.2 Hz, 1H; H2), 3.91–3.97 (m, 1H; H5), 4.17, 4.29 (2dd, $J_{5,6} = 2.4$, $J_{5,6'} = 5.6$, $J_{6,6'} = 12.4$ Hz, 2H; H6,6′), 4.76 (br d, 1H; NH), 5.12 (dd, 1H; H3), 5.37–5.50 (br m, 2H; H1,4), 7.08–7.10, 8.20–8.22 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $CDCl_3$): $\delta = 20.64$, 20.67, 20.72 (3C, $3COCH_3$), 28.18 (3C, $C(CH_3)_3$), 55.68, 62.05, 68.38, 71.44, 72.33, (5C, C2,3,4,5,6), 80.63 (1C, $C(CH_3)_3$), 98.29 (1C, C1), 116.65, 125.77, 143.07, 161.59 (6C, *p*NP), 169.48, 170.46, 170.49 (3C, 3C=O); HRMS (ESI$^+$) *m/z* 549.1687 [*M*+Na]$^+$, requires 549.1697 ($C_{23}H_{30}N_2NaO_{12}$); elemental analysis calcd (%) for $C_{23}H_{30}N_2O_{12}$: C 52.47, H 5.74, N 5.32; found: C 52.51, H 5.85, N 5.28.

**p-Nitrophenyl 3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (6)**: A suspension of the acetylated carbamate **8** (4.74 g, 9.00 mmol) in TFA (6 mL) was stirred at RT for 30 min. The resulting solution was concentrated, and the oily residue was dissolved in $CH_2Cl_2$ then washed with $K_2CO_3$ (3×70 mL, 0.5 M) and water (2× 70 mL), dried ($MgSO_4$), and concentrated. Chromatography ($CHCl_3$/MeOH 98:2→96:4) afforded the amine **6** as a pale yellow foam (3.62 g, 94%). $[\alpha]_D^{23} = -53.7°$ ($c = 0.87$, $CHCl_3$) (lit. $[\alpha]_D^{20} = -42°$ ($c =$ 0.37, MeOH)[36]); $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 2.07$, 2.09, 2.13 (3s, 9H; $3CH_3$), 3.28 (dd, $J_{1,2} = 8.4$ Hz, 1H; H2), 3.89–3.93 (m, 1H; H5), 4.15, 4.31 (2dd, $J_{5,6} = 1.9$, $J_{5,6'} = 5.6$, $J_{6,6'} = 12.4$ Hz, 2H; H6,6′), 4.98 (d, 1H; H1), 5.09–5.14 (m, 2H; H3,4), 7.10–7.12, 8.22–8.24 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $CDCl_3$): $\delta = 20.67$, 20.75, 20.81 (3C, $3CH_3$), 55.69, 62.08, 68.39, 72.38, 74.62 (5C, C2,3,4,5,6), 101.35 (1C, C1), 116.45, 125.79, 143.05, 161.43 (6C, *p*NP), 169.75, 170.53, 170.69 (3C, 3C=O).

**p-Nitrophenyl 2-deoxy-2-sulfoamino-β-D-glucopyranoside, sodium salt (1)**

*1)* p-*Nitrophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-sulfoamino-β-D-glucopyranoside, sodium salt (**9**)*: A suspension of amine **6** (100 mg, 0.236 mmol) and $SO_3$·$NMe_3$ (98.3 mg, 0.706 mmol) in anhydrous pyridine (7 mL) was stirred under $N_2$ for 16 h. The reaction was stopped by the addition of $NaHCO_3$ (1.4 mL, 1 M), and the resulting suspension was concentrated (≤35 °C). The crude yellow residue was suspended in MeOH and filtered, and the filtrate was chromatographed on a Sephadex LH-20 column (2×22 cm, eluted with MeOH/$H_2O$ 1:1). The carbohydrate-containing fractions were then applied to a Dowex 50W×8–400 column ($Na^+$ form, 2×5 cm, eluted with MeOH/$H_2O$ 1:1). Concentration (≤35 °C) gave the acetylated *N*-sulfate **9** (92.8 mg, 76%) as a pale yellow amorphous solid. $^1$H NMR (400 MHz, $CD_3OD$): $\delta = 2.02$, 2.03, 2.07 (3s, 9H; $3CH_3$), 3.68 (dd, $J_{1,2} = 7.9$, $J_{2,3} = 9.6$ Hz, 1H; H2), 4.04 (ddd, $J_{4,5} = 9.2$, $J_{5,6} = 2.4$, $J_{5,6'} = 5.1$ Hz, 1H; H5), 4.14, 4.29 (2dd, $J_{6,6'} = 12.4$ Hz, 2H; H6,6′), 5.07 (t, $J_{3,4} = 9.6$ Hz, 1H; H3), 5.34 (dd, 1H; H4), 5.39 (d, 1H; H1), 7.26–7.28, 8.19–8.22 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $CD_3OD$): $\delta = 20.75$, 20.79, 21.24 (3C, $3CH_3$), 59.73, 63.44, 70.25, 73.13, 74.79 (5C, C2,3,4,5,6), 100.51 (1C, C1), 118.14, 126.58, 144.09, 163.87 (6C, *p*NP), 171.50, 172.42, 173.04 (3C, 3C=O).

*2)* p-*Nitrophenyl 2-deoxy-2-sulfoamino-β-D-glucopyranoside, sodium salt (**1**)*: A small piece of sodium metal was added to the acetylated *N*-sulfate **9** in MeOH (25 mL), and the mixture was stirred until deacetylation was complete. The reaction was stopped by acidification with Amberlite IR-120 ($H^+$ form) resin, the resin was removed by filtration, and the filtrate was immediately neutralized with sat. aq. $NaHCO_3$ and concentrated (≤35 °C). The crude material was suspended in MeOH/$H_2O$ (1:1) and chromatographed as described above (Sephadex LH-20 followed by Dowex 50W×8–400) to afford the 2-*N*-sulfate **1** as a yellow amorphous solid (38.1 mg, 54%). $^1$H NMR (400 MHz, $D_2O$): $\delta = 3.38$ (dd, $J_{1,2} = 8.4$, $J_{2,3} = 9.8$ Hz, 1H; H2), 3.57 (dd, $J_{3,4} = 9.2$ Hz, 1H; H3), 3.69 (ddd, $J_{4,5} = 9.8$, $J_{5,6} = 5.6$, $J_{5,6'} = 1.9$ Hz, 1H; H5), 3.76 (dd, 1H; H4), 3.77 (dd, $J_{6,6'} = 12.3$ Hz, 1H; H6), 3.96 (dd, 1H; H6′), 5.39 (d, 1H; H1), 7.24–7.28, 8.26–8.29 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $D_2O$): $\delta = 60.36$, 61.13, 70.30, 74.75, 76.63 (5C, C2,3,4,5,6), 99.38 (1C, C1), 117.14, 126.66, 143.09, 162.56 (6C, *p*NP); HRMS (ESI$^+$) *m/z* 379.0440 [*M*]$^-$, requires 379.0453 ($C_{12}H_{15}N_2O_{10}S$).

