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Doctor of Philosophy

MODIFIED SUBSTRATES IN β-*N*-ACETYLHEXOSAMINIDASE-CATALYZED SYNTHESIS

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I hereby declare that this thesis is based on my own research carried out in the Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic, between October 2002 and April 2006, except where due acknowledgment has been made in the text.

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ABSTRACT

This Ph. D. thesis is aimed as a systematic study of the substrate specificity and the synthetic potential of β-*N*-acetylhexosaminidases (EC 3.2.1.52) using unnatural substrates with various structural modifications.

Three main goals are pursued in this work. Firstly, gaining new information on the specificity of the active site of these enzymes, also with regard to their particular source. This is essential for seeking specific competitive inhibitors (in collaboration with Prof. Robina, Seville, Spain) and for proposing suitable candidates for cloning and directed mutagenesis (in collaboration with Doc. Bezouška). The second purpose is the optimization of the synthetic procedures using unnatural substrates under the catalysis by β-*N*-acetylhexosaminidases, and, thirdly, the preparation of novel hexosamine structures in transglycosylation reactions. These substances are strong ligands of activation receptors of natural killer cells. Therefore, they are applied in the development of multivalent glycomimetics with immunomodulatory effect, intended for cancer therapy (in collaboration with Doc. Bezouška). Another application of hexosamine structures is the design of competitive inhibitors of β-*N*-acetylhexosaminidases.

In the scope of this work, four areas of substrate modifications (a-d) were thoroughly exploited. The modifications in the glycon part of substrates comprised (a) the introduction of various *N*-acylamido groups at the C-2 position, (b) oxidation of the C-6 hydroxyl to an aldehyde and a uronic acid, and (c) the introduction of a cyano moiety to the C-6 position. A fundamental modification in the aglycon part (d) represented the substitution of the traditional *p*-nitrophenyl (C-*O* glycosidic linkage) by an azide moiety, thus creating a C-*N* linkage. In summary, thirteen modified substrates were synthesized in a combination of chemical and enzymatic (galactose oxidase) methods, seven of them were prepared for the first time. They were tested for hydrolysis and transglycosylation by over thirty β-*N*-acetylhexosaminidases from fungal strains available in culture collections at Charles University and at the Institute of Microbiology, Academy of Sciences of the Czech Republic. The conclusions from hydrolytic screening were considered in relation to the results of molecular modeling (β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066). As a result, possible reasons for untypical affinity of the enzymes to some substrates (*e. g.*, no cleavage of 2-acetamido-2-deoxy-β-D-galactopyranosyl azide) were suggested. Eight oligosaccharidic structures (six of

them were novel) were prepared by semi-preparative transglycosylation reactions (tens of miligrams), isolated and fully characterized. The isolated yields were mostly in the range of 16–37%, with the best yield being 78%. Further upscaling was feasible. β-*N*-Acetylhexosaminidase from *Talaromyces flavus* CCF 2686 showed the highest tolerance to the substrate modifications tested.

Two of the compounds prepared, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid and 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid-(1→4)-2-acetamido-2-deoxy-D-glucopyranose, proved to be among the best known ligands for the natural killer cell activation receptors tested, particularly for the CD69 protein. Another compound, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosiduronitrile, acted as a good competitive inhibitor (K_i = 7.6 μM with β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066). All the modified substrates presented in this work have been used in the synthesis by β-*N*-acetylhexosaminidases for the first time. Moreover, the application of glycosyl azides as efficient and practical donors for glycosidases (good water solubility necessary to supress the unwanted water activity, stability, and easy synthesis) is a completely new concept in the enzymatic synthesis. This approach is promising especially for *N*-acetyl-D-hexosamine structures, whose fluorides, which are used as popular glycosyl donors with other glycosidases, are very unstable.

In summary, this Ph. D. thesis demonstrates the efficacy of applying modified substrates in β-*N*-acetylhexosaminidase-catalyzed synthesis and discloses several novel compounds with noteworthy properties like immunoactivity and inhibitory potential. Its findings are directly applicable in further research, namely the synthetic methodology using glycosyl azides, and development of competitive inhibitors of glycosidases. New immunoactive compounds will be used as core building blocks for the preparation of multivalent glycomimetics for cancer therapy. β-*N*-Acetylhexosaminidase from *Talaromyces flavus* CCF 2686 was identified as an enzyme with an exceptionally broad substrate specificity. It is currently being purified in our laboratory and is a prospective candidate for cloning and directed mutagenesis.

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1 INTRODUCTION

1.1 Oligosaccharides

1.1.1 Properties and Biological Functions

Oligosaccharides – carbohydrates typically consisting of two to ten glycosidically linked monosaccharide units – belong to the most abundant structures in nature. Their impressive structural variety predestine them for a number of functions,[1,2,3] *e. g.*, molecular recognition and signalization, cell proliferation, and tissue organisation. As important markers of the cell surface, they enable the natural detection of defect molecules and cancerogenic cells, and as a vital component of the immune system, they stimulate defence mechanisms in living organisms.

1.1.2 Applications of Oligosaccharides

Oligosaccharides are employed in chemical, textile, food and biotechnological industry. Their immense information potential is especially valuable in medicine, both for diagnostic purposes and for drug development. Specific complex carbohydrate structures are applied in structure-activity relationship studies and in the design of glycomimetics with improved pharmacokinetics and binding affinity.[2] One prospective application of such glycomimetics is the elicitation of the immune response of an organism by stimulating the activation receptors of natural killer cells.

Natural killer cells are a separate class of lymphocytes, which have an important role in host defence against tumor and virally infected cells. The stimulation of their surface activation receptors (*e. g.*, NKR-P1 (rat), and CD69 (human)), if not stopped by an inhibition signal, iniciates the killing process of the contacted damaged cell ('kiss of death'). A systematic study of the rat NKR-P1 activation receptor revealed some patterns of its binding affinity.[4,5,6] An ideal ligand (Figure 1) is a (β1-4)-linked tetrasaccharide composed of *N*-acetyl-D-hexosamines (preferably ManNAc,[a] GalNAc, and GlcNAc). In addition, the presence of negatively charged groups (COOH, SO_3H) at the C-6 position strongly improves the binding affinity, possibly due to the interaction with Ca^{2+} ions (relation to C-type lectins). The construction of such a ligand

[a] All the carbohydrates mentioned in this work are D-enantiomers if not indicated otherwise.

structure is a challenging task and an efficient synthetic procedure is pivotal for further research leading to multivalent immunostimulating glycodendrimers.

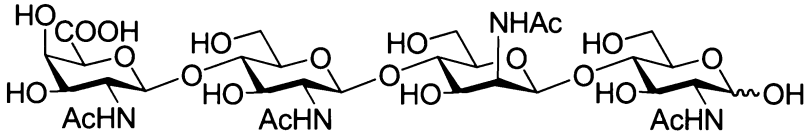


Figure 1 One of the proposed structures of an ideal binding ligand to NKR-P1 activation receptor of natural killer cells.

1.1.3 Approaches to Oligosaccharide Synthesis

Most oligosaccharides are commercially available only in minute quantities at extremely high prices and their isolation from biological structures is inefficient, costly and time consuming.[3] Higher oligosaccharides are readily obtained by chemical or enzymatic hydrolysis of polysaccharides, shorter carbohydrate chains are preferably synthesized *de novo*. Generally, there are two approaches to oligosaccharide synthesis: chemical and enzymatic methods. Although astonishing results are achievable by modern synthetic chemistry,[7] a number of protection, activation, and deprotection steps are required – even several dozens for complex oligosaccharides. This causes negligible yields, considerable material and time consumption. As a result, the enzymatic synthesis, ideally stereo- and regiospecific, comes in play. Two enzyme groups, glycosyltransferases (EC 2.4)[8] and glycosidases (EC 3.2.1), are employed in this field. Each of these methods, be it classical organic synthesis or enzyme catalysis, has its specific features, which predestine its applicability for a particular aim. Very good results can be accomplished by their combination (Section 1.4.4). This introduction is aimed to present the basic features of glycosidases, and particularly β-*N*-acetylhexosaminidases, which form the core of the presented thesis. More details can be found in the publications cited as well as in our own review on glycosidases (Appendix A1).

1.2 Glycosidases

1.2.1 Basic Characteristics

Glycosidases (*O*-glycoside hydrolases; EC 3.2.1) cleave oligo- and polysaccharides by transferring the glycosyl to a water molecule *in vivo*. If a more efficient nucleophile than water is present in a sufficient concentration in the reaction mixture, these enzymes are also able to form a new glycosidic linkage. The synthetic capability of glycosidases can be supported by a variety of strategies including the reduction of water activity (Section 1.2.2) and the use of

glycosyl donors activated by a good leaving group (Paragraph 1.2.5.2). Exoglycosidases (cleaving the oligosaccharidic chain from the non-reducing end) are more commonly used in the synthesis than endoglycosidases (cleaving internal glycosidic linkages). Nowadays, the sub-subclass of glycosidases encompasses 140 valid entries in IUBMB enzyme nomenclature system (International Union of Biochemistry and Molecular Biology, last update on Jan 6, 2006). Another classification, reflecting the amino acid sequence similarities, is described in the frequently updated CAZY (Carbohydrate-Active EnZYmes) database[9] (almost 100 families). β-*N*-Acetylhexosaminidases (EC 3.2.1.52) belong to family 20 of glycosidases. They are closely characterized in Part 1.3.

Glycosidases are readily available from natural sources like seeds, microorganisms, fungi and some higher organisms. Commercial crude enzyme preparations constitute another practical source. Availability, stability and easy handling are the main advantages of glycosidases over glycosyltransferases. They have also been the first enzymes to be used for the glycosidic bond synthesis.[10] Glycosidases are absolutely stereoselective and show a decent degree of enantioselectivity,[11,12] however, not as pronounced as lipases.[13] Importantly, they are rather undemanding in the choice of substrates (Section 1.2.5), which greatly broadens their use compared to glycosyltransferases. Their synthetic applications face drawbacks like low regioselectivity and yields, which can be minimized by an apt reaction design (Section 1.2.4).

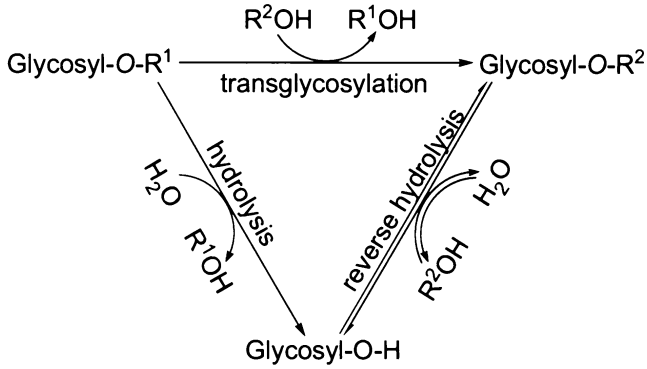
1.2.2 Reverse Hydrolysis and Transglycosylation

Glycosidases synthesize the glycosidic bond in two ways, depending on the substrate structure and the composition of the reaction medium.

- 'Reverse hydrolysis' – thermodynamically controlled equilibrium process

In this process (Scheme 1), a free monosaccharide is combined with a nucleophile under the exclusion of a water molecule. This condensation reaction is traditionally referred to as 'reverse hydrolysis'[10] though this term is chemically incorrect and is illogical like, *e. g.*, 'reverse mountain climbing.' The equilibrium constant of this process strongly favors hydrolysis over glycoside formation and can be shifted towards synthesis in several ways: (a) by decreasing the water activity by high reactant concentrations (*e. g.*, 80–90% w/w total sugar concentration),[14,15] or by adding salts;[16] (b) by removing the product from the reaction mixture (*e. g.*, on an active carbon column)[17] or (c) by reaction medium engineering, *e. g.*, using

organic solvents[18] or microwave field.[19] Generally, increased reaction temperature (50–60 °C) is necessary to bring the reaction to equilibrium on a reasonable time scale.[14,15] Reaction times are days or even weeks and yields do not exceed 15%. Although not as widely used as transglycosylation (see below), this approach resulted in several noteworthy products, such as non-reducing sugars[20] and thioglycosides.[21] Reverse hydrolysis is widely used for the glycosylation of alcohols (Paragraph 1.2.5.1).



