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NEW MICROBIAL GLYCOSIDASES

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NEW MICROBIAL GLYCOSIDASES

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DECLARATION

I hereby declare that this thesis is based on my own research carried out in the Laboratory of Biotransformation, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, except where due acknowledgement has been made in the text.

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Devoted to all I love and to all love me.

SUMMARY

Glycosidases from fungi are useful in the preparation of various glycosides mainly by transglycosylation or reversed glycosylation. The lack of any requirement for protection-deprotection sequences, mild conditions and easier synthesis of thermodynamically not preferred glycosidic bonds are the main advantages of glycosidase-catalysed synthesis of glycosides.

In this thesis we concentrated on exoglycosidases, mainly β-*N*-acetylhexosaminidases, α-galactosidases and α-L-rhamnosidases. For the enzyme preparation taxonomically characterised fungal strains from public collections were used. A library comprising more than 200 various glycosidases was developed by modification of cultivation conditions and by the use of specific inducers. The enzymes were used for screening of substrate specificity and stability in organic solvents and subsequently for synthesis and modification of various substrates.

Both β-*N*-acetylgalactosaminidase and β-*N*-acetylglucosaminidase activities in the series of β-*N*-acetylhexosaminidases were determined. Saccharides with strong immunomodulation activity β-D-Gal*p*NAc-(1→4)-D-Glc*p*NAc and β-D-Gal*p*NAc-(1→6)-D-Glc*p*NAc were synthesised by transglycosylation using β-*N*-acetylhexosaminidase from *Penicillium oxalicum* CCF 2430, enzyme having the highest β-*N*-acetylgalactosaminidase activity. An uncommon isomer of chitobiose β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc was synthesised by reverse hydrolysis with 2-acetamido-2-deoxy-D-glucopyranose using β-*N*-acetylhexosaminidase from *Aspergillus tamarii* CCF 1665, *P. funiculosum* CCF 2325 and *P. funiculosum* CCF 1994. Unique, non-reducing disaccharide β-D-Glc*p*NAc-(1→1)-β-D-Gal*p* and trisaccharides β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→1)-β-D-Gal*p*, β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc, and β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→1)-β-D-Glc*p*NAc were synthesised under the catalysis of β-*N*-acetyl-hexosaminidase from the *A. flavofurcatus* CCF 3061 using D-galactose and lactose as acceptors.

For subsequent screening of application of modified substrates in enzymatic synthesis selectively acylated glycosides and *p*-nitrophenylglycosides were synthesised by enzymatic esterification. α-Galactosidase from *Talaromyces flavus* CCF 2686 was found to have unusually broad substrate specificity. Using this α-galactosidase α-D-Gal*p*-(1→3)-6-*O*-Ac-α-D-Gal*p*-*O*-*p*-NP was prepared.

From a large panel of the enzymes tested only α-L-rhamnosidase from *Aspergillus niger* K2 was found to be able to modify selectively desglucoruscin (releasing L-rhamnose and forming desrhamnodesglucoruscin). Using NMR kinetic measurement α-L-rhamnosidases from filamentous fungi were determined to be glycosidases of the inverting-type.

SOUHRN

Glykosidasy produkované vláknitými houbami jsou užitečné při přípravě různých glykosidů, a to především transglykosylací nebo reversní glykosylací. Výhodou enzymových syntéz je, že zde není zapotřebí protekčních a deprotekčních kroků, mírné reakční podmínky a jejich možné využití při syntéze termodynamicky znevýhodněných glykosidových vazeb.

V této práci jsme se zaměřili na exoglykosidasy, především β-*N*-acetylhexosaminidasy, α-galaktosidasy a α-L-rhamnosidasy. Pro jejich přípravu byly použity taxonomicky charakterizované sbírkové kultury vláknitých hub. Volbou kultivačních podmínek a především použitých induktorů byla vytvořena knihovna více než 200 různých glykosidas, které byly dále použity pro screening substrátové specifity a stability v organických rozpouštědlech a následně při syntézách a modifikacích různých substrátů.