**p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (10)**: A small piece of sodium metal was added to a solution of the *p*-nitrophenyl triacetate **5** (1.93 g, 5.64 mmol) in MeOH, and the solution was stirred at 40 °C until deacetylation was complete. The reaction was stopped by acidification with Amberlite IR-120 ($H^+$ form) resin, and the resin was removed by filtration. The filtrate was concentrated, and the resulting precipitate was used without further purification (1.93 g, 96%); alternatively, it could be recrystallized to afford the triol **10** as colorless needles. M.p. 200–201 °C (MeOH) (lit. 205, 206.2–206.7 °C[37]); $[\alpha]_D^{21} = -16.1°$ ($c = 0.87$, $H_2O$/acetone 1:1) (lit. $[\alpha]_D^{22} = -18°$ (c = 1.0, $H_2O$/acetone 1:1), $[\alpha]_D^{22} = -14.7°$ (c = 0.3, $H_2O$)[38]); $^1$H NMR (400 MHz, $CD_3OD$): $\delta = 1.98$ (s, 3H; $CH_3$), 3.43 (dd, $J_{2,3} = 9.9$, $J_{3,4} = 8.8$ Hz, 1H; H3), 3.51 (ddd, $J_{4,5} = 9.8$, $J_{5,6} = 1.9$, $J_{5,6'} = 5.9$ Hz, 1H; H5), 3.59 (dd, 1H; H4), 3.72 (dd, $J_{6,6'} =$ 12.2 Hz, 1H; H6), 3.93 (dd, 1H; H6′), 3.96 (dd, $J_{1,2} = 9.9$ Hz, 1H; H2), 5.20 (d, 1H; H1), 7.16–7.19, 8.20–8.23 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $CD_3OD$): $\delta = 23.08$ (1C, $CH_3$), 57.25, 62.59, 71.83, 75.79, 78.68 (5C, C2,3,4,5,6), 100.14 (1C, C1), 117.80, 126.81, 144.12, 163.86 (6C, *p*NP), 174.08 (1C, C=O).

**p-Nitrophenyl 2-acetamido-2-deoxy-6-*O*-*tert*-butyldimethylsilyl-β-D-glucopyranoside (11)**: A solution of triol **10** (1.36 g,

3.97 mmol), imidazole (548 mg, 8.05 mmol) and TBSCl (783 mg, 5.20 mmol) in anhydrous DMF (6 mL) was stirred under $N_2$ for 16 h. The reaction was quenched with water and concentrated to afford a yellow oil that was chromatographed ($CHCl_3$/MeOH 93:7) to give the silyl ether **11** as a colorless amorphous solid (1.05 g, 58%). M.p. 163.5–164.5 °C (corrected); $[\alpha]_D^{25} = -25.2°$ ($c = 0.735$, MeOH) (lit. $[\alpha]_D^{20} - 29°$ ($c = 0.56$, $H_2O$)[14]); $^1$H NMR (400 MHz, $CD_3OD$): $\delta = 0.00$, 0.04 (2 s, 6 H; 2 $SiCH_3$), 0.89 (s, 9 H; $C(CH_3)_3$), 1.98 (s, 3 H; OAc), 3.43 (dd, $J_{3,4} = 8.4$, $J_{4,5} = 9.8$ Hz, 1 H; H4), 3.51 (ddd, $J_{5,6} = 5.9$, $J_{5,6'} = 1.9$ Hz, 1 H; H5), 3.59 (dd, $J_{2,3} = 10.4$ Hz, 1 H; H3), 3.81 (dd, $J_{6,6'} = 11.6$ Hz, 1 H; H6), 3.94 (dd, $J_{1,2} = 8.4$ Hz, 1 H; H2), 4.03 (dd, 1 H; H6′), 5.19 (d, 1 H; H1), 7.16–7.19, 8.17–8.21 (AA′XX′, 4 H; *p*NP); $^{13}$C NMR (100.5 MHz, $CD_3OD$): $\delta = -5.06$, −4.99 (2 C, $SiCH_3$), 19.35 (1 C, *C*-$(CH_3)_3$), 23.09 (1 C, $CH_3$), 26.52 (3 C, $C(CH_3)_3$), 57.29, 64.00, 71.77, 75.91, 78.79 (5 C, C2,3,4,5,6), 100.01 (1 C, C1), 117.91, 126.73, 144.08, 163.85 (6 C, *p*NP), 174.06 (1 C, C=O).

***p*-Nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-*tert*-butyldimethylsilyl-β-D-glucopyranoside (12)**: A solution of silyl ether **11** (1.46 g, 3.19 mmol), pyridine (7 mL) and $Ac_2O$ (4 mL) was stirred until acetylation was complete. The resulting suspension was diluted with EtOAc, washed with 1 M HCl (3×70 mL) and sat. aq. $NaHCO_3$ (2×70 mL), dried ($MgSO_4$), and concentrated. Recrystallization gave the acetylated silyl ether **12** as colorless needles (1.49 g, 86%). M.p. 215–216.5 °C (corrected; EtOH); $[\alpha]_D^{23} = -19.5°$ ($c = 0.91$, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta = -0.05$, −0.02 (2 s, 6 H; 2 $SiCH_3$), 0.85 (s, 9 H; $C(CH_3)_3$), 1.94, 2.04, 2.05 (3 s, 9 H; Ac), 3.62–3.74 (m, 2 H; H6,6′), 3.71–3.76 (m, 1 H; H5), 4.12 (dt, $J_{1,2} = 8.4$, $J_{2,3} = 10.8$, $J_{NH,2} = 8.8$ Hz, 1 H; H2), 5.03 (dd, $J_{3,4} = 9.2$, $J_{4,5} = 9.6$ Hz, 1 H; H4), 5.31 (d, 1 H; H1), 5.37 (dd, 1 H; H3), 5.62 (d, 1 H; NH), 7.06–7.08, 8.13–8.16 (AA′XX′, 4 H; *p*NP); $^{13}$C NMR (100.5 MHz, $CDCl_3$): $\delta = -5.31$, −5.16 (2 C, 2 $SiCH_3$), 18.46 (1 C, *C*$(CH_3)_3$), 20.93, 23.59 (3 C, 3 $CH_3$), 25.98 (1 C, $C(CH_3)_3$), 54.92, 62.55, 68.90, 72.30, 75.77 (5 C, C2,3,4,5,6), 98.41 (1 C, C1), 116.89, 125.94, 143.16, 161.19 (6 C, *p*NP), 169.61, 170.58, 171.22 (3 C, 3 C=O); elemental analysis calcd (%) for $C_{24}H_{36}N_2O_{10}Si$: C 53.32, H 6.71, N 5.18; found: C 53.22, H 6.73, N 5.20.

***p*-Nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (13)**: Hydrogen fluoride–pyridine complex (70%, 2.3 mL) was added portion-wise to a solution of the acetylated silyl ether **12** (0.99 g, 1.8 mmol) in THF (18 mL) in a polyethylene vessel at 0 °C, and the solution was warmed to RT. After 3 h, the solution was diluted with EtOAc, washed with sat. aq. $NaHCO_3$ (4×100 mL), dried ($MgSO_4$) and concentrated. Recrystallization gave the 6-hydroxy compound **13** as colorless needles (720 mg, 75%). M.p. 225.5–226 °C (decomp.; corrected; MeOH) (lit. 210–211, 234–236 °C[39,40]); $[\alpha]_D^{26} = -2.6°$ ($c = 0.81$, MeOH) (lit. $[\alpha]_D^{20} = -10°$ ($c = 0.69$, $CHCl_3$/acetone 1:1)[41]); $^1$H NMR (400 MHz, $CDCl_3 + [D_6]$DMSO): $\delta = 1.72$, 1.88, 1.92 (3 s, 9 H; 3 $CH_3$), 3.45 (ddd, $J_{1,2} = 5.9$, $J_{2,3} = 9.2$, $J_{NH,2} = 8.4$ Hz, 1 H; H2), 3.59 (ddd, $J_{4,5} = 9.9$, $J_{5,6} = 6.4$, $J_{5,6'} = 1.9$ Hz, 1 H; H5), 4.02 (dd, $J_{3,4} = 8.8$ Hz, 1 H; H4), 4.09 (dd, $J_{6,6'} = 11.9$ Hz, 1 H; H6), 4.26 (dd, 1 H; H6′), 4.97 (dd, 1 H; H3), 5.07 (d, 1 H; H1), 5.12 (d, 1 H; NH), 6.89–6.92, 7.97–7.99 (AA′XX′, 4 H; *p*NP); $^{13}$C NMR (100.5 MHz, $CDCl_3 + [D_6]$DMSO): $\delta = 20.57$, 20.75, 22.84 (3 C, 3 $CH_3$), 53.24, 63.06, 68.04, 74.07, 74.83 (5 C, C2,3,4,5,6), 98.09 (1 C, C1), 116.36, 125.25, 142.28, 161.74 (6 C, *p*NP), 170.28, 170.43, 170.68 (3 C, 3 C=O).