Scheme 1 Reactions catalyzed by glycosidases.

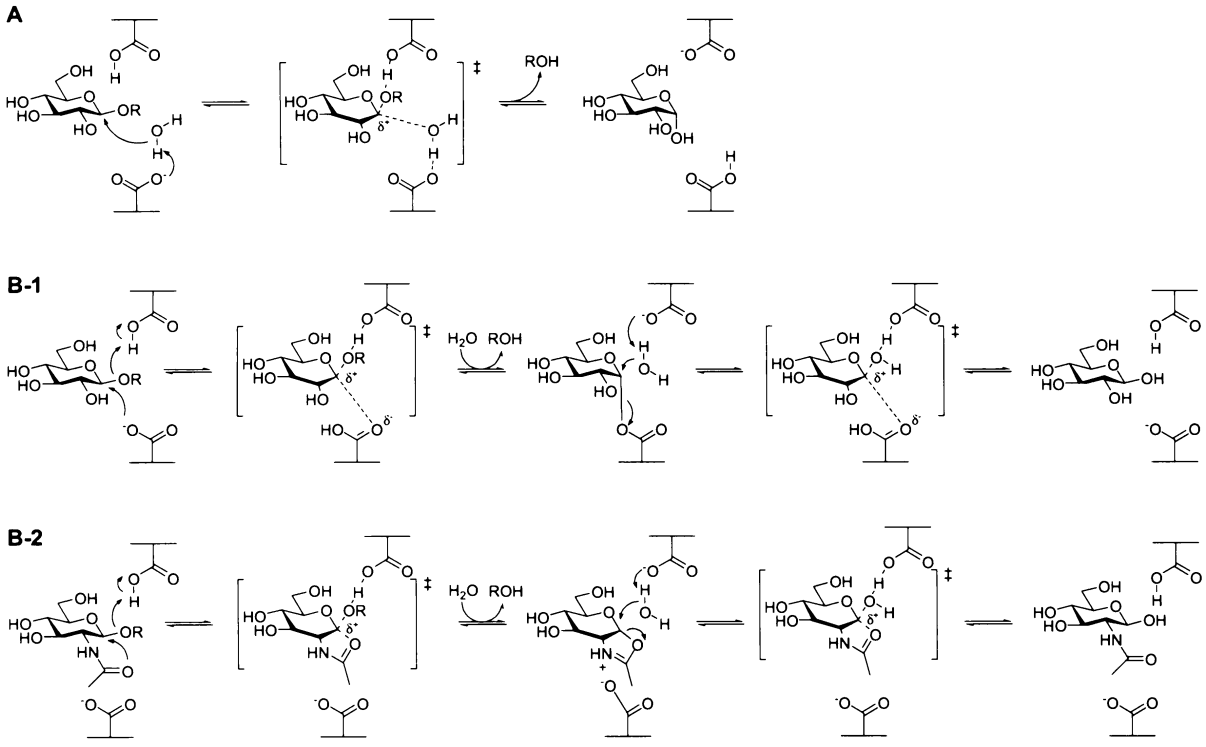
- Transglycosylation – kinetically controlled process

Transglycosylation (Scheme 1) employs an activated glycoside as a glycosyl donor. Similar methods[19,22,23] to those described with reverse hydrolysis are employed to reduce water activity and to increase the reaction conversion. In addition, the success of a transglycosylation reaction strongly depends on the choice of an activating leaving group (Paragraph 1.2.5.2). The product can accumulate at much higher concentrations than in the equilibrium process and, as a result, the reaction gives higher yields,[24] generally of 20–40%. Most glycosidase-catalyzed reactions are performed in this way. Water acts as a competing nucleophile and causes parasitic hydrolysis of the reactant. This problem was fully removed by mutant glycosidases, the so-called glycosynthases.[25] The substitution of the catalytic nucleophile (mostly glutamate) in the active site by an uncharged residue like alanine, serine or glycine (for glycosidase mechanism see below) leads to a complete abolishment of hydrolytic activity and to quantitative transglycosylation yields, when suitable donors like glycosyl fluorides are used.

1.2.3 Reaction Mechanism

There are many studies dealing with the molecular mechanism of glycosidase action.[26,27] In summary, the glycosidic bond hydrolysis can proceed in two ways, resulting in the net

inversion of configuration at the anomeric carbon (inverting enzymes) or *vice versa* (retaining enzymes). Both ways involve similar oxocarbenium-ion-like transition states (Scheme 2). Inverting glycosidases (Scheme 2A) act by a single-step, acid/ base catalyzed mechanism, in which the leaving group is directly displaced by the nucleophilic water molecule. Retaining glycosidases (Scheme 2B-1), which often have transglycosylating abilities, hydrolyze *via* a double-displacement mechanism. The catalytic machinery involves two catalytic carboxylates: an acid/ base and a nucleophile. In the first step (glycosylation), the former carboxylate provides an acid-catalyzed leaving group departure simultaneously with a nucleophilic attack by the other residue to form the glycosyl-enzyme intermediate. In the second step (deglycosylation), the acid/ base carboxylate acts as a general base to activate the incoming nucleophile (water or another acceptor), which hydrolyzes the glycosyl-enzyme intermediate yielding a new glycosidic linkage. β-*N*-Acetylhexosaminidases (Scheme 2B-2) utilize a double-displacement mechanism, in which the nucleophile is not donated by the enzyme but by the 2-acetamido group of the substrate itself, forming an oxazoline intermediate.



Scheme 2 Hydrolysis mechanisms of an inverting (A) and retaining (B-1) glycosidase and a β-*N*-acetylhexosaminidase, which uses a modified retaining mechanism (B-2).

1.2.4 Regioselectivity

Contrary to glycosyltransferases, glycosidases exhibit a rather poor regioselectivity. If more than one acceptor hydroxyl is present, the transglycosylation reaction mostly results in a complex mixture, difficult to separate (Section 1.4.3). In general, the primary hydroxyl group reacts preferentially to the secondary ones, giving yield to (1-6)-linked products. If a free hexopyranose is used as an acceptor, the preference of individual hydroxyls for the glycosidic bond formation mostly declines in this order:[28] 6-OH >> 4-OH ≥ 3-OH > 2-OH >> 1-OH. Hence, although it is rather improbable, the glycosyl moiety can also be transferred to C-1 of an acceptor and a non-reducing sugar can be formed.[29] We also observed this behavior in *Aspergillus* β-*N*-acetylhexosaminidases.[20,30]

Though less common, regioselective glycosidases can be found in nature, *e. g.*, β-galactosidases from *Escherichia coli*[24] and *Aspergillus oryzae*[31] (β1-6), from *Diplococcus pneumoniae* (β1-4)[32] and from bovine testes (β1-3).[33] If several regioisomers are formed, the regioselectivity may be enhanced by modification of several factors, *e. g.*, reaction time[34] and the acceptor anomeric substitution (the so-called 'remote anomeric effect').[35,36,37] The use of C-6 protected acceptors can also lead to a regioselectivity enhancement.[38,39,40]

1.2.5 Substrate Specificity

1.2.5.1 Specificity towards Glycosyl Acceptors

The specificity of glycosidases towards glycosyl acceptors is very broad. Theoretically, any substance containing at least one hydroxyl or a thiol group can be glycosylated, even an oxime.[41] The tolerance of glycosidases towards acceptors differs according to the enzyme source.[3] Many complex and sensitive compounds can be selectively glycosylated, like cardiac genins,[42] nucleosides,[43] UDP-sugars,[44] antibiotics,[45] ergot alkaloids,[46] and hydroxy amino acids.[47] A large group of common glycosyl acceptors are alcohols.[48] The originated glycosides are widely applicable as, *e. g.*, non-ionic detergents.[49] The formation of thioglycosides (with sulphur replacing the glycosidic oxygen atom) by *O*-glycosidases is rather rare. First thioglycosylations were reported in the mid-1990s.[50,51] and other examples followed.[21,52] The originated thioglycosides were practically resistant to further hydrolysis.[21,51] A reliable approach leading to thioglycosides represent thioglycoligases, the acid/ base mutants of retaining glycosidases.[53]

1.2.5.2 Glycosyl Donors Substituted at C-1

Kinetically controlled transglycosylation reactions require the presence of a glycosyl donor, suitably activated by a leaving group at its anomeric position. A good glycosyl donor generally has two main features: it binds strongly to the enzymatic active site and enables a fast formation of the glycosyl-enzyme intermediate. The high affinity of enzyme to the glycosyl donor (*i. e.*, low K_m) and a fast reaction (*i. e.*, high k_{cat}) minimize the risk of product hydrolysis. The efficacy of a certain enzyme-donor system is commonly expressed as k_{cat}/K_m. Apart from these characteristics, the cleavage rate of the glycosyl donor should be simply and fast detectable (Section 1.4.1).

The structure of the leaving group is a decisive factor for donor properties. Natural substrates for glycosidases are polysaccharidic chains and the corresponding disaccharides are still used in transglycosylations, as they are efficient, easily available and cheap (*e. g.*, *N*,*N*′-diacetylchitobiose for β-*N*-acetylhexosaminidases).[34] However, the use of a saccharide leaving group sometimes leads to low yields due to an insufficient activation and side hydrolytic and autocondensation reactions. In many applications, disaccharidic substrates are substituted by synthetic donors. Their synthesis is sometimes time-consuming, but this drawback is fully outweighed by their high efficacy (K_m).[54] Nitrophenyl glycosides are among the most efficient donors known. Their outstanding efficiency is mainly due to the stabilizing interaction ('stacking') of the planar phenyl with the hydrophobic active-site amino acids, and due to the stabilization of the enzyme-substrate complex by the charged nitro group and the π-electron density.

The substitution at the aromatic ring is an important feature, too – an electron-withdrawing substituent enhances the electrophilic character of a leaving group, *e. g.*, a phenyl glycoside is a worse substrate than the corresponding *p*- or *o*-nitrophenyl derivative. On the other hand, electron-donating substitutents can render the substrate even inert to enzyme hydrolysis, *e. g.*, *p*-aminophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside is not hydrolyzed by fungal β-*N*-acetylhexosaminidases (Fialová and Křen, unpublished results). The key factors influencing the reactivity of a leaving group (number and position of aryl substituents, their character) reflect on pK_a of the corresponding phenol derivative.[55,56] As a result, the plot of log (k_{cat}/K_m) *vs.* pK_a (Brönsted plot) showed a relatively high correlation (Table 1, Figure 2). Its concave-downward shape is typical for a two-step hydrolytic mechanism of retaining

glycosidases. Naturally, other interactions in the active site may occur and therefore, the final relations cannot be foreseen with absolute security.

Table 1 Kinetical parameters of hydrolysis of substituted phenyl β-D-glucopyranosides by β-glucosidases from almond[55] and *Agrobacterium sp.*[56] (for plot see Figure 2).

Phenyl substituent	pK_a †	log $(k_{cat}/K_m)^{rel}$ ‡	
		almond β-glucosidase	*Agrobacterium sp.* β-glucosidase
2,4-dinitro	3.96	3.27	3.03
3,4-dinitro	5.36	3.45	3.33
4-nitro	7.18	2.41	2.93
3-nitro	8.39	2.10	2.34
4-cyano	8.49	2.18	2.36
4-chloro	9.38	0.90	1.25
H	9.99	0	0

† pK_a values of the respective substituted free phenols
‡ $(V_{max}/K_m)^{rel}$ is the ratio of V_{max}/K_m of the respective substituted phenyl β-D-glucopyranoside related to that of phenyl β-D-glucopyranoside.

