Charles University in Prague

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Summary of a Ph. D. thesis

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

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1 INTRODUCTION

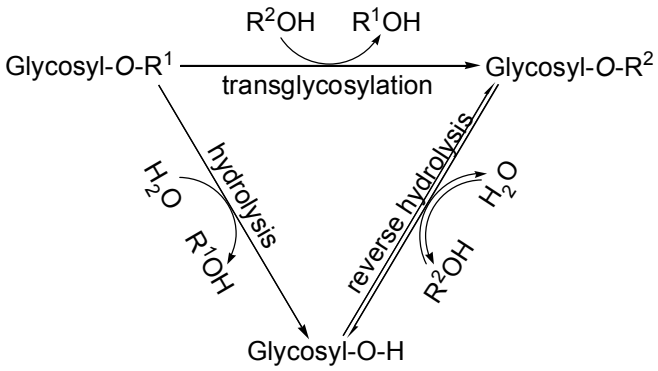
1.1 Glycosidases

Glycosidases (*O*-glycoside hydrolases; EC 3.2.1) cleave oligo- and polysaccharides by transferring the glycosyl to a water molecule *in vivo*. In the presence of a good nucleophile, they can also form a new glycosidic linkage. Their main advantages are good availability, stability and easy handling, as well as the absolute stereoselectivity and broad donor and acceptor specificities.[1] Their major drawback is low regioselectivity - the transglycosylation reaction mostly results in a complex mixture of regioisomers. The regioselectivity may be enhanced by, *e. g.*, selective acceptor substitution.[2]

β-*N*-Acetylhexosaminidases (EC 3.2.1.52, glycosidase family 20) are exoglycosidases hydrolyzing terminal β-D-Glc*p*NAc and β-D-Gal*p*NAc residues in nature. They are obtained from crude commercial enzyme preparations,[3] and, preferably, from fungal strains (extracellular production).[4] Some of them are inducible[4] by, *e. g.*, chitobiose, chitooligomers, and GlcNAc.

1.2 Reverse Hydrolysis and Transglycosylation

During reverse hydrolysis (Scheme 1), a free monosaccharide is combined with a nucleophile under the exclusion of a water molecule. The equilibrium constant of this condensation process favors hydrolysis and must be shifted artificially.[5] Reaction times are days or weeks and yields do not exceed 15%. Reverse hydrolysis is widely used for the glycosylation of alcohols.[6]



Scheme 1 Reactions catalyzed by glycosidases.

Transglycosylation (Scheme 1) employs an activated glycoside as a glycosyl donor. This kinetically controlled process enables higher product accumulation and, as a result, higher yields,[7] generally of 20–40%. Water acts as a competing nucleophile and causes parasitic hydrolysis of the reactant. Good glycosyl donors minimize the risk of product hydrolysis by a high affinity to the enzyme (*i. e.*, low K_m) and a fast reaction (*i. e.*, high k_{cat}).

Apart from disaccharides like *N*,*N*′-diacetylchitobiose,[8] nitrophenyl glycosides are popular and efficient glycosyl donors. However, their limited water solubility lowers the synthesis/ hydrolysis ratio. Therefore, new better soluble donors are sought for.[9,10] A novel alternative are glycosyl azides (C-N bond hydrolyzed). Their cleavage was first observed in *Agrobacterium sp.* β-glucosidase.[11] We were the first to use glycosyl azides as donors in transglycosylations. Apart from β-*N*-acetylhexosaminidases (Section 3.4), good results have been obtained with, *e. g.*, β-galactosidases.

1.3 Modified Substrates for Glycosidases

The C-6 primary hydroxyl of glycosidase donors enables many modifications. The first results on this topic were published by Kimura,[12] followed by MacManus,[13] Hušáková[2] and Weingarten *et al.*[14] The introduction of a reactive moiety like an aldehyde into the C-6 position (*e. g.*, in *p*-nitrophenyl β-D-*galacto*-hexodialdo-1,5-pyranoside) affords further modifications such as coupling to large structures. We used a range of C-6 oxidized nitrophenyl glycosides as donors in β-*N*-acetylhexosaminidase-catalyzed synthesis (Section 3.2).

The 2-acetamido group is crucial for accepting a substrate by β-*N*-acetylhexosaminidases. Their tolerance towards substrate modifications at C-2 has been studied since the 1970s.[15] We disclosed the β-*N*-acetylhexosaminidases with broad specificity towards *N*-acyl modified substrates (Section 3.1).