***p*-Nitrophenyl 2-acetamido-2-deoxy-6-*O*-sulfo-β-D-glucopyranoside, sodium salt (4)**: A suspension of the primary alcohol **13** (167 mg, 0.392 mmol) and $SO_3$·pyr. (250 mg, 1.57 mmol) in a mixture of anhydrous DMF and pyridine (1:1, 2.5 mL) was stirred under nitrogen at 50 °C for 4 h. The reaction was stopped by the addition of $NaHCO_3$ (3 mL, 1 M), and the resulting suspension was concentrated (≤30 °C). The crude material was dissolved in MeOH, applied to a Sephadex LH-20 column (2×22 cm), and eluted with MeOH/$H_2O$ (1:1). The carbohydrate-containing fractions were then immediately applied to a cation-exchange column (Dowex 50W×8–400, $Na^+$ form) and eluted with MeOH/$H_2O$ (1:1). Concentration (≤30 °C) gave the acetylated 6-sulfate **14** as a pale yellow gum, which was dissolved in MeOH, and a small piece of sodium metal was added. After deacetylation was complete, the reaction was acidified with Amberlite IR-120 ($H^+$ form) resin, the resin was removed by filtration, and the filtrate was immediately neutralized with $NaHCO_3$ (1 M), then concentrated (≤35 °C). The crude material was suspended in MeOH/$H_2O$ (1:1) and filtered, and the filtrate was chromatographed as described above (Sephadex LH-20 followed by Dowex 50W×8–400) to afford the 6-sulfate **4** as a colorless amorphous solid (70.3 mg, 40%). $[\alpha]_D^{23} = -67.0°$ ($c = 0.790$, $H_2O$) (lit. $[\alpha]_D^{23} = -31°$ ($c = 0.57$, $H_2O$)[14]); $^1$H NMR (400 MHz, $D_2O$): $\delta = 2.12$ (s, 3 H; $CH_3$), 3.62 (dd, $J_{3,4} = 9.2$, $J_{4,5} = 9.8$ Hz, 1 H; H4), 3.71 (dd, $J_{2,3} = 10.4$ Hz, 1 H; H3), 3.94 (ddd, $J_{5,6} = 2.4$, $J_{5,6'} = 5.8$ Hz, 1 H; H5), 4.05 (dd, $J_{1,2} = 8.8$ Hz, 1 H; H2), 4.25 (dd, $J_{6,6'} = 11.4$ Hz, 1 H; H6), 4.41 (dd, 1 H; H6′), 5.31 (d, 1 H; H1), 7.17–7.19, 8.22–8.24 (AA′XX′, 4 H; *p*NP); $^{13}$C NMR (100.5 MHz, $D_2O$): $\delta = 22.68$ (1 C, $CH_3$), 55.81, 67.49, 69.98, 73.83, 74.71 (5 C, C2,3,4,5,6), 99.21 (1 C, C1), 117.13, 126.71, 143.29, 162.23 (6 C, *p*NP), 175.54 (1 C, C=O).

**4-*tert*-Butylbenzaldehyde dimethyl acetal**: A solution of 4-*tert*-butylbenzaldehyde (10 mL, 60 mmol), trimethylorthoformate (7.0 mL, 64 mmol) and TsOH (cat.) in MeOH (100 mL) was heated at reflux for 4 h. The reaction mixture was concentrated to remove excess MeOH, and the resultant crude residue was distilled under reduced pressure to afford the 4-*tert*-butylbenzaldehyde dimethyl acetal as a colorless liquid. B.p. 245 °C; $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 1.32$ (s, 9 H; $C(CH_3)_3$), 3.33 (s, 6 H; 2 $OCH_3$), 5.36 (s, 1 H; CH), 7.38 (s, 4 H; Ar); $^{13}$C NMR (100.5 MHz, $CDCl_3$): $\delta = 31.33$ (3 C, $C(CH_3)_3$), 34.58 (1 C, *C*-$(CH_3)_3$), 52.79 (2 C, $OCH_3$), 103.34 (1 C, CH), 125.11, 126.35, 135.12, 151.37 (6 C, Ar). The $^1$H and $^{13}$C NMR data are in agreement with those previously reported.[42]

***p*-Nitrophenyl 2-acetamido-4,6-*O*-(4-*tert*-butylbenzylidene)-2-deoxy-β-D-glucopyranoside (16)**: A suspension of the triol **10** (374 mg, 1.09 mmol), 4-*tert*-butylbenzaldehyde dimethyl acetal (0.3 mL, 0.27 g, 1.3 mmol) and TsOH (cat.) in MeCN was stirred under nitrogen at 40 °C. After 2 h, triethylamine (0.3 mL) was added, and the solution was concentrated and triturated with petroleum spirits then ethanol to yield the benzylidene acetal **16** as colorless flakes (285 mg, 38%). $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 1.30$ (s, 9 H; $C(CH_3)_3$), 2.03 (s, 3 H; $CH_3$), 3.32–3.36 (br m, 1 H; H5), 3.61, 3.79 (2 t, 2 H; H3,4), 3.68–3.74 (m, 2 H; H2,6), 4.36–4.39 (m, 1 H; H6′), 5.55 (s, 1 H; benzylidene-CH), 5.68 (d, $J_{1,2} = 8.4$ Hz, 1 H; H1), 5.89 (d, $J_{NH,2} = 6.8$ Hz, 1 H; NH), 7.07–7.09, 8.19–8.21 (AA′XX′, 4 H; *p*NP), 7.39–7.44 (m, 4 H; Ar); $^{13}$C NMR (100.5 MHz, $CDCl_3$): $\delta = 23.66$ (1 C, $CH_3$), 31.27 (3 C, $C(CH_3)_3$), 34.71 (1 C, *C*$(CH_3)_3$), 58.19, 66.49, 68.40, 70.17, 81.33 (5 C, C2,3,4,5,6), 97.59 (1 C, C1), 102.12 (1 C, benzylidene-CH), 116.51, 125.41, 125.88, 125.99, 133.84, 143.03, 152.63, 161.44 (12 C, Ar), 171.49 (1 C, C=O).

**Trifluoromethylsulfonyl azide (triflyl azide)**: ***CAUTION!*** As for many low-molecular-weight azides, care should be taken in the handling of $TfN_3$ to avoid the risk of explosion. $Tf_2O$ (1.95 mL, 11.6 mmol) was added dropwise to a solution of sodium azide (1.51 g, 23.2 mmol) in a mixture of $H_2O$ (4.5 mL) and $CH_2Cl_2$ (4.5 mL) at 0 °C. After 2 h, sat. $NaHCO_3$ (2 mL) was added, and the mixture was stirred for a further 5 min at RT. The aqueous phase was separated and extracted with $CH_2Cl_2$ (2×3 mL), and the combined organic extracts containing triflyl azide were used immediately in the next step.