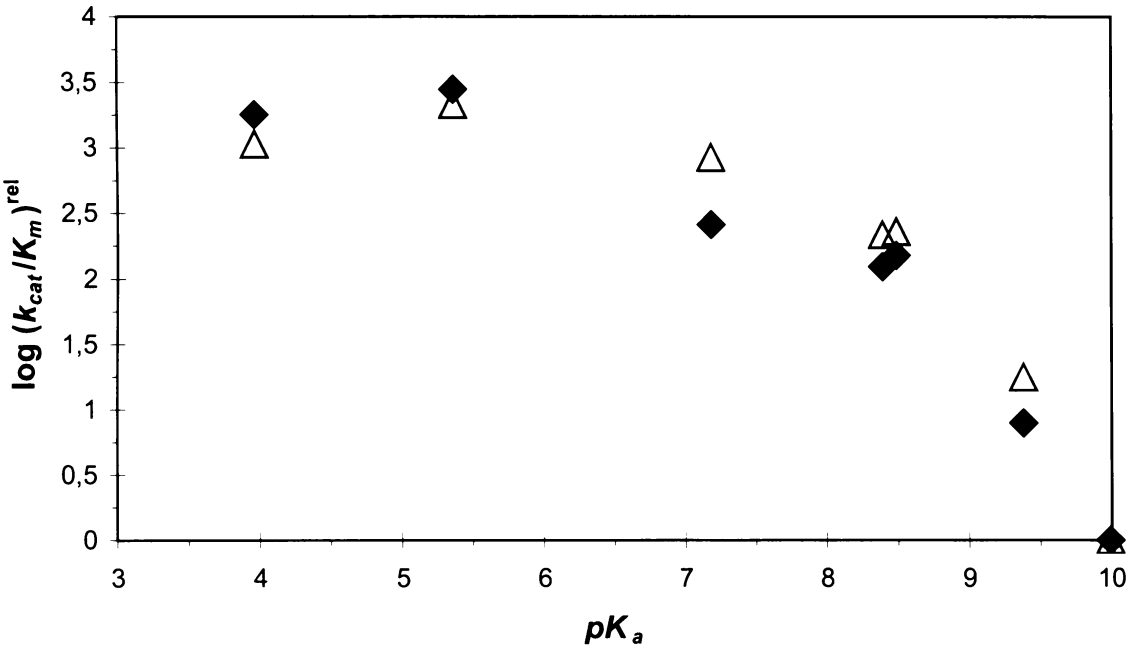


Figure 2 Brönsted plot for the hydrolysis of various phenyl β-D-glucopyranosides by β-glucosidases from almond[55] (◆) and *Agrobacterium sp.*[56] (△). $(k_{cat}/K_m)^{rel}$ is the ratio of k_{cat}/K_m of the substituted phenyl β-D-glucopyranoside related to that of phenyl β-D-glucopyranoside (*i. e.*, $\log(k_{cat}/K_m)^{rel}$ for the latter is 0). For pK_a values of the respective substituted free phenols, see Table 1.

A relatively high donor concentration is essential for effective transfer with a low risk of side hydrolysis. Hydrophobic leaving groups, though providing more efficient substrate cleavage, often cause solubility problems. Organic cosolvents (Section 1.4.2) can sometimes be a solution; however, new effective and highly soluble glycosyl donors are still sought for: 3-nitro- and 5-nitro-2-pyridyl glycosides,[57] vinyl glycosides,[58] and also other donors than *O*-glycosides.

Glycosyl fluorides are established donors both in mechanistic studies and in syntheses[59] although their use is not universal (*e. g.*, 2-acetamido-2-deoxy-D-glycosyl fluorides are very unstable).[60] The main advantages of fluorine as a leaving group is its small size (causing minimum steric hindrance in the active site), its ready detection by ^{19}F NMR spectroscopy, and its electron-withdrawing potential, which facilitates its easy departure during cleavage. Glycosyl fluorides are readily synthesized[61] and their efficacy is comparable to the best aryl glycoside donors known. Their main drawback is a rather low stability (weeks).

A novel alternative to aryl glycosides and glycosyl fluorides are glycosyl azides. They combine some advantages of the above donors, *e. g.*, the small size of a leaving group, strong nucleophilic character and delocalized π-electron density. Additionally, they are exceptionally stable and perfectly water-soluble. They represent a good alternative especially for *N*-acetyl-D-hexosamine structures, whose corresponding fluorides are unstable.[60] Cleavage of glycosyl azides was first observed in β-glucosidase from *Agrobacterium sp.*[62] We were the first to use glycosyl azides as donors in transglycosylation reactions (Appendix A5) and all the aspects of this methodology are described in Section 4.2. Apart from β-*N*-acetylhexosaminidases (Appendix A5), good results have been obtained with β-galactosidases and β-glucosidases (Petrásková, Fialová, Křen, unpublished results). β-D-Glucopyranosyl azide has recently been used as a donor in a coupled screening for thioglycoligase activity.[63]

Several other compounds appeared in the literature as donors for glycosidases, however, their broader utility is to be demonstrated yet – *e. g.*, oxazolines,[64,65,66] and D-glycals.[67,68]

1.2.5.3 Glycosyl Donors Substituted at C-6

The first results on the use of C-6 modified glycosyl donors with glycosidases were published in the late 1990s by Wong's group.[69] *p*-Nitrophenyl β-D-*galacto*-hexodialdo-1,5-pyranoside

was used in transglycosylations catalyzed by β-galactosidase from *Bacillus circulans*. MacManus *et al.*[70] and Hušáková *et al.*[38] elaborated this concept using other C-6 substituted *p*-nitrophenyl glycosides.

Transglycosylations with modified glycosyl donors enable to introduce a reactive substituent to a complex molecule, which can be further modified (*e. g.*, reduced, oxidized or conjugated to amines or hydrazides). We synthesized a novel immunoactive disaccharide of β-D-Gal*p*NAcA-(1→4)-D-Glc*p*NAc from the aldehyde by transglycosylation and subsequent chemical oxidation (Appendix A3). Details are given in Paragraph 4.1.2.1. Weingarten *et al.*[71] showed another use of *p*-nitrophenyl β-D-*galacto*-hexodialdo-1,5-pyranoside on β-galactosidase from *Bacillus circulans*.

1.2.5.4 Glycosyl Donors Substituted at Other Hydroxyls

A high tolerance towards a change in the C-4 hydroxyl configuration is a typical feature of β-*N*-acetylhexosaminidases. They hydrolyze and transfer both β-D-Glc*p*NAc and β-D-Gal*p*NAc structures, though with different affinities. The *gluco*-structures are generally more willingly accepted – the β-D-Glc*p*NAc-ase/β-D-Gal*p*NAc-ase activity ratio commonly ranges among 1.5 and 4.0.[54] Some enzymes, *e. g.*, from *Penicillium oxalicum*, even exhibit a clear preference for β-D-Gal*p*NAc structures.[72] The aglycon moiety also plays a surprisingly important part – we observed practically no hydrolysis of 2-acetamido-2-deoxy-β-D-galactopyranosyl azide by β-*N*-acetylhexosaminidases, in contrast to its *p*-nitrophenyl analogue (Appendix A5).

Enzymatic recognition of glycosyl donors modified at a secondary hydroxyl has been studied since the early 1970s.[73] An important group of secondary hydroxyl modifications represent fluoroglycosides,[59] which are used as mechanism-based inhibitors with retaining glycosidases,[74] for example 2,4-dinitrophenyl 2-deoxy-2-fluoro-D-glycosides,[75] and 5-fluoro-D-glycosyl fluorides,[76] suitable even for β-*N*-acetylhexosaminidases[77] (the unsubstituted C-2 position is occupied by 2-acetamido moiety, essential for β-*N*-acetylhexosaminidase substrates).

The C-2 amino group of hexosaminides offers a wide range of modifications. The 2-acetamido group is a crucial structural feature for accepting the substrate by β-*N*-acetylhexosaminidases

and they are quite sensitive towards its changes. Studies of the tolerance towards *N*-acyl modified substrates as well as a search after new 'acylhexosaminidase' activities have taken place since the 1970s.[78,79] The affinity of various β-*N*-acetylhexosaminidases towards *N*-acyl modified substrates was examined by Molodtsov and Vafina.[80,81] Our recent study (Appendix A2) disclosed several β-*N*-acetylhexosaminidases (especially from *Penicillium oxalicum* and *Aspergillus oryzae*) with a broad substrate specificity towards *N*-acyl modified substrates.

1.3 β-*N*-Acetylhexosaminidases

1.3.1 Basic Characteristics

β-*N*-Acetylhexosaminidases (EC 3.2.1.52, family 20 of glycosidases; systematic name 2-acetamido-2-deoxy-β-D-hexopyranoside acetamidodeoxyhexohydrolases) are exoglycosidases, which catalyze the hydrolysis of terminal β-D-Glc*p*NAc and β-D-Gal*p*NAc residues in nature. Some microbial, human and animal enzymes show a high degree of homology, which suggests a common evolutionary ancestor.[82] Fungal enzymes are mostly heavily glycosylated and occur as dimers, whereas bacterial ones are monomeric.[82] The reaction mechanism, regioselectivity and substrate specificity of β-*N*-acetylhexosaminidases are described in Sections 1.2.3 – 1.2.5.

β-*N*-Acetylhexosaminidases are widely distributed in plants, animals, fungi, and microorganisms. They belong among the most active lysosomal glycosidases[83] and they are fundamental for chitin degradation.[82] In human organism, β-*N*-acetylhexosaminidase activity and isoenzyme pattern are clinically important markers of various disorders,[84] especially in nephrology, urology, and pediatry, notably also of Tay-Sachs and Sandhoff diseases.[83] Their industrial applications[82] comprise structural characterization of the glycosylation patterns of glycolipids and glycoproteins, synthesis of glycostructures by transglycosylation, and design of antifungal agents in medicine and agriculture.

β-*N*-Acetylhexosaminidases are readily obtained as minor components from crude commercial enzyme preparations, *e. g.*, the amylase preparation Takadiastase,® and crude β-D-galactosidase from *Aspergillus oryzae* (Sigma). The main problem of these sources is the low specific activity of the desired enzyme (< 0.3 U/mg protein) and its variable content in different batches of the preparation.[85] Fungi constitute a more reliable source of β-*N*-acetylhexosaminidases, especially *Aspergillus* and *Penicillium* genera.[82] Most

microorganisms produce extracellular β-*N*-acetylhexosaminidase, easy to isolate from the reaction medium. They are constitutive or inducible and the production of the latter can significantly be enhanced by adding inducers[82] to the reaction medium, *e. g.*, chitobiose, higher chitooligosaccharides, GlcNAc, and GalNAc.

1.3.2 Inhibitors of β-*N*-Acetylhexosaminidases

Apart from common inhibitory substances like urea and heavy metals, a range of other compounds affects β-*N*-acetylhexosaminidase activity.[82] Many β-*N*-acetylhexosaminidases show competitive inhibition by acetate – hence the reason for using citrate-phosphate buffer and not acetate buffer as a reaction medium.[84] Some free monosaccharides[73] also exhibit inhibitory effect, *e. g.*, GlcNAc (K_i = 19 mM for *Aspergillus oryzae* β-*N*-acetylhexosaminidase). β-*N*-Acetylhexosaminidases are also often inhibited by high substrate concentrations. Interestingly, this inhibition strongly depends on the aglycon type, *e. g.*, 2-acetamido-2-deoxy-β-D-glucopyranosyl azide shows no inhibitory effect, in contrast its *p*-nitrophenyl analogue (Appendix A5).

Competitive inhibitors of β-*N*-acetylhexosaminidases are important for mechanistic and structure-activity relationship studies – fluoroglycosides (Paragraph 1.2.4.4) are a typical example. Inhibitors of fungal β-*N*-acetylhexosaminidases[54,84] are applied in the therapy of fungal infections in human and veterinary medicine and in agriculture. They are mainly structural analogues of the enzyme substrates or hydrolytic intermediates, *e. g.*, of GlcNAc, GalNAc or oxazolines. Typical modifications comprise formal oxidation or reduction at the anomeric centre (2-acetamido-2-deoxy-D-glucono-1,5-lactone), substitution at C-1 (nagstatin) or a change of the ring heteroatom (2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucopyranose) – see Figure 3. K_i Values of the most efficient competitive inhibitors varies in the 1–30 nM range.