1.4 Oligosaccharides and Their Applications

Oligosaccharides have a variety of functions in nature.[16] Their immense information potential is especially valuable for diagnostic purposes and for drug development, *e. g.*, in the design of glycomimetics with improved pharmacokinetics. 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 (*e. g.*, NKR-P1 (rat), and CD69 (human)), and thus iniciating the killing process of the contacted defect cell. An ideal ligand[17] to these receptors (Figure 1) is a (β1-4)-linked tetrasaccharide composed of *N*-acetyl-D-hexosamines, containing negatively charged groups (COOH, SO_3H) at the C-6 position.

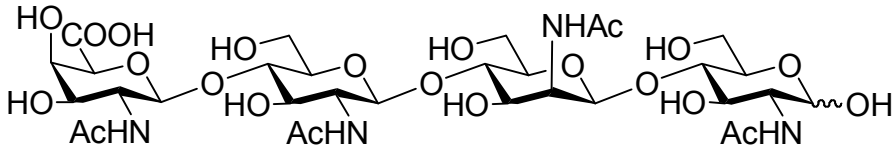


Figure 1 An ideal binding ligand to NKR-P1 activation receptor of natural killer cells.

Competitive inhibitors of glycosidases, such as fluoroglycosides, are important for mechanistic and structure-activity relationship studies. Inhibitors of fungal β-*N*-acetylhexosaminidases[18] also find applications in the therapy of fungal infections in medicine and agriculture. K_i of the best competitive inhibitors varies in the 1–30 nM range. Figure 2 shows some typical inhibitor structures.

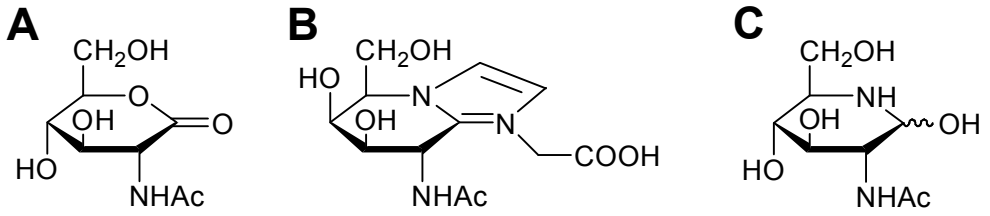


Figure 2 Competitive inhibitors of β-*N*-acetylhexosaminidases: 2-acetamido-2-deoxy-D-glucono-1,5-lactone (A), nagstatin (B), and 2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucopyranose (C).

2 WORKING HYPOTHESIS

This Ph. D. thesis is 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 fungal β-*N*-acetylhexosaminidases towards selected substrate modifications**. The substrate modifications studied (at C-1, C-2, and C-6) will be chosen respective to the interest for structure-activity relationship studies and the applicability of prepared products. Large screening data will be collected and the findings will be correlated to molecular modeling results (β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066).

(b) **To optimize the synthesis with modified substrates**. A novel methodology on glycosyl azides as donors in β-*N*-acetylhexosaminidase-catalyzed synthesis will be studied. Glycosyl azides should be a suitable alternative to the currently used glycosyl fluoride donors.

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

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

(a) **Structure-activity relationship studies** 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 (immunomodulatory glycomimetics, competitive inhibitors).

3 RESULTS AND DISCUSSION

3.1 Substrates Modified at C-2

The 2-acetamido group is a crucial feature of β-*N*-acetylhexosaminidase substrates as it is essential for the formation of the oxazoline intermediate in substrate hydrolysis. Neither complex structure-activity relationship studies nor syntheses were published with *N*-acyl modified substrates. Therefore, we prepared five *N*-acyl modified derivatives of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**, the standard substrate) – see Figure 3 – and subjected them to a hydrolysis and transglycosylation screening with 35 fungal β-*N*-acetylhexosaminidases of *Aspergillus*, *Penicillium* and *Talaromyces* genera (culture collections at Charles University (CCF) and at the Institute of Microbiology, Czech Academy of Sciences (CCIM)).

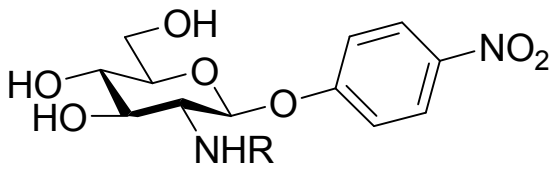
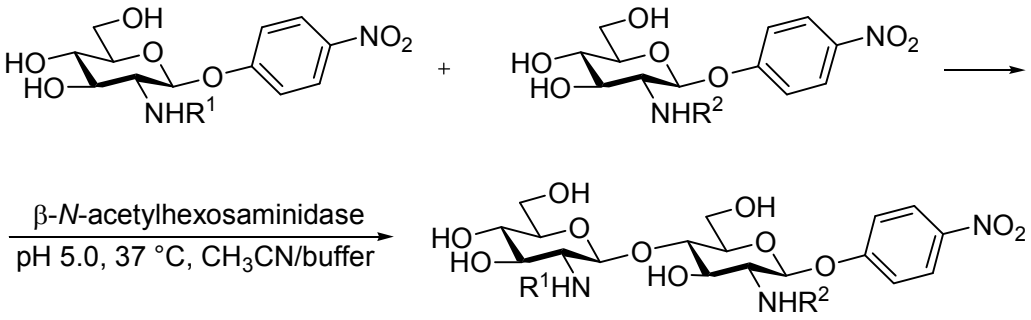