***p*-Nitrophenyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranoside (18)**: The freshly prepared solution of $TfN_3$ (1.5 equiv) was added to a mixture of the amine **6** (1.65 g, 3.87 mmol), $NEt_3$ (0.8 mL, 5.8 mmol) and catalytic $CuSO_4$, MeOH (1 mL) was then added dropwise and the solution was stirred overnight. The resulting green solution was diluted with EtOAc, washed with $H_2O$ (2× 100 mL), sat. $NaHCO_3$ (3×70 mL), sat. NaCl (100 mL), dried ($MgSO_4$) and concentrated. Chromatography (4:6→6:4 EtOAc/pet. spirits) yielded the acetylated 2-azido sugar **18** as colorless needles (1.64 g, 93%). M.p. 130.5–132 °C (corrected), $[\alpha]_D^{24} = -0.32°$ (c 0.99); IR (thin film): $\tilde{\nu} = 2111.1$ ($N_3$), 1748.2 (C=O), 1520.2, 1343.8 ($NO_2$), 1222.9 (C–O), 1044.5 (Ar) $cm^{-1}$. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta = 2.05$, 2.09, 2.12 (3 s, 9H; $CH_3$), 3.86 (dd, 1H; $J_{1,2} = 8.5$ Hz, H2), 3.89 (ddd, 1H; $J_{4,5} = 9.5$, $J_{5,6} = 1.9$, $J_{5,6'} = 5.5$ Hz, H5), 4.16 (dd, 1H; $J_{6,6'} = 12.5$ Hz, H6), 4.30 (dd, 1H; H6′), 5.04 (d, 1H; H1), 5.08, 5.12 (2 t, $J_{3,4} = 8.9$ Hz, 2H; H3,4), 7.12–7.14, 8.23–8.25 (AA′XX′, 4H; *p*NP); $^{13}C$ NMR (100.5 MHz, $CDCl_3$): $\delta = 20.77$, 20.86, 20.89 (3C, 3$CH_3$), 61.94, 63.56, 68.19, 72.29, 72.62 (5C, C2,3,4,5,6), 99.73 (1C, C1), 116.96, 126.09, 143.67, 161.10 (6C, *p*NP), 169.77, 170.04, 170.59 (3C, 3C=O); elemental analysis calcd (%) for $C_{18}H_{20}N_4O_{10}$: C 47.79, H 4.46, N 12.39; found: C 47.90, H 4.51, N 12.37.

***p*-Nitrophenyl 2-azido-2-deoxy-β-D-glucopyranoside (19)**: A small piece of sodium metal was added to a suspension of azide **18** (1.64 g, 3.62 mmol) in MeOH (150 mL), and the mixture was stirred until deprotection was complete. The solution was acidified by the addition of Amberlite IR-120 ($H^+$ form) resin, the resin was removed, and the filtrate was concentrated to afford the crude triol **19** (1.07 g, 91%), which was used without further purification. M.p. 142–143 °C (corrected); $[\alpha]_D^{23} = -35.8°$ (c = 0.910, MeOH); $^1H$ NMR (500 MHz, $D_2O$): $\delta = 3.57$ (dd, $J_{2,3} = 9.5$, $J_{3,4} = 8.9$ Hz, 1H; H3), 3.62 (dd, $J_{4,5} = 9.9$ Hz, 1H; H4), 3.67–3.70 (m, 1H; H5), 3.73 (dd, $J_{1,2} = 8.5$ Hz, 1H; H2), 3.78 (dd, $J_{5,6} = 4.9$, $J_{6,6'} = 12.5$ Hz, 1H; H6), 3.95 (dd, $J_{5,6'} = 1.9$ Hz, 1H; H6′), 5.36 (dd, 1H; H1), 7.26–7.29, 8.28–8.29 (AA′XX′, 4H; *p*NP); $^{13}C$ NMR (100.5 MHz, $D_2O$): $\delta = 60.89$, 65.83, 69.76, 74.69, 76.91 (5C, C2,3,4,5,6), 99.24 (1C, C1), 117.22, 126.73, 143.39, 161.86 (6C, *p*NP); IR (thin film): $\tilde{\nu} = 3361.1$ (br, OH), 2111.1 ($N_3$), 1514.3, 1343.8 ($NO_2$), 1246.7, 1074.2 $cm^{-1}$ (Ar).

***p*-Nitrophenyl 2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (20)**: A suspension of triol **19** (950 mg, 2.91 mmol), benzaldehyde dimethyl acetal (1.2 mL, 8.70 mmol) and catalytic TsOH in $CHCl_3$ (15 mL) was refluxed under $N_2$ with the liberated MeOH removed by using a Dean–Stark apparatus. After 4 h, $NEt_3$ (0.2 mL) was added, and the solution was concentrated and purified through a silica plug ($CHCl_3$→EtOAc) to give the benzylidene acetal **20** as a beige amorphous powder (1.05 g, 87%). M.p. 170.5–172.5 °C (corrected), $[\alpha]_D^{22} = -0.39°$ (c = 1.06, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$): $\delta = 3.61$ (dt, $J_{4,5} = 9.6$, $J_{5,6} = 1.6$, $J_{5,6'} = 4.8$ Hz, 1H; H5), 3.68 (t, 1H; $J_{2,3} = 9.4$ Hz, H3), 3.74 (dd, $J_{1,2} = 7.6$ Hz, 1H; H2), 3.81 (dd, 1H; H6), 3.83 (t, 1H; H4), 4.41 (dd, 1H; H6′), 5.09 (d, 1H; H1), 5.59 (s, 1H; benzylidene-CH), 7.13–7.15, 8.24–8.26 (AA′XX′, 4H; *p*NP), 7.39–7.41, 7.48–7.51 (2m, 5H; Ar); $^{13}C$ NMR (100.5 MHz, $CDCl_3$): $\delta = 66.22$, 66.88, 68.46, 72.21, 80.32 (5C, C2,3,4,5,6), 100.19 (1C, C1), 102.38 (1H; benzylidene-CH), 116.92, 126.14, 126.45, 128.68, 129.79, 136.66, 161.21 (12C, *p*NP,Ph); IR (thin film): $\tilde{\nu} = 2115.1$ ($N_3$), 1520.3, 1345.8 ($NO_2$), 1244.7 (C–O), 1090.1 $cm^{-1}$ (Ar); microanalysis calcd (%) for $C_{19}H_{18}N_4O_7$: C 55.07, H 4.38, N 13.52; found: C 55.03, H 4.33, N 13.48.

***p*-Nitrophenyl 2-azido-4,6-*O*-benzylidene-3-*O*-chloroacetyl-2-deoxy-β-D-glucopyranoside (21)**: A solution of benzylidene acetal **20** (1.55 g, 3.73 mmol), 2,6-lutidine (4 mL), $(ClAc)_2O$ (2.58 g, 13.6 mmol) and DMAP (97.3 mg, 0.796 mmol) in $CH_2Cl_2$ (30 mL) was stirred at room temperature for 30 min. The resulting yellow solution was diluted with $CH_2Cl_2$ (150 mL) and washed with HCl (3×50 mL, 1 M) and sat. aq. $NaHCO_3$ (3×50 mL), dried ($MgSO_4$) and concentrated. Purification of the crude material through a silica plug eluted with $CH_2Cl_2$ gave the 3-*O*-chloroacetylated benzylidene derivative **21** as a pale yellow foam (1.79 g, 98%). Recrystallization gave pale beige needles. M.p. 110.5–113 °C (corrected; MeOH); $[\alpha]_D^{24} = -0.44°$ (c = 0.81, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$): $\delta = 3.71$ (dt, $J_{5,6} = 9.6$, $J_{5,6'} = 4.8$ Hz, 1H; H5), 3.79 (t, $J_{3,4} = 9.2$ Hz, 1H; H4), 3.84 (dd, $J_{6,6'} = 10.6$ Hz, 1H; H6), 3.88 (dd, $J_{1,2} = 7.6$, $J_{2,3} = 9.9$ Hz, 1H; H2), 4.12–4.20 ($AB_q$, 2H; $CH_2Cl_2$), 4.42 (dd, 1H; H6′), 5.19 (d, 1H; H1), 5.29 (dd, 1H; H3), 5.53 (s, 1H; benzylidene-CH), 7.14–7.16, 8.24–8.27 (AA′XX′, 4H; *p*NP), 7.36–7.38, 7.41–7.44 (2m, 5H; Ar); $^{13}C$ NMR (100.5 MHz, $CDCl_3$): $\delta = 40.71$ (1C, $CH_2Cl_2$), 64.39, 67.03, 68.37, 72.65, 78.13 (5C, C2,3,4,5,6), 100.35 (1C, C1), 101.95 (1C, benzylidene-CH), 116.98, 128.56, 136.43, 160.94 (6C, *p*NP), 126.19, 126.29, 129.59, 143.79 (6C, Ar), 166.34 (1C, C=O); IR (thin film): $\tilde{\nu} = 2111.1$ ($N_3$), 1770.0 (C=O), 1518.3, 1343.8 $cm^{-1}$ ($NO_2$); elemental analysis calcd (%) for $C_{21}H_{19}ClN_4O_8$: C 51.39, H 3.90, N 11.41; found: C 51.29, H 3.83, N 11.33.