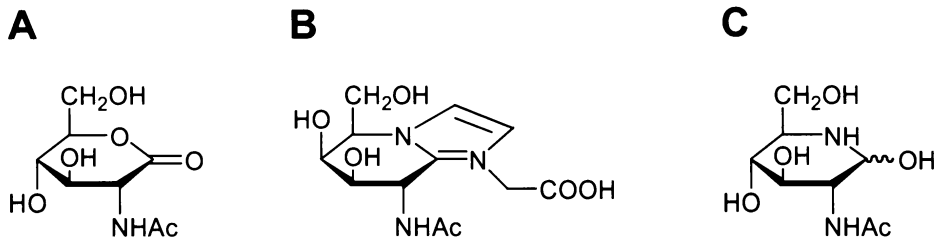


Figure 3 Competitive inhibitors of β-*N*-acetylhexosaminidases: 2-acetamido-2-deoxy-D-glucono-1,5-lactone (A), nagstatin (B), and 2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucopyranose (C).

1.4 Glycosidase-Catalyzed Reactions

1.4.1 Monitoring of Reaction Progress

Kinetical studies and large activity screenings require a fast, simple and reproducible method for measuring the reaction progress with minimum false-negative and false-positive signals. Generally, there are two possibilities of measuring the cleavage rate: assay of declining donor concentration or assay of increasing concentration of the hydrolyzed monosaccharide or the leaving group. Universal methods comprise TLC, GC or HPLC, which are, however, quite laborious, measure discontinuously and allow rather a qualitative assessment. GC and HPLC may give reasonable information on quantity if a calibration is available. Monosacharide concentration can be measured, *e. g.*, in coupled assays.[86]

Therefore, the reaction progress is preferably monitored as the increasing concentration of a suitable leaving group. The most common method is spectrophotometry (*e. g.*, for phenyl glycosides). It is relatively cheap and enables either continuous (real-time) or discontinuous (end-point)[87] assay. There are other methods like fluorimetry[88] and chemiluminometry.[89] Besides the cheap colorimetric assay of *p*-nitrophenol, fluorimetry is widely used, also due to its high sensitivity. A helpful modification is the microtiter plate format, which saves both time and material. Another useful monitoring technique, ^{1}H NMR,[90] is able to follow even slow reactions *in situ*. It is also commonly used to determine the glycosidase character (retaining/ inverting) by monitoring the anomeric configuration of the released monosaccharide (Appendix A5).

1.4.2 Unconventional Reaction Designs

Aqueous solution, the natural milieu for the enzyme action, has many limitations, such as the restriction to hydrophilic reagents, reduced yields due to product and activated donor hydrolysis, unfavorable shift of thermodynamic equilibrium, unwanted side reactions, and risk of microbial contamination. Medium engineering[91,92] (*i. e.*, tailoring of the reaction environment using cosolvents) can solve some of the above problems. Apart from the common monophasic and biphasic aqueous-organic systems and non-aqueous systems, other media are described in the literature, *e. g.*, ionic liquids,[93] plasticized glass phase,[23] ice media[94] and supercritical fluids.[95]

Glycosidases are scarcely used in purely non-aqueous solvent systems.[96] More popular are homogenous water-solvent media. In such media, the nature and the amount of cosolvent are

crucial for the enzyme activity and stability and the effects are only hardly predictable. Low concentrations of cosolvents (5–25% v/v) are known to enhance the initial reaction rate as well as the enzyme stability compared to the neat water medium (Appendix A2). By contrast, cosolvent concentrations over 70–80% v/v mostly cause rapid enzyme inactivation. Synthesis by glycosidases is typically performed in a monophasic solvent-water system to increase the solubility of hydrophobic substrates, to reduce water activity (ideally, to 0.7–0.8) and to shift the equilibrium in reverse hydrolysis. Examples include reactions in media containing acetonitrile,[45,67,97] acetone,[67,98] diethyl ether,[98] dioxane,[45] and *tert*-butyl alcohol.[99]

To better resist the detrimental effect of low-water media, the enzyme molecule can be modified *e. g.*, by covalent attachment to a polymeric support,[100] by cross-linking,[101] non-covalent coating[102] or encapsulation into micelles.[103] This leads to heterogeneous reaction systems.[92,104] Stabilization can also be achieved by genetic manipulation.[105]

1.4.3 Purification of Products of Glycosidase Catalysis

The low regioselectivity of glycosidases (Section 1.2.4) usually results in a mixture of regioisomeric products, whose separation is difficult due to their similar chromatographic behavior. Additionaly, use of nitrophenyl glycosides brings about the necessity of removing the high amount of nitrophenol, released during transglycosylation (extraction with diethyl ether is necessary). This complication is avoided using glycosyl fluorides and azides, as the released small ions do not complicate the separation, contrary to the bulky nitrophenol. Complex saccharidic mixtures are preferably pre-purified[106] before the 'polishing' gel chromatography step – *e. g.*, by trapping on XAD resins, ion-exchangers[107] or by charcoal-celite chromatography.[17] This can help to avoid the laborious separations by preparative HPLC. Peracetylation followed by silica gel chromatography is applicable if the compounds are better distinguishable in the peracetylated state.

Another pre-purification can be done by selective glycosidases, which cleave the unwanted regioisomers[34,108,109] However, this method may be relatively costly, as the unwanted, though often expensive 'contaminating' oligosaccharide product is cleaved to monosaccharides. Therefore, a better solution, at least for *N*-acetyl-D-hexosamine structures, is the chromatography on polyolic gels in borate buffer.[110] During the separation, borate makes a

stable complex with the saccharides of *manno*-configuration, which are then eluted prior to the uncomplexed *gluco*- and *galacto*-structures.

1.4.4 Glycosidases in Multi-Enzyme Reactions

Complex carbohydrate structures can be synthesized by a combination of several enzymes in one-pot or sequential mode. Crude intermediate products may be either directly processed by the next enzyme, or a simple purification step may be included, such as desalting or concentrating. This approach saves both time and costs, however, the enzymes used should be quite specific, regioselective and high yielding.

A typical multi-enzyme reaction comprises the use of a glycosidase and a glycosyltransferase. Probably the first example is a one-pot reaction by Herrmann *et al.*[111] He coupled β-galactosidase from *Bacillus circulans* and α-2,6-sialyltransferase from pig liver to prepare *N*-acetyl-α-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-galactopyranose (26%). In addition, CMP-Neu5Ac, the sialyl donor, was enzymatically regenerated *in situ*. Křen and Thiem[33] adopted an analogous approach in the synthesis of a sialylated T-antigen. Other approaches include the use of sialidases[32] and transsialidase from *Trypanosoma cruzi*.[112]

Another useful enzyme combination is a tandem of galactose oxidase–glycosidase. For instance, *p*-nitrophenyl β-D-glucopyranoside was oxidized to an aldehyde at C-6 to increase the efficacy of transglycosylation with *Bacillus circulans* β-galactosidase.[69] The resultant disaccharide was reduced back by $Na[BH_4]$ yielding the target methyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside. In our recent work (Appendix A3), galactose oxidase from *Dactylium dendroides*, β-*N*-acetylhexosaminidase from *Talaromyces flavus* and *in situ* chemical oxidation were combined to prepare an immunoactive disaccharide of β-D-Gal*p*NAcA-(1→4)-D-Glc*p*NAc.

2 WORKING HYPOTHESIS

2.1 Background Research

The increasing demand for new carbohydrate materials, applicable both in the industry and research, is directly related to the need for efficient synthetic procedures. In the design of various oligosaccharide derivatives, the enzymatic synthesis with modified substrates is a clear method of choice. Although this topic has been thoroughly studied with glycosyltransferases,[113] the potential of glycosidases was long neglected. Their substrate specificity was just examined in terms of the hydrolytic potential.[73,78,79,80,81,114] The first publications on the synthesis with structurally modified substrates catalyzed by glycosidases appeared only in the late 1990s.[38,69,70] Glycosidases have excellent eligibility for wide synthetic applications with modified substrates – they are stable, cheap and readily available, and their substrates (glycosides) are much cheaper than those of glycosyltransferases (nucleotide-activated sugars).

2.2 Aims and Envisaged Outputs

This Ph. D. thesis is aimed as a systematic study of the **substrate specificity** and the **synthetic potential** of **β-*N*-acetylhexosaminidases** with **modified substrates**. There are two **main targets** in this work:

(a) **To investigate the tolerance of β-*N*-acetylhexosaminidases of various origins towards selected substrate modifications**. For this aim, a range of modified substrates will be prepared by chemical or chemo-enzymatic synthesis. The modifications studied (at C-1, C-2, and C-6) will be chosen with respect to the interest for structure-activity relationship studies, the potential applicability of synthesized oligosaccharides, and the feasibility based on previous research. Extensive screening data will be collected from the Culture Collection of Fungi, available in the Laboratory of Biotransformation. The experimental results will be correlated to molecular modeling results (β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066).

(b) **To optimize the synthesis with modified substrates** concerning, *e. g.*, transglycosylation yields, and problems with substrate solubility. Glycosyl azides will be tested as substrates in β-*N*-acetylhexosaminidase-catalyzed synthesis. This will be an entirely new synthetic concept, never before tested with glycosidases. Glycosyl azides should be a suitable alternative to the

currently used glycosyl fluoride donors, which would be practicable even with β-*N*-acetylhexosaminidases (the respective fluorides are unstable).

The properties of the prepared modified oligosaccharides will be examined concerning their biological activity (namely, the affinity to natural killer cell activation receptors) and their inhibition potential towards β-*N*-acetylhexosaminidases.

The **main outputs** of this work should be:

(a) **Structure-activity relationship studies** of a large set of β-*N*-acetylhexosaminidases and modified substrates. Their results will be used, *e. g.*, for selecting suitable candidates for planned cloning and directed mutagenesis, and for designing new competitive inhibitors.

(b) **Optimized synthetic methodologies** with modified substrates.

(c) Novel, fully characterized **hexosamine structures** with direct applications in further research (multivalent glycomimetics with immunomodulatory effect, competitive inhibitors).

3 METHODS

In the scope of this Ph. D. thesis, the methodologies from the following areas have been combined: chemical synthesis, enzymology, carbohydrate purification and structural analysis, and immunology. An alphabetical list of the methods used is given below. All the details can be found in the experimental sections of the full-text publications included as Appendices A2–A5.

3.1 Analytical Methods

- Analytical High-Performance Liquid Chromatography
- Circular Dichroism
- Mass Spectrometry
- Molecular Modeling
- Nuclear Magnetic Resonance
- Optical Rotation
- Thin-Layer Chromatography

3.2 Separation Methods

- Flash Chromatography on Silica Gel
- Gel Permeation Chromatography
- Ion-Exchange Chromatography
- Preparative High-Performance Liquid Chromatography
- Solid-Phase Extraction on Amberlite XAD-4 Resin

3.3 Enzymological Methods

- Bradford Protein Assay
- Cultivation of Fungal Strains and Preparation of Crude β-*N*-Acetylhexosaminidases
- End-Point Activity Assay of β-*N*-Acetylhexosaminidases
- Hydrolytic and Transglycosylation Screening
- Kinetic Measurements (Determination of V_{max}, K_m, K_i)
- Oxidation by Galactose Oxidase in a Batch Reactor with Bubble-Free Aeration
- Preparative Transglycosylation Reactions

3.3 Assays of Affinity to Natural Killer Cell Activation Receptors

Quantitative Binding and Inhibition Assays on Microtiter Plates: determination of the affinity of tested ligands to natural killer cell activation receptors – NKR-P1 and CD69.

3.4 Methods of Chemical Synthesis

In the scope of this work, the following substrates for β-*N*-acetylhexosaminidases have been synthesized chemically or chemo-enzymatically. The numbers in brackets indicate the compound numbering in Chapter 4 of this thesis and do not necessarily correspond to the numbering in the respective publications.

3.4.1 Substrates Modified at C-2 (Appendix A2)

p-Nitrophenyl 2-amino-2-deoxy-β-D-glucopyranoside (**2**), *p*-nitrophenyl 2-deoxy-2-formamido-β-D-glucopyranoside (**3**), *p*-nitrophenyl 2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (**4**), *p*-nitrophenyl 2-deoxy-2-glycoloylamido-β-D-glucopyranoside (**5**), *p*-nitrophenyl 2-deoxy-2-propionamido-β-D-glucopyranoside (**6**).