Figure 3 *N*-Acyl modified substrates.
R = Ac (**1**, standard), H (**2**), CHO (**3**), $COCF_3$ (**4**), $COCH_2OH$ (**5**), $COCH_2CH_3$ (**6**)

The enzymes fairly tolerated shorter or longer acyls and a hydroxyl instead of a hydrogen at the C-2 position, contrary to the electronegative trifluoroacetyl and the charged free amino group. The results of docking experiments (β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066) indicated good binding of the substrates into the enzymatic active site. Therefore, we concluded that the lower hydrolysis rate compared to **1** may have been the result of the destabilization of the oxazolinium reaction intermediate.

Despite the lower hydrolytic potential, transglycosylation products **7–9** (Scheme 2) were prepared from donors **3**, **5**, and **6** in high yields (78% for **8**). A surprising result was the formation of product **10**. Here, β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 exhibited a

higher transglycosylation affinity to the modified substrate **6** than to standard **1** and transferred solely the modified glycosyl, using standard **1** as an acceptor.



Product	**R**	Source of enzyme	CH_3CN [%]	Yield [%]
7	$R^1 = R^2 = CHO$	*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 2 Transglycosylation reactions with *N*-acyl modified substrates.

This study demonstrated that besides cleavage, fungal β-*N*-acetylhexosaminidases are able to catalyze transglycosylations with *N*-acyl modified substrates in considerable yields. In summary, four novel oligosaccharides **7**–**10** were prepared.

3.2 Substrates Oxidized at C-6

Glycosidases accept substrates with modifications of the C-6 hydroxyl and use them both for hydrolysis and synthesis. As a result, various functionalities can be introduced that enable further modifications of the oligosaccharide molecule including conjugation to complex structures.[19] Moreover, oligosaccharides with a β-D-Gal*p*NAcA moiety show strong immunoactivation properties (binding to natural killer activation receptors). 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 4) by chemoenzymatic synthesis. A selective C-6 oxidation of

p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**11**) to aldehyde **12** was performed by galactose oxidase from *Dactylium dendroides* in an optimized batch reactor.

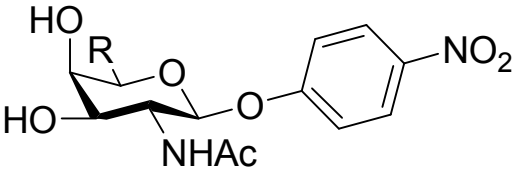


Figure 4 C-6 Oxidized substrates.
R = CH_2OH (**11**, standard), $CH(OH)_2$ (**12**), COOH (**13**), COOMe (**14**).

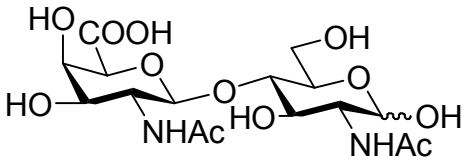
We studied the enzymatic recognition of these substrates by 36 fungal β-*N*-acetylhexosaminidases (CCF, CCIM). Aldehyde **12** was readily cleaved, contrary to both **13** and **14**. Molecular modeling with β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 revealed that **12** bound to the enzyme as a *geminal* diol, whereas **13** and **14** bound weakly due to a loss in hydrogen bonding, probably rather for sterical reasons. This shows the limitations of the use of C-6 modified substrates with β-*N*-acetylhexosaminidases.

A novel disaccharide of β-D-Gal*p*NAcA-(1→4)-D-Glc*p*NAc (**15**) was synthesized by transglycosylation with **12** as a donor (β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686) followed by a chemical oxidation at C´-6 *in situ* to a uronic acid (37% yield). All the compounds were tested for binding to natural killer cell activation receptors: NKR-P1A (rat) and CD69 (human). Both **13** and **15** proved to be high-affinity ligands of these receptors, particularly for CD69 (among the best described). This increase was obviously due to the presence of a carboxy moiety (Table 1).