***p*-Nitrophenyl 2-azido-3-*O*-chloroacetyl-2-deoxy-β-D-glucopyranoside (22)**: Aqueous TFA (0.6 mL, 70%) was added dropwise to a solution of 2-azido chloroacetate **21** (0.54 g, 1.1 mmol) in $CH_2Cl_2$ (2 mL) at 0 °C, and the mixture was allowed to warm to RT. After 2 h, the mixture was concentrated, azeotroped with toluene and triturated with petroleum spirits (5×3 mL) to afford the crude 2-azido-3-*O*-chloroacetate **22** (quant.) as a colorless suspension that was used immediately in the next step, or recrystallized to give a beige amorphous solid. M.p. 166.5–169 °C (corrected; EtOH); $[\alpha]_D^{22} = -25.9°$ (c = 1.13, MeOH); $^1H$ NMR ($CDCl_3$ + [$D_6$]DMSO): $\delta =$ 3.47–3.53 (m, 2H), 3.66 (dd, $J_{1,2} = 7.9$, $J_{2,3} = 9.9$ Hz, 1H; H2), 3.68 (dd, 1H), 3.75–3.84 (m, 2H), 4.09–4.19 ($AB_q$, 2H; $CH_2Cl_2$), 4.98 (dd, 1H; H3), 5.03 (d, 1H; H1), 5.09 (d, $J_{H4\text{-}OH} = 5.6$ Hz, 1H; 4-OH), 7.04–7.08, 8.11–8.15 (AA′XX′, 4H; *p*NP); $^{13}C$ NMR (100.5 MHz, $CDCl_3$ + [$D_6$]DMSO): $\delta = 40.75$ (1C, $CH_2Cl$), 60.60, 63.23, 67.55, 76.13, 76.69 (5C, C2,3,4,5,6), 99.09 (1C, C1), 116.39, 125.48, 142.68, 160.97 (6C, *p*NP), 166.41 (1C, C=O); elemental analysis calcd (%) for $C_{14}H_{15}ClN_4O_8$: C 41.75, H 3.75, N 13.91; found: C 41.80, H 3.78, N 14.03.

***p*-Nitrophenyl 4,6-di-*O*-acetyl-2-azido-3-*O*-chloroacetyl-2-deoxy-β-D-glucopyranoside (23)**: A solution of the crude 2-azido-3-*O*-chloroacetate **22** (402 mg, 1.0 mmol), $Ac_2O$ (4 mL), 2,6-lutidine (2.5 mL) and DMAP (30 mg) in $CH_2Cl_2$ (11 mL) was stirred at RT for 30 min. The yellow solution was diluted with $CH_2Cl_2$ (100 mL), washed with HCl (3×30 mL, 1 M) and sat. aq. $NaHCO_3$ (3×30 mL), dried ($MgSO_4$), and concentrated. The yellow oil was chromatographed (EtOAc/petroleum spirits 3:7→1:1) to afford the acetylated 2-azido-3-*O*-chloroacetate **23** as a pale yellow foam (0.49 g, 91% over 2 steps). $[\alpha]_D^{24} = -31.4°$ (c = 0.58, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$): $\delta = 2.06$, 2.09 (2 s, 6H; 2$CH_3$), 3.91 (dd, $J_{1,2} =$ 7.9 Hz, 1H; H2), 3.94 (ddd, $J_{5,6} = 2.4$, $J_{5,6'} = 5.4$ Hz, 1H; H5), 4.11 ($AB_q$, 2H; $CH_2Cl_2$), 4.19 (dd, $J_{6,6'} = 12.4$ Hz, 1H; H6), 4.31 (dd, 1H; H6′), 5.11 (d, 1H; H1), 5.10–5.19 (m, 2H; H3,4), 7.13–7.17, 8.23–8.26 (AA′XX′, 4H; *p*NP); $^{13}C$ NMR (100.5 MHz, $CDCl_3$): $\delta = 20.69$, 20.82 (2C, 2$CH_3$), 40.53 (1C, $CH_2Cl_2$), 61.83, 63.40, 68.00, 72.52, 73.97 (5C, C2,3,4,5,6), 99.74 (1C, C1), 116.97, 126.06, 143.74, 161.01 (6C, *p*NP), 166.74, 169.76, 170.52 (3C, 3C=O); IR (thin film): $\tilde{\nu} = 2115.1$ ($N_3$), 1744.3 (C=O), 1518.3, 1343.8 ($NO_2$), 1228.9 $cm^{-1}$ (C–O); HRMS (ESI$^+$): *m/z* 509.0687 [*M*+Na]$^+$, requires 509.0687 ($C_{18}H_{19}ClNaN_4O_{10}$).

***p*-Nitrophenyl 2-acetamido-4,6-di-*O*-acetyl-3-*O*-chloroacetyl-2-deoxy-β-D-glucopyranoside (24)**: $PPh_3$ (258 mg, 0.985 mmol) in anhydrous $CH_2Cl_2$ (1 mL) was added dropwise over a period of 30 min to a solution of the acetylated 2-azido-3-*O*-chloroacetate **23**

(339 mg, 0.697 mmol) and AcCl (89 μL, 1.3 mmol) in anhydrous $CH_2Cl_2$ (3.5 mL). Nitrogen evolution was immediately observed. After 1 h the solution was diluted with $CH_2Cl_2$ (50 mL), then washed with sat. aq. $NaHCO_3$ (20 mL) and $H_2O$ (2×20 mL), dried ($MgSO_4$), and concentrated. The crude oil was adsorbed onto silica gel and chromatographed (EtOAc/petroleum spirits 6:4→7:3) to give the 3-*O*-chloroacetylated acetamide **24** as colorless needles (226 mg, 64%). M.p. 216–217 °C (corrected); $[\alpha]_D = -55.5°$ ($c$ = 0.750, acetone); $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 1.95 (s, 3H; NAc), 2.07, 2.08 (2s, 6H; 2OAc), 3.99 (ddd, $J_{5,6}$ = 2.4, $J_{5,6'}$ = 5.6 Hz, 1H; H5), 4.04–4.08 (m, 1H; H2), 4.05 ($AB_q$, 2H; $CH_2Cl_2$), 4.18 (dd, $J_{6,6'}$ = 12.4 Hz, 1H; H6), 4.29, (dd, 1H; H6′), 5.17 (dd, 1H; H3 or 4), 5.59–5.64 (m, $J_{1,2}$ = 8.4 Hz, 2H; H1 and 3 or 4), 5.80 (d, $J_{NH,2}$ = 8.4 Hz, 1H; NH), 7.06–7.09, 8.18–8.21 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $CDCl_3$): $\delta$ = 20.86, 20.92, 23.55 (3C, $3CH_3$), 40.65 (1C, $CH_2Cl_2$), 55.27, 62.11, 68.35, 72.45, 73.54 (5C, C2,3,4,5,6), 97.51 (1C, C1), 116.78, 125.99, 143.37, 161.57 (6C, *p*NP), 167.57, 169.68, 170.65, 170.94 (4C, 4C=O); elemental analysis calcd (%) for $C_{20}H_{23}ClN_2O_{11}$: C 47.77, H 4.61, N 5.57; found: C 47.81, H 4.67, N 5.61.