3.4.2 Substrates Oxidized at C-6 (Appendix A3)

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid (**13**), methyl (*p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosid)uronate (**14**).

3.4.3 Substrates Modified at C-6 (Appendix A4)

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosiduronitrile (**16**), *p*-nitrophenyl 2-acetamido-2,6-dideoxy-β-D-*gluco*-heptopyranosyluronononitrile (**17**), 2-acetamido-2,6-dideoxy-β-D-*gluco*-heptopyranosyluronononitrile azide (**18**).

3.4.4 Substrates Modified at C-1 (Appendix A5)

2-Acetamido-2-deoxy-β-D-glucopyranosyl azide (**19**), 2-acetamido-2-deoxy-β-D-galactopyranosyl azide (**20**).

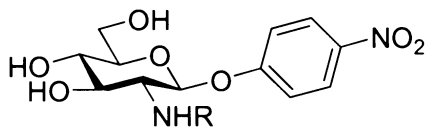
4 RESULTS AND DISCUSSION

4.1 β-*N*-Acetylhexosaminidases and Substrates Modified in the Glycon Part

4.1.1 Modifications at C-2 (Appendix A2)

The presence of the acetamido group at the C-2 position of the pyranose ring is a crucial structural feature of all β-*N*-acetylhexosaminidase substrates. These enzymes are also considered to be rather sensitive towards its modifications. Moreover, the substrate acetamido group plays an essential role both in the hydrolytic and synthetic mechanism (Scheme 2B-2), forming the oxazoline reaction intermediate. Although some examples of the hydrolysis of *N*-acyl modified substrates were published (Paragraph 1.2.5.4), nobody has ever presented any complex structure-activity relationship studies related to the enzyme source, either any synthetic applications.

For these reasons, we selected five *N*-acyl modified derivatives of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**[b], the standard substrate) – see Figure 4 – and subjected them to a hydrolysis and transglycosylation screening with a large set (35) of fungal β-*N*-acetylhexosaminidases. The tested C-2 modifications included a charged group (2-amino), a highly electronegative group (2-trifluoroacetamido), and some sterically more (2-propionamido, 2-glycoloylamido) or less (2-formamido) demanding groups than the standard 2-acetamido moiety. For the substrate synthesis, see Appendix A2. The β-*N*-acetylhexosaminidases were mainly produced by the strains of *Aspergillus*, *Penicillium* and *Talaromyces* genera, available from culture collections at Charles University (Culture Collection of Fungi, CCF) and Institute of Microbiology, Czech Academy of Sciences.[c]



1: R = Ac (standard substrate)
2: R = H (2-amino)
3: R = CH=O (2-formamido)
4: R = $COCF_3$ (2-trifluoroacetamido)
5: R = $COCH_2OH$ (2-glycoloylamido)
6: R = $COCH_2CH_3$ (2-propionamido)

Figure 4 *N*-Acyl modified substrates tested with β-*N*-acetylhexosaminidases.

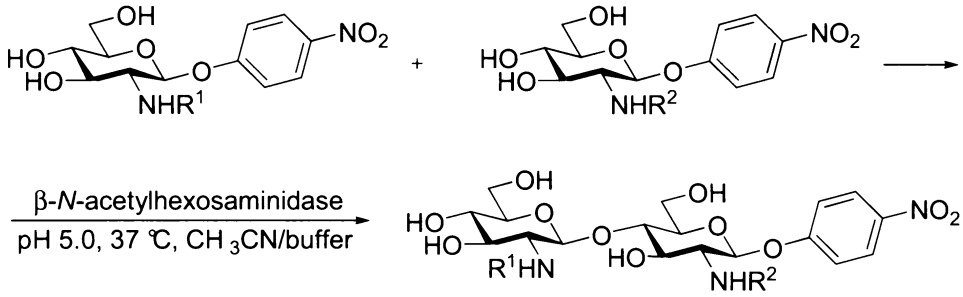
[b] For the sake of unity, new compound numbering was introduced in this Chapter, which does not necessarily correspond to the numbering in the respective publications.

[c] If not indicated otherwise, β-*N*-acetylhexosaminidases used in this work were crude ammonium sulfate precipitates (20–80% sat.). Other glycosidase activities were determined to be negligible in the samples.

In the hydrolytic screening, the enzymes fairly tolerated certain sterical changes at C-2 (shorter or longer acyls, a hydroxyl instead of a hydrogen) – in over 50% cases, the hydrolysis rates exceeded 20%[d] related to **1**; in the case of substrate **6**, the rates were even comparable to the standard. Nevertheless, neither the highly electronegative trifluoroacetyl, nor the charged free amino group were accepted (hydrolysis rate lower than 1% related to the standard substrate). In molecular modeling experiments, all the substrates were docked into the active site of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF[e] 1066 and the interaction energies of the enzyme-substrate complexes were calculated. The results indicated that the modified substrates bind relatively well (comparably to standard **1**) into the enzymatic active site and that the lower conversion to the hydrolytic product is rather a result of the destabilization of the oxazolinium reaction intermediate, or it is on another level of the 'dynamic' part of the hydrolytic process.

The best hydrolyzing enzymes were subjected to transglycosylation assays with the substrates, whose hydrolysis was feasible (**3**, **5**, and **6**). Despite the lower hydrolytic potential, transglycosylation products (Scheme 3, products **7–9**) were prepared in high yields (78% for product **8**). This was probably due to the fact that the transglycosylation product, also a poor substrate, was not readily hydrolyzed either and once formed, it accumulated in the reaction mixture. In these reactions, acetonitrile was used as a cosolvent (for its concentrations, see Scheme 3) due to the limited water solubility of the modified sugars. A surprising result was the formation of product **10**. In this case, β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 preferred to hydrolyze and transfer the modified 2-propionamido-2-deoxy-β-D-glucopyranosyl moiety and used the intact standard substrate **1** as an acceptor. The other product, which we expected (*i. e.*, 2-deoxy-2-acetamido-β-D-glucopyranosyl transferred to the intact modified acceptor **6**), was not detected at all in the reaction mixture.

[d] As a rule of thumb, this is a good result in the hydrolysis of modified substrates by glycosidases and it is fully sufficient for transglycosylation reactions.
[e] CCF Culture Collection of Fungi, deposited at Charles University, Faculty of Science, Department of Botany.



Product	R	Source of enzyme	CH_3CN [%]	Yield [%]
7	$R^1 = R^2 = CH{=}O$	*Talaromyces flavus* CCF 2686	45	16
8	$R^1 = R^2 = COCH_2OH$	*Talaromyces flavus* CCF 2686	5	78
9	$R^1 = R^2 = COCH_2CH_3$	*Penicillium oxalicum* CCF 2315	45	24
10	$R^1 = COCH_2CH_3$ $R^2 = Ac$	*Aspergillus oryzae* CCF 1066	45	1.8

Scheme 3 Transglycosylation reactions with *N*-acyl modified substrates.

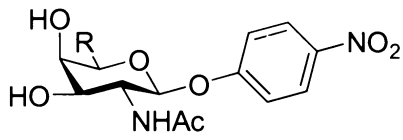
This study clearly demonstrated that besides cleavage, fungal β-*N*-acetylhexosaminidases are able to catalyze synthetic transglycosylation reactions with *N*-acyl modified substrates in high yields. Out of the five modifications tested, three of them (2-formamido, 2-glycoloylamido, and 2-propionamido) were decently accepted; the two modifications comprising charge and electronegativity changes (2-amino, 2-trifluoroacetamido) were not tolerated. Enzymes from *Penicillium oxalicum* CCF 2316 and from *Talaromyces flavus* CCF 2686 showed broad substrate specificity towards *N*-acyl modified substrates, combined with the highest transglycosylation yields. In summary, four novel oligosaccharides **7–10** were prepared.

4.1.2 Modifications at C-6 (Appendices A3 and A4)

4.1.2.1 C-6 Oxidized Compounds with Immunomodulatory Effect (Appendix A3)

Glycosidases are known to accept substrates that carry even substantial modifications at the C-6 position of the pyranose ring (Paragraph 1.2.5.3), and use them both for hydrolysis and synthesis. This relative 'benevolence' towards the changes in the primary hydroxyl opens a promising field for the introduction of various reactive functionalities into the oligosaccharidic structures that would enable further modifications, such as reduction, oxidation or conjugation (*e. g.*, with amines or hydrazides).[115] Thus, target oligosaccharides can be prepared and embodied into large biological structures.

One research project in the Laboratory of Biotransformation is aimed at the synthesis of ligands binding to activation receptors of natural killer cells, especially to rat NKR-P1 and human CD69 proteins (Section 1.1.2). Previous results[5] identified the oligosaccharides with 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid as particularly prospective for this aim. Therefore, we prepared a set of C-6 oxidized *p*-nitrophenyl glycosides (*p*-nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranoside (**12**), the respective uronic acid **13** and its methyl ester **14**; Figure 5) by chemoenzymatic synthesis. As starting material, we used *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**11**), which was also the standard compound in screening assays. Selective oxidation of the C-6 primary hydroxyl to an aldehyde can theoretically be accomplished, *e. g.*, by laccase-TEMPO oxidation,[116] however, a mixture of the aldehyde and the uronic acid is usually formed. Therefore, we decided to use galactose oxidase from *Dactylium dendroides*, highly specific for the carbohydrate C-6 position.[115,117] This enzyme needs continuous oxygen supply, however, a simple diffusive gas transport through the solution surface[71,118] led to poor yields in our hands. Bubbling by air or oxygen quickly deactivates the enzyme, probably due to the denaturation in the gas/ liquid interphase area. Therefore, in collaboration with Prof. Elling (Aachen, Germany), we optimized their methodology for the preparative oxidation in a batch reactor with bubble-free oxygen supply by doubling the batch and by twelve times reducing the amount of enzyme used. This method enabled a quantitative oxidation on a 100-mg scale with a considerably lower consumption of enzymes and time compared to literature.[71,115,118] The relatively unstable[119] aldehyde was purified by Dowex 50W-X8 in Ca^{2+} cycle. For the details and the chemical synthesis of the uronic acid and of its methylester, see Appendix A3.



11: R = CH_2OH (standard substrate)
12: R = $CH(OH)_2$ (aldehyde)
13: R = COOH (uronic acid)
14: R = COOMe (methyl uronate).

Figure 5 C-6 Oxidized substrates tested with β-*N*-acetylhexosaminidases.

We studied the enzymatic recognition of substrates **12–14** by a set (36) of fungal β-*N*-acetylhexosaminidases mainly of *Aspergillus*, *Penicillium* and *Talaromyces* genera. All tested enzymes readily cleaved aldehyde **12** (ca. 20% related to **11**), contrary to both uronic acid **13** and methyl uronate **14**. Molecular modeling with β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 revealed that aldehyde **12** binds to the enzyme in the form of a

geminal diol, which is also the prevailing form in the water solution. On the other hand, uronic acid **13** and methyl uronate **14** bound weakly to the active site due to a loss in hydrogen bonding, in which the steric parameters of the carboxy group seemed to hamper more than its charge. This shows the limitations of the use of C-6 modified substrates with β-*N*-acetylhexosaminidases.

Using aldehyde **12** as a glycosyl donor, a novel disaccharide of β-D-Gal*p*NAcA-(1→4)-D-Glc*p*NAc (**15**, for structure see Figure 6), a LacNAc analogue, was synthesized by transglycosylation (β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686) followed by selective chemical oxidation at C′-6 *in situ*, in an overall yield of 37%. For the scheme see Appendix A3.

All the prepared compounds were tested for binding to two representative natural killer cell activation receptors: NKR-P1A (rat) and CD69 (human). Both uronic acid **13** and disaccharide **15** proved to be high-affinity ligands of these receptors. The introduction of a carboxy moiety led to a ca. 10-fold increase in the binding potency to NKR-P1 protein, compared to the respective unoxidized compounds. With the CD69 protein (see Figure 6), the affinity increase was even more profound – up to four orders of magnitude, and thus, disaccharide **15** became the best ligand ever described for this receptor. For further details, see Appendix A3.