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 will be applied in the development of therapeutically useful glycomimetics.

Table 1 Affinity to CD69 – human NK cell activation receptor.

Tested ligands	–log IC_{50} †
GlcNAc	3.3
Standard **11**	3.8
Aldehyde **12**	5.0
Uronic acid **13**	**6.9**
Methyl uronate **14**	4.9
β-D-Gal*p*NAc-(1→4)-D-Glc*p*NAc	3.9
Disaccharide **15**	**7.8**

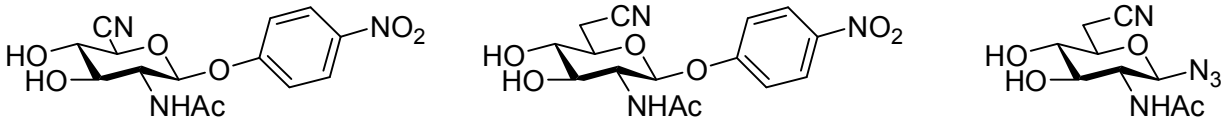


Disaccharide **15**

† –log IC_{50} is the negative decimal logarithm of the concentration of the respective ligand, at which the CD69 receptor reached the same saturation as with the standard ($GlcNAc_{23}BSA$).

3.3 Sugar Nitriles – Competitive Inhibitors

The relatively broad substrate specificity of glycosidases towards substrates modified at the C-6 position (Sections 1.3 and 3.2) drew our attention to another class of C-6 modified substrates – sugar nitriles **16-18** (Figure 5).



16 **17** **18**

Figure 5 Sugar nitriles tested with β-*N*-acetylhexosaminidases.

Compound **16** was prepared from **1** in three steps (66% overall yield), compound **17** in four steps (18.5% overall yield),. Azide **18** was prepared from **19** in 15% overall yield. Compounds **16–18** were subjected to a hydrolysis screening with 33 fungal β-*N*-acetylhexosaminidases (CCF). None of the compounds proved to be a good substrate for the enzymes tested and therefore, transglycosylations were not feasible.

As the docking of compounds **16–18** into the active site of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 indicated good binding, we suggested compounds **16–18** being competitive inhibitors of this enzyme. Indeed, the decline in the enzyme activity towards standard **1** in the presence of **16** and **17** (Table 2) as well as the inhibition kinetic studies proved

17 (K_i = 0.37 mM) and particularly **16** (K_i = 7.6 μM) to be competitive inhibitors of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066. Thus, compound **16**, though a structural analogue of **17**, exhibited a 50-fold stronger inhibition effect. As a result, conversion of the C-6 hydroxyl into a cyano moiety, such as in compound **16,** appears to be a route to other efficient glycosidase inhibitors.

Table 2 Inhibition of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 by **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.

3.4 Glycosyl Azides – Novel Glycosyl Donors

Common nitrophenyl glycoside donors sometimes have solubility problems (especially if modified), which lowers transglycosylation yields and requires the use of organic cosolvents. Furthermore, they are quite expensive, and *p*-nitrophenol, released during transglycosylation, complicates the purification. Therefore, we developed a new methodology based on glycosyl azides (C-N bond cleaved; Figure 6) as donors for β-*N*-acetylhexosaminidases. They are stable, well water-soluble, can be prepared easily and in a high yield. Moreover, the azide ion is easily removable from the reaction mixtures.

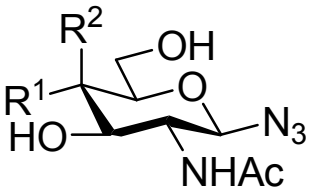


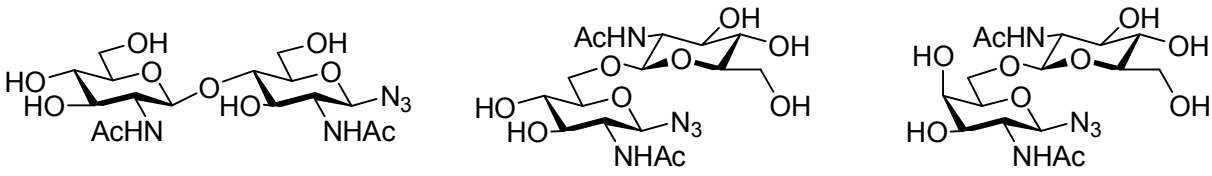
Figure 6 Glycosyl azides.
19: R^1 = OH, R^2 = H
20: R^1 = H, R^2 = OH

The hydrolysis of azides **19** and **20** was tested with 20 fungal β-*N*-acetylhexosaminidases (CCF). Due to the lack of a chromophore moiety, the common

colorimetric assay[20] was not applicable and an HPLC determination method was developed.