***p*-Nitrophenyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (25)**: A suspension of the chloroacetylated acetamide **24** (267 mg, 0.531 mmol) and thiourea (62.4 mg, 0.820 mmol) in MeOH (15 mL) was refluxed for 6 h, and the resulting yellow solution was concentrated and adsorbed onto silica gel. Chromatography ($CHCl_3$/MeOH 95:5→9:1) afforded the 3-hydroxy derivative **25** as fine colorless needles (139 mg, 61%). M.p. 196–197 °C (corrected); $[\alpha]_D^{23} = -55.9°$ ($c$ = 0.675, MeOH); $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 2.05, 2.07, 2.14 (3s, 9H; $3CH_3$), 3.71 (ddd, $J_{1,2}$ = 8.4, $J_{2,3}$ = 9.8, $J_{NH,2}$ = 6.4 Hz, 1H; H2), 3.89 (ddd, $J_{4,5}$ = 9.9, $J_{5,6}$ = 2.4, $J_{5,6'}$ = 5.9 Hz, 1H; H5), 4.17 (dd, $J_{6,6'}$ = 12.4 Hz, 1H; H6), 4.23 (dd, $J_{3,4}$ = 9.4 Hz, 1H; H3), 4.29 (dd, 1H; H6′), 4.95 (dd, 1H; H4), 5.55 (d, 1H; H1), 5.99 (d, 1H; NH), 7.05–7.09, 8.17–8.21 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $CDCl_3$): $\delta$ = 20.94, 21.09, 23.78 (3C, $3CH_3$), 58.56, 62.51, 71.66, 72.02, 72.62 (5C, C2,3,4,5,6), 97.24 (1C, C1), 116.74, 125.99, 143.28, 161.58 (6C, *p*NP), 170.74, 170.93, 172.03 (3C, 3C=O); elemental analysis calcd (%) for $C_{18}H_{22}N_2O_{10}$: C 50.70, H 5.20, N 6.57; found: C 50.67, H 5.24, N 6.63.

***p*-Nitrophenyl 2-acetamido-2-deoxy-3-*O*-sulfo-β-D-glucopyranoside, sodium salt (2)**: A suspension of the 3-hydroxy derivative **25** (139 mg, 0.326 mmol) and $SO_3$·pyr (212 mg, 1.33 mmol) in anhydrous DMF/pyridine (1:1, 2 mL) was stirred under $N_2$ at 50 °C for 4 h. The reaction was stopped by the addition of $NaHCO_3$ (2.5 mL, 1 M), and the resulting suspension was concentrated (≤35 °C). The crude yellow residue was resuspended in MeOH and filtered, and the filtrate was chromatographed on a Sephadex LH-20 column (2×22 cm, eluted with MeOH/$H_2O$ 1:1). The carbohydrate-containing fractions underwent cation exchange by being passed through a Dowex 50W×8–400 column ($Na^+$ form, 2×5 cm, eluted with MeOH/$H_2O$ 1:1) to give the acetylated 3-sulfate **26** as a pale yellow glassy solid. The acetylated 3-sulfate was taken up in MeOH (20 mL), and a small piece of sodium metal was added. After deacetylation was complete, the reaction was stopped by acidification with Amberlite IR-120 ($H^+$ form) resin, the resin was removed by filtration, and the filtrate was immediately neutralized with 1 M $NaHCO_3$ and concentrated (≤35 °C). The crude material was resuspended in MeOH/$H_2O$ (1:1) and filtered, and the filtrate was chromatographed as described previously (Sephadex LH-20 followed by Dowex 50W×8–400) to afford the 3-sulfate **2** as a colorless amorphous solid (63.1 mg, 44%, over 2 steps). $^1$H NMR (400 MHz, $D_2O$): $\delta$ = 2.00 (s, 3H; $CH_3$), 3.72–3.79 (m, 2H; H4,5), 3.82 (dd, $J_{5,6}$ = 5.2, $J_{6,6'}$ = 11.9 Hz, 1H; H6), 3.97 (dd, $J_{5,6'}$ = 1.2 Hz, 1H; H6′), 4.14 (dd, $J_{1,2}$ = 8.8, 1H; H2), 4.52 (dd, $J_{2,3}$ = 10.4, $J_{3,4}$ = 8.4 Hz, 1H; H3), 5.44 (d, 1H; H1), 7.15–7.19, 8.18–8.22 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $D_2O$): $\delta$ = 22.85 (1C, $CH_3$); 54.65, 61.01, 69.07, 76.41, 81.48 (5C, C2,3,4,5,6), 98.67 (1C, C1), 117.19, 126.71, 143.29, 162.23 (6C, *p*NP), 175.49 (1C, C=O).

***p*-Nitrophenyl 3-*O*-acetyl-2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (27)**: A solution of benzylidene acetal **20** (1.14 g, 2.75 mmol), pyridine (3 mL) and $Ac_2O$ (1.5 mL, 15 mmol) was stirred until acetylation was complete. The mixture was diluted with EtOAc then washed with HCl (3×25 mL, 1 M), sat. aq. $NaHCO_3$ (2×25 mL) and sat. aq. NaCl (25 mL), dried ($MgSO_4$), and concentrated. The resulting colorless amorphous solid, the 3-*O*-acetylated benzylidene acetal **27** (1.09 g, 86%), was used without further purification. M.p. 142–144 °C (corrected); $[\alpha]_D^{23} = -0.53°$ ($c$ = 0.985, $CHCl_3$); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 2.16 (s, 3H; $CH_3$), 3.68 (ddd, $J_{4,5}$ = 9.24, $J_{5,6}$ = 1.6, $J_{5,6'}$ = 4.9 Hz, 1H; H5), 3.73 (dd, $J_{3,4}$ = 9.5 Hz, 1H; H4), 3.79–3.85 (m, 2H; H2,6), 4.38 (dd, $J_{6,6'}$ = 10.7 Hz, 1H; H6′), 5.14 (d, $J_{1,2}$ = 8.5, 1H; H1), 5.28 (t, $J_{2,3}$ = 9.9 Hz, 1H; H3), 5.52 (s, 1H; benzylidene-CH), 7.09–7.11, 8.19–8.22 (AA′XX′, 4H; *p*NP), 7.33–7.36, 7.43–7.44 (2m, 5H; Ar); $^{13}$C NMR (100.5 MHz, $CDCl_3$): $\delta$ = 21.02 (1C, $CH_3$), 64.58, 67.16, 68.42, 70.98, 78.37 (5C, C2,3,4,5,6), 100.28 (1C, C1), 101.89 (1C, benzylidene-CH), 116.97, 128.52, 136.63, 161.03 (6C, *p*NP), 126.16, 126.31, 129.49, 143.71 (6C, Ar), 169.75 (1C, C=O); IR (thin film): $\tilde{\nu}$ = 2111.1 ($N_3$), 1750.2 (C=O), 1518.3, 1343.8 ($NO_2$), 1238.8, 1218.9 $cm^{-1}$ (C–O); elemental analysis calcd (%) for $C_{21}H_{20}N_4O_8$: C 55.26, H 4.42, N 12.28; found: C 55.34, H 4.41, N 12.17.