This concept demonstrated the potential of combining the multienzyme and chemical approaches and it is the first systematic study of the structure-activity relationship with a complete series of C-6 oxidized substrates. Moreover, the very high affinity of the novel disaccharide **15** to CD69 protein represents an entirely novel observation, which will be applied in the development of therapeutically useful glycomimetics.

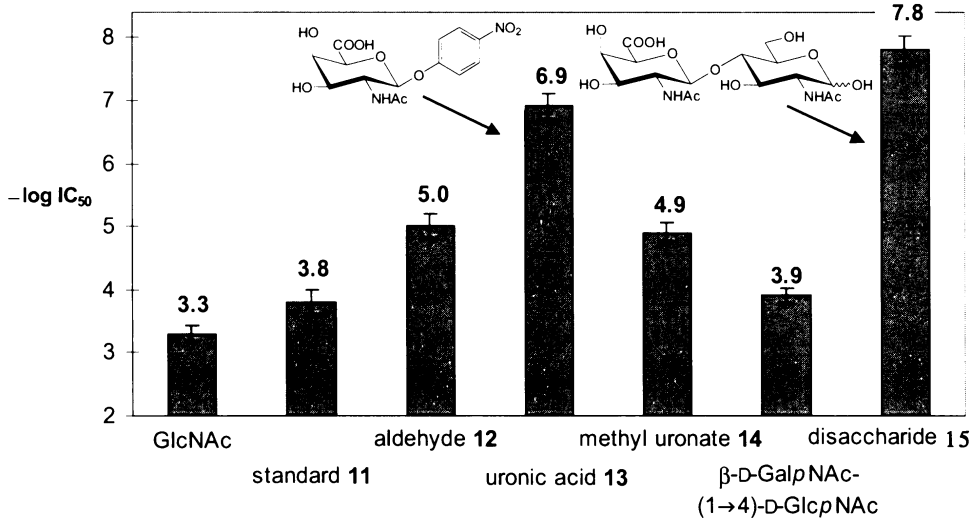


Figure 6 Affinity of new ligands towards the human natural killer cell activation receptor, CD69, expressed in the logaritmic scale (−log IC_{50}). The higher value, the better ligand. The affinity increase brought by the carboxy group is seen when comparing −logIC_{50} of uronic acid **13** (6.9) and disaccharide **15** (7.8) to the values of respective unoxidized compounds (3.8 and 3.9, respectively). The '−log IC_{50}' value represents the negative decimal logarithm of the concentration of the respective sugar derivative, at which the degree of saturation of the CD69 receptor by this derivative was the same as by the standard ($GlcNAc_{23}BSA$ conjugate).

4.1.2.2 Sugar Nitriles – Inhibitors of β-N-Acetylhexosaminidases (Appendix A4)

The relatively broad substrate specificity of glycosidases towards substrates modified at the C-6 position (Paragraphs 1.2.5.3. and 4.1.2.1) drew our attention to another class of C-6 modified substrates – sugar nitriles: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosiduronitrile (**16**), *p*-nitrophenyl 2-acetamido-2,6-dideoxy-β-D-*gluco*-heptopyranosylurononitrile (**17**), and 2-acetamido-2,6-dideoxy-β-D-*gluco*-heptopyranosylurononitrile azide (**18**). For structures see Figure 7. The highly versatile functionality such as a nitrile group brings about the possibility of further modifications,[120] *e. g.*, reduction to an amino group followed by conjugation with aldehydes or isothiocyanates or hydrolysis to the respective carboxylic acid.

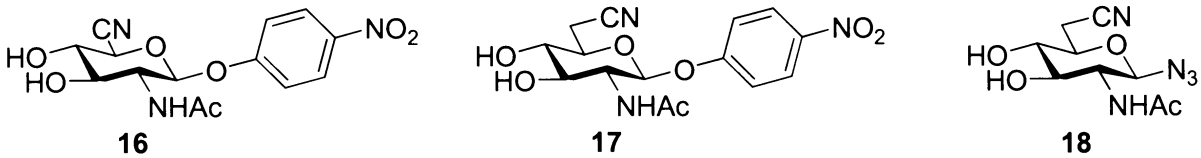


Figure 7 Sugar nitriles tested with β-*N*-acetylhexosaminidases.

The synthesis of *p*-nitrophenyl glycosides started from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**), used also as a standard compound in the hydrolysis screening. After several unsuccessful tries (see Appendix A4), compound **17** was prepared by four subsequent steps, comprising protection of C-3 and C-4 hydroxyls by butanediacetal, iodination at C-6, displacement of the C-6 iodine by tetrabutylammonium cyanide and deprotection in aqueous trifluoroacetic acid, in an overall yield of 18.5%. Azide **18** was prepared in 15% overall yield from 2-acetamido-2-deoxy-β-D-glucopyranosyl azide (**19**). The synthetic route was analogous to that for nitrile **17**, only 6-*O*-triflation was used instead of iodination. For details and schemes of both syntheses see Appendix A4. Nitrile **16** (66% overall yield) was prepared by C-6 Swern oxidation of the respective bis-acetal, followed by a transformation of the C-6 aldehyde to a nitrile group in the presence of iodine and ammonia in tetrahydrofuran and acidic deprotection (for the scheme see Appendix A4). All three compounds were prepared and characterized for the first time.

Compounds **16–18** were subjected to a hydrolysis screening comprising 33 fungal β-*N*-acetylhexosaminidases (mainly *Aspergillus*, *Penicillium* and *Talaromyces* genera). Owing to the lack of a chromophore moiety, the hydrolysis of compound **18** was analyzed by TLC. None of the compounds proved to be a good substrate for the enzymes tested – the best results were obtained with compound **17** and β-*N*-acetylhexosaminidases from *Talaromyces flavus* CCF 2686 (3.3% relative to **1**), *Penicillium pittii* CCF 2277 (3.0%) and *Hamigera avellanea* CCF 2923 (2.9 %). With compound **16**, *Fusarium oxysporum* CCF 377 (3.4%) proved to be the best source. In other cases, negligible or no hydrolysis was observed (lower than 1% relative to **1**). With such a low hydrolytic potential, transglycosylation reactions were not feasible due to a very high enzyme consumption.

However, the docking of compounds **16–18** into the active site of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 and the calculation of the enzyme-substrate complex interaction energies (Table 2) brought rather surprising results. It is known from other experiments (Appendices A2, A3, and A5) that the total interaction energy of a poor substrate with this enzyme is typically lower than ca. –170 kJ/mol and that it ranges between ca. –300 and –240 kJ/mol for well hydrolyzed substrates. Thus, our results (Table 2) clearly indicate that substrates **16–18** bind quite well to the active site, although azide **18** binds slightly weaker than nitrophenyl glycosides **16** and **17**, similarly to azide **19** (Appendix A5). Considering these

facts, we proposed a hypothesis that compounds **16–18** may be competitive inhibitors of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066.

Table 2 Interaction energies of compounds **16–18**, compared to the underivatized, well hydrolyzed substrates **1** and **19**. Substrates were docked into the active site of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066.

Substrate	Interaction energy [kJ/mol] [†]		
	Total	Steric	Electrostatic
p-Nitrophenyl standard **1**	−300	−84	−216
16	−238	−96	−142
17	−208	−65	−143
Azide standard **19**	−257	−57	−200
18	−249	−86	−163

[†] Drop in the enzyme-substrate complex interaction energy reflects better substrate binding into the enzymatic active site.

To verify this assumption, we determined the hydrolytic activity of this enzyme in the presence of compounds **16–18**. The results (Table 3) as well as the inhibition kinetic studies showed that compound **16** is a strong competitive inhibitor of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 (K_i = 7.6 μM with K_m for **1** being 0.75 mM – see Appendix A5). Inhibitory properties were also demonstrated in compound **17** (K_i = 0.37 mM). Thus, compound **16**, though a structural analogue of **17**, exhibits a 50-fold stronger inhibition effect, which can be explained by stronger binding into the enzymatic active site (Table 2). Compound **18** is not an inhibitor, probably because it binds more weakly into the enzymatic active site (Table 2).

Table 3 Residual activities of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 in the presence of **8–10**.

Inhibitor concentration [mM]	Substrate/inhibitor ratio	Residual activity [%] [†]		
		Compound **8**	Compound **9**	Compound **10**
0	–	100	100	100
0.2	10	88	100	16
0.4	5	82	100	10
1	2	60	100	3
2	1	44	100	1

[†] Residual activity was determined as the ratio of hydrolytic activities towards standard **1** (2 mM) in the presence and absence of inhibitors **16–18** in the respective concentration.

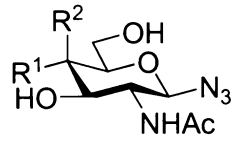
In the scope of this study, three novel nitriles **16-18** were synthesized. These compounds proved to be bad substrates for the β-*N*-acetylhexosaminidases tested. However, compound **17** and particularly compound **16** were shown to be potent competitive inhibitors of a

representative β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066. These results were in full accordance with the molecular modeling experiments that implied good binding of the compounds into the active site of this enzyme. As a result, conversion of C-6 hydroxyl into a cyano moiety, such as in compound **16,** appears to be a promising route to other efficient glycosidase inhibitors.

4.2 β-*N*-Acetylhexosaminidases and Glycosyl Azides (Appendix A5)

Nitrophenyl glycosides are widely used donors in transglycosylations (Paragraph 1.2.5.2). Nevertheless, despite their good reactivity, they often have solubility problems (especially if modified, see Appendix A2), which lowers transglycosylation yields and requires the use of organic cosolvents. Furthermore, commercially available nitrophenyl glycosides are quite expensive and nitrophenol, released during the reaction, complicates the purification (Section 1.4.3). A good alternative are glycosyl fluorides, which, however, cannot be used with β-*N*-acetylhexosaminidases as the respective fluoride substrates are unstable.[60]

Therefore, we developed a new methodology using glycosyl azides as donors (C-N bond cleaved) for β-*N*-acetylhexosaminidases. Glycosyl azides (Figure 8) can be prepared easily and in a high yield, they are stable and well water-soluble. Moreover, the azide ion is easily removable, which facilitates the purification of transglycosylation reaction mixtures.



19: R^1 = OH, R^2 = H
20: R^1 = H, R^2 = OH

Figure 8 Glycosyl azides tested with β-*N*-acetylhexosaminidases.

The hydrolysis of azides **19** and **20** was tested with 20 fungal β-*N*-acetylhexosaminidases, selected on the basis of our previous studies (Appendices A2–A4). The results were compared to the standard *p*-nitrophenyl glycosides **1** and **11**, respectively. Due to the lack of a chromophore moiety, the common colorimetric assay[87] was not applicable, and an HPLC determination method was developed (Polymer IEX H^+ column).

Surprisingly, the azide aglycon had a great influence on the substrate specificity of β-*N*-acetylhexosaminidases. Typically, these enzymes tolerate both the equatorial and axial

configurations of the C-4 hydroxyl and accept both β-D-Glc*p*NAc and β-D-Gal*p*NAc structures (Paragraph 1.2.5.4); however, with the azide substrate, we observed practically no hydrolysis of azide **20** (*galacto*-configuration), whereas **19** (*gluco*-) was readily hydrolyzed (ca. 10% related to standard **1**). Thus, the enzymes became selective β-*N*-acetylglucosaminidases. This behavior is absolutely unique and to our knowledge, it has not been observed with other aglycons.

This unexpected experimental result correlated well with the conclusions drawn from molecular modeling (Figure 9). Both glycosyl azides were docked into the active site of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 and the interaction energies of the enzyme-substrate complex were calculated, considering both resonance structures of the azido group ($-N=N^+=N^-$ and $-N^--N^+\equiv N$, see Appendix A5). The interaction energies for the binding of azide **19** (*gluco*-) ranged around –260 kJ/mol, which indicates good binding into the active site (see also Appendices A2–A4). However, the binding of azide **20** (*galacto*-) showed an insufficient drop in the total interaction energy (–165 kJ/mol), which corresponds to weak or no binding. Additionally, a too large distance between **20** and the glutamic acid 519 resulted in a substantial loss in hydrogen bonding in the active site (Figure 9B).