U knihovny β-*N*-acetylhexosaminidas byla určena jejich aktivita, jak β-*N*-acetylgalaktosaminidasová, tak i β-*N*-acetylglukosaminidasová. Nejvyšší β-*N*-acetylgalaktosaminidasovou aktivitu vykazovala β-*N*-acetylhexosaminidasa z *Penicillium oxalicum* CCF 2430; transglykosylací za použití tohoto enzymu byly syntetizovány sacharidy se silnou imunomodulační aktivitou β-D-Gal*p*NAc-(1→4)-D-Glc*p*NAc a β-D-Gal*p*NAc-(1→6)-D-Glc*p*NAc. Za použití β-*N*-acetylhexosaminidas z *Aspergillus tamarii* CCF 1665, *P. funiculosum* CCF 2325 a *P. funiculosum* CCF 1994 byl reversní hydrolýzou s 2-acetamido-2-deoxy-D-glukopyranosou syntetizován vzácný izomer chitobiosy β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc. Unikátní neredukující disacharid β-D-Glc*p*NAc-(1→1)-β-D-Gal*p* a trisacharidy β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→1)-β-D-Gal*p*, β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc a β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→1)-β-D-Glc*p*NAc byly syntetizovány transglykosylací za katalýzy β-*N*-acetylhexosaminidasy z *A. flavofurcatus* CCF 3061 při použití D-galaktosy a laktosy jako akceptorů.

Pro další screening využití modifikovaných substrátů v enzymové syntéze byly připraveny enzymovou esterifikací selektivně acylované glykosidy a *p*-nitrofenyl-glykosidy. α-D-Galaktosidasa z *Talaromyces flavus* CCF 2686 má neobvykle širokou substrátovou specifitu. Pomocí této α-galaktosidasy byl syntetizován α-D-Gal*p*-(1→3)-6-*O*-Ac-α-D-Gal*p*-*O*-*p*-NP. α-L-Rhamnosidasa z *Aspergillus niger* K2 si zachovala jako jediná svou schopnost selektivně modifikovat desglukoruscin (za odštěpení L-rhamnosy a vzniku desrhamnodesglukoruscinu). U houbových α-L-rhamnosidas byl potvrzen kinetickým měřením NMR invertující typ.

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1. INTRODUCTION

Microorganisms play a fundamental role in nature and in a life of mankind as they belong among the main factors influencing creation and preservation of the environment on the Earth. They are widespread in the whole biosphere. They are able to adapt to most varied and extreme conditions. Activity of microorganisms was already known by ancients. Microorganisms were instrumental in development of biosciences. Extensive part of knowledge in biochemistry was obtained by microorganism study, representing the simplest living model with basic properties of all organisms. Study of the metabolism of microorganisms enabled to decipher numerous metabolic processes common for the most of living systems.

Microorganisms are commonly used in scientific research and in industry. They are easily available and their cultivation is feasible. The advantage of microbial enzymes in comparison with plant or animal ones is their simple isolation, especially of the extracellular ones, cultivation under reproducible laboratory conditions and possibility of the scale up.

Filamentous fungi are often used in production of enzymes for processing of carbohydrate materials, *e.g.* glycosidases, glycoamylases and many others.

Numbers of biologically active compounds are glycosides, *e.g.* hormones, sweeteners, alkaloids, flavonoids and antibiotics. Sometimes, the glycosidic residue is crucial for their activity, in other cases glycosylation only improves pharmacokinetic parameters. New knowledge about the role of saccharides in cell-cell interactions, in immunology and in the initiation of some microbial infections lead to their practical application. Some saccharidic structures can be used as vaccines (synthetic) against some tumours and virus diseases, against unspecific inflammatory processes and for treatment of some immune diseases and can be applied for “drug targeting”.

Application of enzymes in combination with synthetic methods is one of the leading trends in glyco-sciences at present.

Glycoside hydrolases are a widespread group of enzymes, which hydrolyse the glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety.

Glycosidases from fungi proved to be very useful in the preparation of many glycosidic structures, such as β-galactosides, β-glucosides, β-mannosides, β-*N*-acetylglucosaminides and β-*N*-acetylgalactosaminides, by transglycosylation or reversed glycosylation. In addition, biochemical characteristics, *i.e.* production of particular

glycosidases, and their inducibility can reveal new chemotaxonomic features for better characterisation and eventual taxonomic re-classification of the strains.

We concentrated to exoglycosidases, mainly to β-*N*-acetylhexosaminidases, α-D-galactosidases and α-L-rhamnosidases.

β-*N*-Acetylhexosaminidase is widely distributed in nature, and purification procedures from bacterial, fungal, plant and animal sources have been reported. The hexosamines were found to be important ligands for the major activating receptor protein of rat natural killer cells (NKR-P1). An important factor for the binding potency to the receptor is oligosaccharide chain length. Hexosamine oligosaccharide building blocks that can eventually be turned into multivalent neoglycoconjugates can be synthesised by the use of β-*N*-acetylhexosaminidases.