***p*-Nitrophenyl 3-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranoside (28)**: Aqueous TFA (70%, 0.5 mL) was added dropwise to a solution of acetate **27** (1.09 g, 2.38 mmol) in $CH_2Cl_2$ (3 mL) at 0 °C, and the mixture was allowed to warm to RT. After 5 h, the mixture was neutralized with sat. aq. $NaHCO_3$ (4 mL), concentrated and triturated with petroleum spirits (5×3 mL) to afford the 3-*O*-acetate **28** (664 mg, 76%) as a yellow amorphous solid that was used directly in the next step or recrystallized to give colorless needles. M.p. 137–140 °C (corrected; EtOH); $[\alpha]_D^{23} = -0.10°$ ($c$ = 1.09, MeOH); $^1$H NMR (400 MHz, $CDCl_3$ + [D$_6$]DMSO): $\delta$ = 2.13 (s, 3H; $CH_3$), 3.51 (ddd, $J_{4,5}$ = 9.4, $J_{5,6}$ = 3.9, $J_{5,6}$ = 7.6 Hz, 1H; H5), 3.66 (dd, $J_{1,2}$ = 7.9, $J_{2,3}$ = 10.4 Hz, 1H; H2), 3.67 (dd, $J_{3,4}$ = 9.6 Hz, 1H; H4), 3.79–3.87 (m, 2H; H6,6′), 4.96 (dd, 1H; H3), 5.02 (d, 1H; H1), 7.06–7.09, 8.14–8.18 (AA′XX′, 4H; *p*NP); $^{13}$C NMR (100.5 MHz, $CDCl_3$ + [D$_6$]DMSO): $\delta$ = 21.12 (1C, $CH_3$), 61.50, 63.81, 68.53, 74.59, 77.50 (5C, C2,3,4,5,6), 99.55 (1C, C1), 116.69, 125.91, 143.17, 161.32 (6C, *p*NP), 170.53 (1C, C=O); IR (thin film): $\tilde{\nu}$ = 3369.0 (br s, OH), 2115.1 ($N_3$), 1746.3 (C=O), 1518.3, 1345.81 ($NO_2$), 1240.74 (C–O), 1078.2, 1040.5 $cm^{-1}$ (Ar); HRMS (ESI$^+$): *m/z* 391.0860 [*M*+Na]$^+$, requires 391.0866 ($C_{14}H_{16}NaN_4O_8$).

***p*-Nitrophenyl 3,6-di-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranoside (29)**: AcCl (41 μL, 0.57 mmol) was added dropwise to a solution of the crude 3-*O*-acetate **28** (162 mg, 0.439 mmol) in dry 2,4,6-collidine (2.5 mL) at −40 °C under $N_2$. The resulting suspension was kept at −40 °C for 2 h, then was allowed to warm to RT. After a further 2 h, the reaction was quenched with MeOH, and the mixture was diluted with $CH_2Cl_2$ (50 mL), washed with HCl (3×25 mL, 1 M) and sat. aq. $NaHCO_3$ (2×30 mL), dried ($MgSO_4$), and then concentrated. Chromatography of the resulting oil (EtOAc/toluene 3:7) gave the 2-azido-4-hydroxy derivative **29** as a pale yellow oil (115 mg, 64%). $[\alpha]_D = -42.9°$ ($c$ = 1.07, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 2.12, 2.21 (2s, 6H; $2CH_3$), 3.23 (d, $J_{H4,OH}$ = 4.3 Hz, 1H; OH), 3.63 (ddd, $J_{3,4}$ = 9.3, $J_{4,5}$ = 9.5 Hz, 1H; H4), 3.72 (ddd, $J_{5,6}$ = 2.4, $J_{5,6'}$ = 5.2 Hz, 1H; H5), 3.77 (dd, $J_{1,2}$ = 7.9, $J_{2,3}$ = 10.1 Hz, 1H; H2), 4.38 (dd, $J_{6,6'}$ = 12.4 Hz, 1H; H6), 4.47 (dd, 1H; H6′), 4.95 (dd, 1H; H3), 5.05 (d, 1H; H1), 7.11–7.15, 8.21–8.25 (AA′XX′, 4H; *p*NP); $^{13}$C NMR

(100.5 MHz, $CDCl_3$): $\delta = 21.02$, 21.12 (2C, $2CH_3$), 62.91, 63.52, 69.16, 74.95, 75.26 (5C, C2,3,4,5,6), 99.79 (1C, C1), 116.93, 126.07, 143.62, 161.25 (6C, *p*NP), 171.58, 171.65 (2C, 2C=O); IR (thin film): $\tilde{\nu} =$ 3472.2 (br s, OH), 2111.1 ($N_3$), 1742.3 (C=O), 1518.3, 1343.8 ($NO_2$), 1228.9 $cm^{-1}$ (C–O).

***p*-Nitrophenyl 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (30)**: A solution of $PPh_3$ (132 mg, 0.504 mmol) in anhydrous $CH_2Cl_2$ (0.5 mL) was added dropwise to the 2-azido-4-hydroxy derivative **29** (157 mg, 0.382 mmol) and AcCl (50 μL, 0.70 mmol) in anhydrous $CH_2Cl_2$ (3 mL). Nitrogen evolution was immediately observed. After 1.5 h, the solution was diluted with $CH_2Cl_2$ (30 mL), washed with sat. aq. $NaHCO_3$ (20 mL) and $H_2O$ (2× 20 mL), dried ($MgSO_4$), and concentrated. The crude oil was adsorbed onto silica gel and chromatographed ($CHCl_3$/MeOH 98:2→ 96:4) to give the 4-hydroxy derivative **30** as colorless needles (87.7 mg, 54%). M.p. 190–192 °C (decomp.; corrected) (lit. 190–192 °C[39]); $[\alpha]_D^{22} = -30.7°$ ($c = 0.705$, MeOH) (lit. $[\alpha]_D^{22} = -37°$ ($c = 0.9$, MeOH)[39]); $^1H$ NMR (400 MHz, $CDCl_3 + [D_6]$DMSO): $\delta = 1.67$, 1.84, 1.87 (3 s, 9H; $3CH_3$), 3.40 (ddd, $J_{3,4} = 9.2$, $J_{4,5} = 9.6$, $J_{OH,4} = 5.6$ Hz, 1H; H4), 3.54 (ddd, $J_{5,6} = 1.9$, $J_{5,6'} = 5.9$ Hz, 1H; H5), 3.97 (ddd, $J_{1,2} = 8.6$, $J_{2,3} = 10.2$, $J_{NH,2} = 9.2$ Hz, 1H; H2), 4.06 (dd, $J_{6,6'} = 12.0$ Hz, 1H; H6), 4.21 (dd, 1H; H6′), 4.92 (dd, 1H; H3), 5.07–5.09 (m, 2H; H1,4-OH), 6.85–6.89, 7.92–7.96 (AA′XX′, 4H; *p*NP), 7.29 (d, 1H; NH); $^{13}C$ NMR (100.5 MHz, $CDCl_3 + [D_6]$DMSO): $\delta = 20.52$, 20.69, 22.78 (3C, $3CH_3$), 53.21, 63.03, 68.02, 74.05, 74.84 (5C, C2,3,4,5,6), 98.08 (1C, C1), 116.36, 125.20, 142.25, 161.73 (6C, *p*NP), 170.19, 170.33, 170.55 (3C, 3C=O).