The azide aglycon strongly influenced the substrate specificity of β-*N*-acetylhexosaminidases. Their typical tolerance towards both β-D-Glc*p*NAc and β-D-Gal*p*NAc structures disappeared with the azide substrate and the enzymes became selective β-*N*-acetylglucosaminidases. This behavior is quite unique and to our knowledge, it has not been observed in other aglycons. It was concluded from docking experiments (β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066) that azide **20** (*galacto*-) is not accepted due to a substantial loss in hydrogen bonding in the active site caused by a too large distance between **20** and the glutamate 519.

The enzymatic hydrolysis of azide **19** was characterized by basic kinetic constants (K_m, V_{max}, K_i). The results showed that, contrary to the **1**, the enzymes were not inhibited at high concentrations of **19**. This fact, together with the high water solubility, enabled efficient transglycosylations yielding three disaccharides **21**–**23** (32%, 16%, 22% yields, respectively; Figure 7). Thanks to the azido moiety, these transglycosylation products can simply be conjugated to other structures after the reduction to an amine.



21 **22** **23**

Figure 7 Products of transglycosylations with azide **19** as a donor.

These results present azide **19** as a prospective donor for β-*N*-acetylhexosaminidases, a viable alternative to traditional nitrophenyl glycosides.

4 CONCLUSION

This Ph. D. thesis is a systematic study of the substrate specificity and the synthetic potential of β-*N*-acetylhexosaminidases (EC 3.2.1.52) with structurally modified substrates. It comprises four publications in international journals, one review and 17 oral and poster contributions.

The following parts of the substrate molecule were modified: 2-acetamido moiety, the C-6 hydroxyl (oxidations, introduction of a cyano group) and the aglycon part (glycosyl azides – C-N bond hydrolysis). Thirteen modified substrates were synthesized, seven of them were described for the first time. They were tested for hydrolysis and transglycosylation by over thirty fungal β-*N*-acetylhexosaminidases (culture collections at Charles University and at the Institute of Microbiology, Academy of Sciences of the Czech Republic) and the results were discussed in relation to the conclusions of molecular modeling (β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066). Eight oligosaccharidic structures (six of them novel) were prepared by semi-preparative transglycosylation reactions (tens of miligrams), isolated (mostly 16–37% yields, even 78% yield) and fully characterized. Noteworthy properties like immunoactivity (binding to natural killer cell activation receptors) and inhibitory potential were disclosed in four new compounds prepared. An original synthetic methodology with glycosyl azides as donors in β-*N*-acetylhexosaminidase-catalyzed transglycosylations was introduced. β-*N*-Acetylhexosaminidase from *Talaromyces flavus* CCF 2686 was identified as a prospective candidate for cloning and directed mutagenesis due to its exceptional substrate specificity.

The findings of this work will be further applied, *e. g.*, in the synthesis with glycosidases and glycosyl azide donors, the development of glycosidase inhibitors, and the design of multivalent immunomodulatory glycomimetics.

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APPENDIX A. LIST OF PUBLICATIONS

P. Fialová, L. Weignerová, J. Rauvolfová, V. Přikrylová, A. Pišvejcová, R. Ettrich, M. Kuzma, P. Sedmera, V. Křen: *Tetrahedron* **2004**, *60*, 693–701 (IF 2.6).[a]

J. Rauvolfová, L. Weignerová, **P. Fialová**, V. Přikrylová, M. Kuzma, A. Pišvejcová, V. Křen: *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: *Adv. Synth. Catal.* **2005**, *347*, 997–1006 (IF 4.5).[a]

P. Fialová, A. T. Carmona, I. Robina, R. Ettrich, P. Sedmera, V. Přikrylová, L. Petrásková-Hušáková, V. Křen: *Tetrahedron Lett.* **2005**, *46*, 8715–8718 (IF 2.5).[a]

A. T. Carmona, **P. Fialová**, V. Křen, R. Ettrich, L. Martínková, A. J. Moreno-Vargas, C. Gonzales. I. Robina: *Eur. J. Org. Chem.* **2006**, 1876–1885 (IF 2.4).[a]

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

O. Kaplan, V. Vejvoda, O. Plíhal, P. Pompach, D. Kavan, **P. Fialová**, K. Bezouška, M. Macková, M. Cantarella, V. Jirků, V. Křen, L. Martínková: *Appl. Microbiol. Biotechnol., submitted* (IF 2.4).

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

APPENDIX B. LIST OF PRESENTATIONS

B1. 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.

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.

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