α-D-Galactosidase microbial sources – mostly fungal – are useful in various industrial applications, such as hydrolysis of raffinose-type oligosaccharides in sugar beet syrup, soya milk, cowpea flour and modification of galactomannans (gelling properties). α-D-Galactosidase preparations are used as digestive aid for people intolerant to oligosaccharides from legumes or some other vegetables (flatulence). D-Galactopyranose (Gal, **1**) is ubiquitous constituent of oligosaccharide chains of soluble and cell surface bound glycoconjugates with important biological functions.

Many microorganisms have been studied for their potential to produce glycosidases. However, little is known about microorganisms that produce α-L-rhamnosidases.

L-Rhamnose (Rha, **2**) is widely distributed in plants and bacteria as a component of cell walls or of various natural products. Some rhamnosides are important bioactive compounds, *e.g.* cytotoxic saponins, antifungal plant glycoalkaloids and bacterial virulence factors. Glycosides from rhizomes of *Ruscus aculeatus* have important pharmacological properties, in particular the anti-inflammatory activity. One of them, desglucoruscin (**3**), can be used for the treatment of chronic venous insufficiency. Presently this compound is investigated for its cytostatic activity on leukaemia HL-60 cells. Several technological applications of fungal α-L-rhamnosidases in the food industry, such as the debittering of grapefruit juice by hydrolysis of the bitter component naringin, the elimination of hesperidin crystals from orange juice and the enhancement of wine aromas by enzymatic hydrolysis of terpenylglycosides, have been investigated.

2. WORKING HYPOTHESIS

Within recent years the application of enzymes in organic chemistry became a well-established technique. Synthesis and modification of carbohydrates using enzymes belong to one of the most intensively exploited area of enzyme applications. Glycosylation is considered to be an important method for structural modification of compounds with useful biological activities. Glycosides comprise of several important classes of the compounds such as hormones, sweeteners, alkaloids, flavonoids, antibiotics, *etc*. In the heteroglycosides the glycosidic residue can be crucial for their activity or can only improve pharmacokinetic parameters, *e.g.* solubility, toxicity, transport.

Sugar-based drugs are suddenly on a medicine's menu, *e. g.* as vaccines-antimicrobial (against *H. influenzae*) or anticancer or for a direct treatment of some pathologic processes as envisaged for the Le^x antigen.

Which enzymes?

There are two basic approaches in the enzymatic glycosylation: the use of glycosidases or the use of glycosyltransferases. To concentrate our efforts onto our targets we dealt mainly with glycosidases (and some other enzymes, *e.g*. lipases). Glycosyltransferases are more expensive and rare enzymes obtainable from commercial sources or by laborious isolation from scarce and hardly available material (human milk, various animal glands, *etc*.) or by cloning, which is highly cost- and labour-demanding.

Glycosidases are cheap and quite robust enzymes utilising relatively inexpensive donors (compared to sugar nucleotides needed for the glycosyltransferases). They show absolute anomeric selectivity, ensured by the type of enzyme that is one of their main advantages. On the other hand, the main drawbacks for their use in glycoside synthesis are lower yields and sometimes lower regioselectivity. Glycosidases can form new glycosidic bonds by kinetically controlled transglycosylation or by reverse hydrolysis that is regulated thermodynamically.

In the first case, glycosidases use activated glycosides (the most often *p*-nitrophenyl glycosides) or disaccharides as glycosyl donors. In the second case, free sugars in high concentration are employed.

Regioselectivity often depends on the source of an enzyme and, therefore, it can be influenced by the rational choice of an appropriate glycosidase with more pronounced regioselectivity. Other factors influencing regioselectivity are, *e.g.* reaction conditions,

immobilisation of the enzyme and, quite often, anomeric configuration of the acceptor glycoside. Sometimes, also the type of reaction - transglycosylation/reverse glycosylation - influences the product formed. Thus, for the synthesis of pure regioisomers a broad choice of glycosidases is required and, therefore, screening for new, more selective glycosidases is very important. Another possibility is the use of regioselective protection of the acceptor saccharide, quite often by (enzymatic) acylation.

- Development of new enzymes

Large collection of microbial strains will be screened for enzyme producing. Sets of enzymes (enzyme libraries) will be created, which will be used for further screening and for particular task solution. Production of the most promising enzymes will be scaled up.
Enzymatic modification of sugars and glycosidic derivatives will be performed using not only glycosidases, but also using lipases and proteases.