***p*-Nitrophenyl 2-acetamido-2-deoxy-4-*O*-sulfo-β-D-glucopyranoside, sodium salt (3)**: A suspension of the 4-hydroxy derivative **30** (94.0 mg, 0.221 mmol) and $SO_3$·pyr (140 mg, 0.879 mmol) in a mixture of anhydrous DMF and pyridine (1:1, 1.2 mL) was stirred under $N_2$ at 50 °C for 4 h. The reaction was stopped by the addition of $NaHCO_3$ (2 mL, 1 M), and the resulting suspension was concentrated (≤35 °C). The crude yellow residue was suspended in MeOH and filtered, and the filtrate was chromatographed on a Sephadex LH-20 column (2×22 cm, eluted with MeOH/$H_2O$ 1:1). The carbohydrate-containing fractions then underwent cation exchange through a Dowex 50 W×8–400 column ($Na^+$ form, 2×5 cm, eluted with MeOH/$H_2O$ 1:1). Concentration (≤35 °C) of the corresponding fractions gave the acetylated 4-sulfate **31** (50.1 mg, 51%) as a pale yellow glassy solid. The acetylated 4-sulfate was taken up in MeOH (10 mL), a small piece of sodium metal was added, and the solution was stirred until deacetylation was complete. The reaction was stopped by acidification with Amberlite IR-120 ($H^+$ form) resin, the resin was removed by filtration, and the filtrate was immediately neutralized with 1 M $NaHCO_3$ and then concentrated (≤35 °C). The crude material was suspended in MeOH/$H_2O$ (1:1) and filtered, and the filtrate was chromatographed as described above (Sephadex LH-20 followed by Dowex 50W×8–400) to afford the 4-sulfate **3** as a colorless amorphous solid (16.6 mg, 39% over 2 steps). $^1H$ NMR (400 MHz, $D_2O$): $\delta = 2.01$ (s, 3H; $CH_3$), 3.79–3.86 (m, 2H; H5,6), 3.92 (dd, $J_{5,6'} = 5.0$, $J_{6,6'} = 12.0$ Hz, 1H; H6′), 3.99 (dt, $J_{2,3} = 10.6$, $J_{3,4} =$ 9.8 Hz, 1H; H3), 4.12 (dd, $J_{1,2} = 8.2$ Hz, 1H; H2), 4.31 (m, $J_{4,5} = 9.4$ Hz, 1H; H4), 5.33 (d, 1H; H1), 7.16–7.20, 8.22–8.26 (AA′XX′, 4H; *p*NP); $^{13}C$ NMR (100.5 MHz, $D_2O$): $\delta = 22.68$ (1C, $CH_3$), 55.69, 60.89, 72.47, 75.34, 77.19 (5C, C2,3,4,5,6), 99.07 (1C, C1), 117.14, 126.71, 143.31, 162.25 (6C, *p*NP), 175.57 (1C, C=O).

### Hydrolysis of carbohydrate sulfates by sulfatases

*Materials*: Sulfatases from limpet, abalone and snail were commercially available from Sigma. *Pa*AtsA was recombinantly expressed and purified as reported.[43] Potassium *p*-nitrophenyl sulfate was prepared as reported.[44]

*Control assays with* p-*nitrophenyl sulfate*: Assays were performed in 1 cm polyacrylate cuvettes and contained potassium *p*-nitrophenyl sulfate (2 mM) in $K_2HPO_4/KH_2PO_4$/0.05% BSA (50 mM) at pH 6, which also contained α-cyclodextrin (10 mM) to a final volume of 0.5 mL. The cuvettes were incubated at 37 °C in a water bath. Sulfatase (sufficient to give a rate of 10–15 μM $min^{-1}$) was added, and the reaction was monitored by spectrophotometry at 410 nm for 4 min ($\Delta\varepsilon_{410} = 6715\ M^{-1}cm^{-1}$). For the Ba-PEG assay, the reaction mixtures were incubated for 1 h with sufficient sulfatase to give a rate of 8–9 μM $h^{-1}$.

*Coupled assay procedure*: Each cuvette containing the sulfated carbohydrate (2 mM) in $K_2HPO_4/KH_2PO_4$/0.05% BSA (50 mM) at pH 6 plus α-cyclodextrin (10 mM) to a final volume of 0.5 mL was preincubated at 37 °C in a water bath. The coupling enzyme, β-*N*-acetylhexosaminidase from *A. oryzae* (Sigma), was added (sufficient to reach a $V_{max}$ of 150 μM $min^{-1}$, resulting in $t_{0.90} = 10$ min), followed by sulfatase (10–20-fold more than for a 4 min assay of *p*-nitrophenyl sulfate), and the reaction was monitored by spectrophotometry at 410 nm for 5 h ($\Delta\varepsilon_{410} = 6715\ M^{-1}cm^{-1}$). The reaction rate (μM $min^{-1}$) was calculated and compared to those of corresponding assays with *p*-nitrophenyl sulfate, adjusted to the equivalent amounts of sulfatase. The rates in control reactions without sulfatase and, separately, without *A. oryzae* β-*N*-acetylhexosaminidase were measured to assess the contribution to the observed rates of cleavage by the coupling β-*N*-acetylhexosaminidase and by contaminating β-*N*-acetylhexosaminidase activity in each sulfatase preparation, respectively.

*Ba-PEG assay procedure*: Reaction mixtures contained carbohydrate sulfate (2 mM) in HOAc/NaOAc/0.05% BSA (50 mM) pH 5 for abalone and limpet sulfatases or in $K_2HPO_4/KH_2PO_4$/0.05% BSA (50 mM) pH 6 for sulfatases from snail and *Pa*AtsA. Each mixture was preincubated at 37 °C in a water bath prior to initiation of the reaction by addition of sulfatase (10–20-fold more than that used for a 1 h assay of *p*-nitrophenyl sulfate (2 mM) under the same conditions). The total reaction volume was 500 μL. A "time 0" aliquot (150 μL) was taken and frozen at −80 °C. After 18–21 h, two additional aliquots (150 μL) were taken and, in parallel with the "time 0" aliquot and a positive control assay with *p*-nitrophenyl sulfate, were assayed by using the Ba-PEG reagent. The reagent contained $BaCl_2$ (40 mM) and PEG-6000 (25 mM) in water and was seeded with $Na_2SO_4$ (50 mM) in water (2 μL per mL reagent) under vigorous stirring. The extinction coefficient ($\Delta\varepsilon_{600}$) was determined from a calibration curve with $Na_2SO_4$. The seeded reagent was prepared fresh and was used within 2–3 h after seeding. Samples for analysis (150 μL) were diluted to a total of 350 μL with the respective buffer, then HCl (50 μL, 1 M) was added to stop the enzymatic reaction, followed by the addition of seeded Ba-PEG reagent (100 μL) to give a final volume of 0.5 mL. After 5 min, the absorbance at 600 nm was measured. The rate of cleavage (μM $h^{-1}$) was calculated from the calibration curve and compared to the respective control assay with *p*-nitrophenyl sulfate, adjusted to the equivalent amounts of sulfatase. All sulfated compounds were shown to be stable under the assay conditions.

### Hydrolysis of carbohydrate sulfates by fungal β-*N*-acetylhexosaminidases

*General*: Citrate/phosphate buffer (pH 5.0) was prepared by mixing citric acid (24.3 mL, 0.1 M) and $Na_2HPO_4$ (25.7 mL, 0.2 M), diluting with water to 100 mL, and adjusting the pH to 5.0. The fungal strains producing β-*N*-acetylhexosaminidases (EC 3.2.1.52) originat-

ed from the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague (Czech Republic), or from the Culture Collection of the Institute of Microbiology (CCIM), Prague (Czech Republic). The strains were cultivated in submerse media as described previously.[33] Enzymes were obtained by $(NH_4)_2SO_4$ precipitation (80% sat.) of the cultivation media, and the precipitates were directly used for their respective reactions.

*Enzyme activity assay*: Reaction mixtures ($V_{tot} = 55$ μL) containing *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **10** (2 mM) and β-*N*-acetylhexosaminidase (0.6–0.8 mU) in citrate/phosphate buffer at pH 5.0 were incubated in microtitration plates with shaking at 35 °C. After 10 min, the reaction was stopped by adding $Na_2CO_3$ (150 μL, 1 M). Liberated *p*-nitrophenol was detected spectrophotometrically at 420 nm (Sunrise Absorbance Reader, Tecan). One unit of enzymatic activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute under the above conditions. β-*N*-Acetylhexosaminidase activity towards the carbohydrate sulfates was determined in the same way by using 12–16 mU of enzyme. In the case of 3-sulfate, which contained 14% *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (assessed by HPLC-UV), the hydrolysis rate was monitored between 10 and 20 min after the addition of enzyme; this ensured that all the *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **10** present was cleaved before the start of monitoring. The 4-sulfate contained 1.5% *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (assessed by HPLC-UV), and the measured cleavage rate was corrected correspondingly. The 2-*N*- and 6-sulfates were sufficiently pure.

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