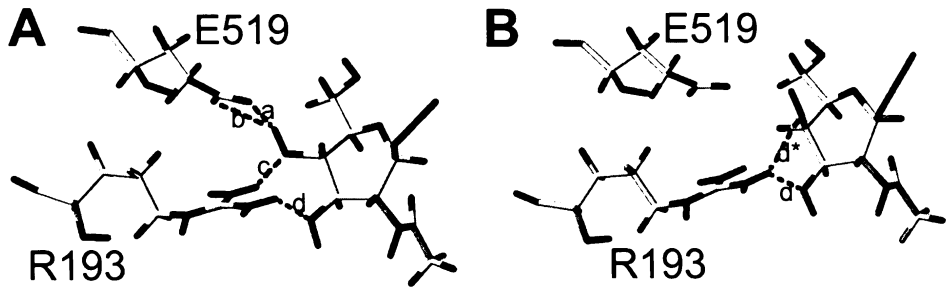


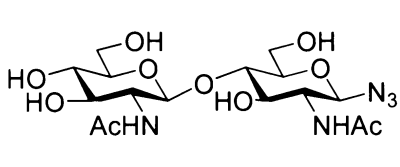
Figure 9 Azides **19** (A) and **20** (B) docked into the active site of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066. Glycosides are fixed by hydrogen bonding (a–d, in green) to glutamic acid 519 and arginine 193. The azido group is depicted as a blue stick.

The hydrolysis of azide **19** and of *p*-nitrophenyl glycoside **1** by two representative enzymes, β-*N*-acetylhexosaminidases from *Aspergillus oryzae* CCF 1066 (used in docking experiments) and from *Talaromyces flavus* CCF 2686 (best in hydrolysis – ca. 16% related to the standard substrate) was characterized by basic kinetic constants (K_m, V_{max}, K_i, see Table 4).

Table 4 Hydrolysis of azide **19** and of *p*-nitrophenyl glycoside **1** by selected enzymes.

Enzyme source	Azide **19**			*p*-Nitrophenyl glycoside **1**		
	K_m [mM]	V_{max} [μM/min]	K_i [mM]	K_m [mM]	V_{max} [μM/min]	K_i [mM]
Aspergillus oryzae CCF 1066	3.1 ± 0.4	3.4 ± 0.2	0	0.75 ± 0.05	56 ± 2	7.0 ± 0.6
Talaromyces flavus CCF 2686	0.75 ± 0.09	4.4 ± 0.1	0	0.36 ± 0.04	67 ± 4	1.3 ± 0.1

The results showed that, contrary to the **1**, the enzymes exhibited no inhibition at high concentrations of azide **19**. This fact, together with the substantially improved water solubility (saturated water solutions of **19** and **1** are 1.1M and 0.02M, respectively), enabled efficient transglycosylation reactions with low enzyme consumption using azide **19** as a donor. The retaining character of β-*N*-acetylhexosaminidases when hydrolyzing the C-N bond was confirmed by ^{1}H NMR experiment. The efficiency of azide **19** as a glycosyl donor was demonstrated in the preparation of three disaccharides **21–23** (Figure 10) in good yields (32%, 16%, 22% yields, respectively; for details see Appendix A5). Thus, although azide **19** was less willingly hydrolyzed than *p*-nitrophenyl glycoside **1** (also considering V_{max}, K_m), it did not inhibit the enzyme at higher concentrations and afforded good transglycosylation yields, probably due to the reduction of water activity by high donor concentration in the reaction mixture. Thanks to the azido moiety, these transglycosylation products can simply be conjugated to other structures after reduction to an amine.



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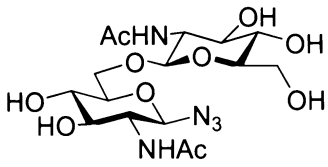

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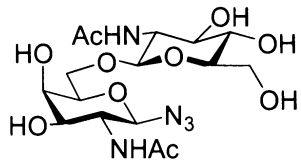

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Figure 10 Products of transglycosylations with azide **19** as a donor.

The use of glycosyl azides as donors in transglycosylations is not limited to β-*N*-acetylhexosaminidases – the respective glycosyl azides were prepared and are recently being tested with other glycosidases, namely α-galactosidases, β-galactosidases, β-glucosidases, α- and β-mannosidases. Several transglycosylation products were isolated and characterized, however, these results are beyond the scope of this Ph. D. thesis. They just support the picture of glycosyl azides as prospective donors for glycosidases, a viable alternative to traditional nitrophenyl glycosides.

5 CONCLUSION

This Ph. D. thesis is a systematic study of the substrate specificity and the synthetic potential of β-*N*-acetylhexosaminidases, family 20 of glycosidases, with their unnatural (modified) substrates. It comprises four publications in international journals, one review and 17 oral and poster contributions in domestic and international conferences. There are three main outputs of this work, described in the following paragraphs:

5.1 β-*N*-Acetylhexosaminidases and Modified Substrates: Structure-Activity Relationship

Four areas of substrate modifications were thoroughly exploited, namely the transformation of the 2-acetamido moiety into various *N*-acylamido groups, oxidation of the C-6 hydroxyl, the introduction of a cyano moiety at the C-6 position, and the substitution of the *p*-nitrophenyl aglycon (C-O glycosidic linkage) by an azide moiety (C-N linkage).

Thirteen modified substrates were synthesized by original methods (compounds **5**, **12–14**, **16–18**) or by literature procedures. They were tested for hydrolysis and transglycosylation by over thirty fungal β-*N*-acetylhexosaminidases (culture collections at Charles University in Prague and at the Institute of Microbiology, Czech Academy of Sciences). All the modified substrates presented have been used in the synthesis by β-*N*-acetylhexosaminidases for the first time. β-*N*-Acetylhexosaminidase from *Talaromyces flavus* CCF 2686 was identified as a prospective candidate for cloning and directed mutagenesis due to its exceptionally broad substrate specificity. The experimental results were discussed in view of the conclusions from molecular modeling (β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066) and possible reasons for some untypical behavior of the enzymes (*e. g.*, no cleavage of 2-acetamido-2-deoxy-β-D-galactopyranosyl azide (**20**)) were proposed.

5.2 Optimized Synthetic Methodologies with Modified Substrates

On the basis of the screening data, semi-preparative transglycosylation reactions were designed and optimized, especially by increasing the synthesis/ hydrolysis ratio. As a result, eight oligosaccharidic structures were prepared, isolated and fully characterized, with the yields ranging between 16 and 37% (best yield: 78%, product **8**). The amount of products isolated

was in the range of tens of miligrams, sufficient for full structural characterization and assessment of potential biological and inhibitory activities. Further upscaling was feasible. Six out of these eight compounds were isolated and characterized for the first time. A new methodology, never before used with glycosidases, was introduced: glycosyl azides as donors for transglycosylations. This concept obviated the problem of the low solubility of common *p*-nitrophenyl donors, the difficult removal of the released *p*-nitrophenol, and it afforded high yielding syntheses. As a result, glycosyl azides were found to be a suitable alternative to glycosyl fluoride donors, practicable even with β-*N*-acetylhexosaminidases (respective fluoride substrates are unstable).

5.3 Novel Hexosamine Structures with Further Applications

Potential biological activity of the prepared oligosaccharides were studied, namely their affinity to natural killer cells activation receptors and their inhibition potential towards β-*N*-acetylhexosaminidases.

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid (**13**) and 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**15**) were among the best known ligands for the tested natural killer cell activation receptors, particularly for the CD69 protein. Two out of three tested sugar nitriles, particularly *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosiduronitrile, exhibited an inhibitory effect on β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066.

In conclusion, this Ph. D. thesis showed the efficacy of β-*N*-acetylhexosaminidase-catalyzed synthesis with modified substrates. Novel compounds with high affinity to natural killer cell receptors and with inhibitory properties were identified and extensive data on structure-activity relationship were collected. The findings of this work will be applied in further research, *i. e.*, the use of glycosyl azides as donors in transglycosylations, the development of glycosidase inhibitors based on sugar nitriles, the preparation of multivalent glycomimetics with immunomodulatory effect, and the purification and characterization of β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686.

6 List of Prepared Compounds

6.1 Compounds Prepared by Chemical Synthesis

p-Nitrophenyl 2-amino-2-deoxy-β-D-glucopyranoside (**2**)[f]	Appendix A2
p-Nitrophenyl 2-deoxy-2-formamido-β-D-glucopyranoside (**3**)	Appendix A2
p-Nitrophenyl 2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (**4**)	Appendix A2
p-Nitrophenyl 2-deoxy-2-glycoloylamido-β-D-glucopyranoside (**5**)[g]	Appendix A2
p-Nitrophenyl 2-deoxy-2-propionamido-β-D-glucopyranoside (**6**)	Appendix A2
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosiduronic acid (**13**)[g]	Appendix A3
Methyl (*p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranosid)uronate (**14**)[g]	Appendix A3
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosiduronitrile (**16**)[g]	Appendix A4
p-Nitrophenyl 2-acetamido-2,6-dideoxy-β-D-*gluco*-heptopyranosyluronononitrile (**17**)[g]	Appendix A4
2-Acetamido-2,6-dideoxy-β-D-*gluco*-heptopyranosyluronononitrile azide (**18**)[g]	Appendix A4
2-Acetamido-2-deoxy-β-D-glucopyranosyl azide (**19**)	Appendix A5
2-Acetamido-2-deoxy-β-D-galactopyranosyl azide (**20**)	Appendix A5

[f] The numbers in brackets indicate the compound numbering in Chapter 4 of this thesis, which does not necessarily correspond to the numbering in the respective publications.
[g] To our best knowledge, the indicated compounds were prepared and characterized for the first time (source: Bailstein, updated in January 2006).

6.2 Compounds Prepared by (Chemo-)Enzymatic Synthesis

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8 ABREVIATIONS AND SYMBOLS

Ac	Acetyl
BSA	Bovine serum albumin
CCF	Cuture Collection of Fungi
C-*i*	The *i*-th carbon in the carbohydrate pyranose ring
CMP-Neu5Ac	Cytidine 5'-(5-acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosylonic acid monophosphate)
COOH	Carboxyl
N,N'-diacetylchitobiose	2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose
GalNAc	2-Acetamido-2-deoxy-D-galactose
β-D-Gal*p*NAc	2-Acetamido-2-deoxy-β-D-galactopyranoside
β-D-Gal*p*NAc-(1→4)-D-Glc*p*NAc	2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose
β-D-Gal*p*NAcA-(1→4)-D-Glc*p*NAc	2-Acetamido-2-deoxy-β-D-galactopyranosyluronic acid-(1→4)-2-acetamido-2-deoxy-D-glucopyranose
GlcNAc	2-Acetamido-2-deoxy-D-glucose
β-D-Glc*p*NAc	2-Acetamido-2-deoxy-β-D-glucopyranoside
GC	Gas chromatography
HPLC	High-performance liquid chromatography
k_{cat}	Turnover number, [s^{-1}]. The quotient of V_{max} and the molar concentration of the enzyme.
K_i	Inhibition constant, [mol/l]. The inhibitor concentration, at which it occupies half of the enzyme sites.
K_m	Michaelis-Menten constant, [mol/l]. Substrate concentration, at which the initial reaction rate is ½ V_{max}.

LacNAc	β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose
$-\log IC_{50}$	Negative decimal logarithm of the concentration of the respective sugar derivative, at which the degree of saturation of the CD69 receptor by this derivative was the same as by the standard ($GlcNAc_{23}BSA$ conjugate)
ManNAc	2-Acetamido-2-deoxy-D-mannose
Me	Methyl
$Na[BH_4]$	Sodium borohydride
NKR-P1	Natural Killer Receptor – Protein 1
NMR	Nuclear magnetic resonance
OH	Hydroxyl
pK_a	Negative decimal logarithm of the acid dissociation constant (K_a)
SO_3H	Sulfo group
TEMPO	2,2,6,6-Tetramethylpiperidine 1-oxyl radical
TLC	Thin-layer chromatography
V_{max}	Maximum reaction rate, [mol/l/s]. The initial rate of a reaction, in which the substrate concentrations are limiting to infinity ($[S] \rightarrow \infty$).

Appendix B. List of Publications

- **P. Fialová**, L. Weignerová, J. Rauvolfová, V. Přikrylová, A. Pišvejcová, R. Ettrich, M. Kuzma, P. Sedmera, V. Křen: Hydrolytic and Transglycosylation Reactions of *N*-Acyl Modified Substrates Catalysed by β-*N*-Acetylhexosaminidases. *Tetrahedron* **2004**, *60*, 693–701 (IF 2.6).[h]

- J. Rauvolfová, L. Weignerová, **P. Fialová**, V. Přikrylová, M. Kuzma, A. Pišvejcová, V. Křen: β-*N*-Acetylhexosaminidase Catalysed Synthesis of Non-Reducing Oligosaccharides. *J. Mol. Catal. B: Enzymatic* **2004**, *29*, 233–239 (IF 1.5).

- **P. Fialová**, L. Elling, D.-J. Namdjou, R. Ettrich, M. Kuzma, V. Přikrylová, J. Rauvolfová, K. Bezouška, V. Křen: Combined Application of Galactose Oxidase and β-*N*-Acetylhexosaminidase in the Preparation of Complex Bioactive *N*-Acetylhexosaminides. *Adv. Synth. Catal.* **2005**, *347*, 997–1006 (IF 4.5).[h]

- **P. Fialová**, A. T. Carmona, I. Robina, R. Ettrich, P. Sedmera, V. Přikrylová, L. Petrásková-Hušáková, V. Křen: Glycosyl Azide – A Novel Substrate for Enzymatic Transglycosylations. *Tetrahedron Lett.* **2005**, *46*, 8715–8718 (IF 2.5).[h]

- A. T. Carmona, **P. Fialová**, V. Křen, R. Ettrich, L. Martínková, A. J. Moreno-Vargas, C. Gonzales. I. Robina: Cyanodeoxy-Glycosyl Derivatives as Substrates for Enzymatic Reactions. *Eur. J. Org. Chem.* **2006**, 1876–1885 (IF 2.4).[h]

- **P. Fialová**, V. Křen: Enzymatic approaches to *O*-glycoside introduction: Glycosidases. In *Comprehensive Glycoscience* (J. P. Kamerling, Ed.); Elsevier: Oxford, 2006, *submitted*.[h]

- O. Kaplan, V. Vejvoda, O. Plíhal, P. Pompach, D. Kavan, **P. Fialová**, K. Bezouška, M. Macková, M. Cantarella, V. Jirků, V. Křen, L. Martínková: Purification and Characterization of a Nitrilase from *Aspergillus niger* K10. *Appl. Microbiol. Biotechnol., submitted* (IF 2.4).

[h] Indicated publications form the core of this Ph. D. thesis.

Appendix C. List of Presentations

C1. Oral Presentations

- **P. Fialová**, L. Hušáková, Z. Huňková, M. Kuzma, L. Weignerová, V. Křen: Substrate Specificity of β-*N*-Acetylhexosaminidases of Various Origin. The 1st Interdisciplinary Meeting of Young Scientists organized by the Sigma-Aldrich Co., Kamenné Žehrovice (Czech Republic); May 17–19, 2001. **Abstract:** *Book of Abstracts*, p. 9.

- **P. Fialová**, L. Weignerová, V. Křen: Modified Substrates for β-*N*-Acetylhexosaminidases. CarbLink III - Workshop on Carbohydrate Chemistry and Glycobiology, Kiel (Germany), June 6–9, 2002. **Abstract:** *Final program*, 4th lecture.

- **P. Fialová**, L. Weignerová, V. Křen: Modified Substrates for β-*N*-Acetylhexosaminidases. 10th Bratislava Symposium on Saccharides, Smolenice (Slovak Republic), September 1–6, 2002. **Abstract:** *Book of Abstracts*, p. 41.

- **P. Fialová**, L. Weignerová, A. Pišvejcová, V. Křen: β-*N*-Acetylhexosaminidases Acting at *N*-Acyl Modified Substrates. 5th German-East-European Carbohydrate Workshop, Güstrow (Germany), March 27–30, 2003. **Abstract:** *Final Program*, 7th lecture.

- K. Bezouška, P. Pompach, O. Plíhal, J. Sklenář, A. Pišvejcová, L. Hušáková, **P. Fialová,** V. Křen: Structural Investigations of Fungal β-*N*-Acetylhexosaminidases Useful in Syntheses of New Unique Oligosaccharides. The 6th International Symposium on Biocatalysis and Biotransformation, Olomouc (Czech Republic) June 28 – July 3, 2003. **Abstract:** *Chem. Listy* **2003**, *97*, 356.

- **P. Fialová**, D. J. Namdjou, L. Elling, V. Křen: Oxidised Glycosides as Substrates for β-*N*-Acetylhexosaminidases. Cukrblik Workshop 2004: Advances in the Chemistry and Biology of Saccharides, Prague (Czech Republic), April 15, 2004.

- V. Křen, **P. Fialová**, A. Pišvejcová, D.-J. Namdjou, N. Nettelstroth, L. Elling: Glycosidases – bizarre substrates and products. 15th Joint Meeting of Dutch and German societies for glycobiology, Wageningen (The Netherlands), November 28–30, 2004.

- **P. Fialová**: New approach to transglycosylations – substrates with glycosidic C-N bond. 4th Meeting of the Working Group COST D25/0001/02, Barcelona (Spain), April 6–7, 2005. **Abstract:** *Final Programme*, 10th lecture.

- **P. Fialová**, A. T. Carmona, I. Robina, P. Sedmera, L. Hušáková, V. Křen: Glycosyl azides – novel substrates for enzymatic transglycosylations. 13th European Carbohydrate Symposium, Bratislava (Slovakia), August 21–26, 2005. **Abstract:** *Abstracts*, OP17.

- **P. Fialová**, L. Petrásková, D. Monti, L. Elling, K. Bezouška, V. Křen: Synthesis of immunoactive oligosaccharides: three enzymes in play. Multi-Step Enzyme Catalysed Processes, Graz (Austria), April 18–21, 2006. **Abstract:** *Programme and Abstract Book*, p. 22.

C2. Poster Presentations

- **P. Fialová**, E. Herkommerová, Z. Huňková, L. Weignerová, V. Křen: Enzymatic Synthesis of Chitooligosaccharides Containing Various *N*-Acyl Groups. The XVIIth Congress of Czech Society for Biochemistry and Molecular Biology, Prague (Czech Republic), September 7–10, 2000. **Abstract:** *Final Program*, S4-07.

- **P. Fialová**, L. Hušáková, Z. Huňková, M. Kuzma, L. Weignerová, V. Křen: Substrate Specificity of β-*N*-Acetylhexosaminidases of Various Origin. 3rd German-East-European Carbohydrate Workshop, Güstrow (Germany), February 28 – March 4, 2001. **Abstract:** *Final Program*, P 13.

- **P. Fialová**, L. Hušáková, Z. Huňková, M. Kuzma, L. Weignerová, V. Křen: Substrate Specificity of β-*N*-Acetylhexosaminidases of Various Origin. Cukrblik Workshop 2001, Prague (Czech Republic), March 22, 2001.

- V. Mylerová, J. Páca, M. Ovesná, V. Přikrylová, **P. Fialová**, J. Smola, V. Křen, L. Martínková: *N*-Acyl Amidase-Catalysed Hydrolysis of 2-Deoxy-2-Acetamido-Saccharides. The 5th International Symposium on Biocatalysis and Biotransformation, Darmstadt (Germany), September 2–7, 2001. **Abstract:** *Book of Abstracts*, p. 303 (P 186).

- **P. Fialová,** L. Hušáková, Z. Huňková, M. Kuzma, L. Weignerová, V. Křen: *N*-Acyl Modified Substrates for β-*N*-Acetylhexosaminidases. The 5th International Symposium on Biocatalysis and Biotransformation, Darmstadt (Germany), September 2–7, 2001. **Abstract:** *Book of Abstracts*, p. 308 (P 191).

- **P. Fialová**, A. Pišvejcová, L. Weignerová, V. Křen: Tolerance of *N*-Acyl Modified Substrates by β-*N*-Acetylhexosaminidases. 5th Carbohydrate Bioengineering Meeting, Groningen (The Netherlands), April 6–9, 2003. **Abstract:** *Book of Abstracts*, p. 58 (P9).

- V. Křen, L. Hušáková, **P. Fialová**, A. Pišvejcová K. Bezouška: Use of Fungal β-*N*-Acetylhexosaminidases for Discrimination of ManNAc-, GlcNAc- and GalNAc-Containing Oligosaccharides. 5th Carbohydrate Bioengineering Meeting, Groningen (The Netherlands), April 6–9, 2003. **Abstract:** *Book of Abstracts*, p. 60 (P11).

- **P. Fialová**, L. Weignerová, A. Pišvejcová, V. Křen: New Transglycosylation Reactions Catalysed by β-*N*-Acetylhexosaminidases. The 3rd Interdisciplinary Meeting of Young Scientists organized by the Sigma-Aldrich Co., Hotel 9 skal, Žďárské vrchy (Czech Republic), July 4–7, 2003. **Abstract:** *Chem. Listy* **2003**, *97*, 290.

- **P. Fialová**, A. Pišvejcová, L. Weignerová, V. Křen: New Transglycosylation Reactions with Modified Substrates Catalysed by β-*N*-Acetylhexosaminidases. The 6th International Symposium on Biocatalysis and Biotransformation, Olomouc (Czech Republic), June 28 – July 3, 2003. **Abstract:** *Chem. Listy* **2003**, *97*, 393 (2003).

- J. Rauvolfová, V. Přikrylová, L. Weignerová, M. Kuzma, M. Macková, **P. Fialová**, A. Pišvejcová, V. Křen: β-*N*-Acetylhexosaminidase-Catalysed Synthesis of Non-Reducing Oligosaccharides. The 6th International Symposium on Biocatalysis and Biotransformation, Olomouc (Czech Republic), June 28 – July 3, 2003. **Abstract:** *Chem. Listy* **2003**, *97*, 515.

- **P. Fialová**, A. Pišvejcová, L. Weignerová, V. Křen: New Transglycosylation Reactions with Modified Substrates Catalysed by β-*N*-Acetylhexosaminidases. 12th European Carbohydrate Symposium, Grenoble (France), July 6–11, 2003. **Abstract:** *Book of Abstracts*, p. 291 (PB 078).

- **P. Fialová**, D.-J. Namdjou, L. Elling, V. Křen: Oxidised Glycosides as Substrates for β-*N*-Acetylhexosaminidases. 22nd International Carbohydrate Symposium, Glasgow (United Kingdom), July 23–27, 2004. **Abstract:** *List of Poster Presentations*, P72.

- **P. Fialová,** A. T. Carmona, I. Robina, R. Ettrich, P. Sedmera, V. Křen: Glycosyl Azides – New Efficient Substrates for Enzymatic Transglycosylations. 6th Carbohydrate Bioengineering Meeting, Barcelona (Spain), April 3–6, 2005. **Abstract:** *Program and Abstracts*, P30.

- A. T. Carmona, **P. Fialová**, V. Křen, A. J. Moreno-Vargas, I. Robina: Cyanodeoxy-Glycosyl Derivatives as Substrates for Enzymatic Transglycosylations. 4th Meeting of the Working Group COST D25/0001/02, Barcelona (Spain), April 6–7, 2005. **Abstract:** *Final Programme*, p. 2.

- **P. Fialová**, L. Elling, D.-J. Namdjou, R. Ettrich, K. Bezouška, V. Křen: Combined Application of Galactose Oxidase and β-*N*-Acetylhexosaminidase - Synthesis of Bioactive *N*-Acetylgalactosaminides. The 7th International Symposium on Biocatalysis and Biotransformations, Delft (The Netherlands), July 2–8, 2005. **Abstract:** *Book of Abstracts*, p. 131 (P131).