- Development of new methods

Novel methods for enzymatic synthesis of bioactive glycosides will be developed using complementary approaches, *e.g.* biphasic systems, cosolvents, high salt concentrations.
New reaction conditions will be tested in enzymatic glycosylation to improve their effectiveness.
Semi-synthetic substrates and sugar mimetics will be used as targets for enzymatic glycosylation.
New synthetic glycosidase donors (*e.g.* azides) will be developed and optimised. Regioselectivity of enzymatic glycosylations will be increased using partly protected sugars by chemoenzymatic acylation.
Advanced NMR methodology and molecular modelling will be used for mechanistic studies of interactions of inhibitors and modified substrates with glycosidases and target proteins (receptors).

One of the major results of this work should be large versatile library of well characterised glycosidases applicable for various tasks.

3. THEORETICAL BACKGROUND

3. 1. Microorganisms in Nature and in Industry

Microorganisms play a fundamental role in nature and in a life of mankind because they belong among the main factors influencing creation and preservation of the environment on the Earth. Communities of various types of microorganisms are able to decompose virtually all organic substances to their total mineralization. Thus they return chemical elements, which are essential components of cell material, to element cycling in nature. Decomposition activity proceeds not only in the soil, where is the cause of enrichment of the soil, but also in waterways, in stagnant water and in the sea. This activity is the main part of so-called self-purification of streams (stream sanitation) and in industry it is used in waste treatment plants.

Microorganisms are widespread in the whole biosphere. They are virtually everywhere. We can find them in the air, soil and water all around the world – in tropical places as well as in polar parts of the Earth. They are in food we eat, in water we drink and in the air we breathe. They live inside human, animal and plant bodies. They are able to adapt to most varied and extreme conditions – low or high temperature, pressure (atmospheric, hydrostatical, and osmotic), concentration of nutrients, *etc*. The most of thermophilic bacteria have the optimal growth temperature from 70 °C to 100 °C. In the Pacific Ocean near Philippines bacteria live in about 11 000 m deep under hydrostatic pressure 100 MPa. Bacteria of family *Halobacteriaceae* demand for their growth 8 – 15 % NaCl in surrounding at least, optimal is 20 - 26 % NaCl (3.5 – 4.5 M NaCl). They live in salty lakes, in seawater, salt-mines and in salt-works.

Microorganisms accompany us through the all life from our birth to the death. Some of them are useful for us and some of them are pathogenic. Very fast reproduction of microorganisms and insufficient hygiene can lead to large epidemic („pandemic“). Other negative effect of microorganisms is undesired decomposition of food, food material, textile, paper, leather, wood, organic paint and some plastic materials. Nevertheless, some other microorganisms can inhibit the growth of the unwanted ones; *e.g.* lactic fermentation of vegetables, which inhibits decay of these materials, production of cheese, milk or fermented drinks.

3. 2. Microbiology in the Development of Biosciences

Activity of microorganisms was already known by ancients who use them for production of alcohols, bread, cheese and other food. Microorganisms as such found A. van Leeuwenhoek in 17. century, who constructed the first microscope. L. Pasteur and his equals proved that microorganisms are responsible for disintegrative processes, which proceed in nature and for methods traditionally used for production of fermented food and drinks.

Microorganisms were instrumental in development of biosciences. Extensive part of knowledge in biochemistry was obtained by microorganism study, representing the simplest living model with basic properties of all organisms. Study of the metabolism of microorganisms enabled to decipher numerous metabolic processes common for the most of living systems. Very short doubling time and possibility of cultivation and analysis of huge populations under reproducible conditions make the microorganism an ideal model for genetic studies.

3. 3. Microorganisms as a Powerful Production Tool

Microorganisms are commonly used in scientific research and in industry. They are easily available and their cultivation is feasible. The advantage of microbial enzymes in comparison with plant or animal ones is their simple isolation, especially of the extracellular ones, cultivation under reproducible laboratory conditions and possibility of the scale up.

Activity of microorganisms is used not only in well-known fermenting industry and dairying, but also in pharmaceutical industry producing a large amount of antibiotics, as well as for fermentative production of organic acids, vitamins and enzymes, in chemical industry, and also in bioenergetics and biometallurgy.

3. 4. Advantages and Drawbacks of Using Filamentous Fungi

Filamentous fungi are often used in production of enzymes for processing of carbohydrate materials, *e.g.* glycosidases, glycoamylases and many others (*see* **3. 7.** *Glycoside Hydrolases*).

Nutrients are absorbed over the whole surface of the mycelium, making for the efficient utilization of natural resources and also making possible the cultivation of fungi in synthetic media. Prior to the use of respective fungal strain for experimental work it is necessary to ensure a stable source of the organisms. This involves three problems: keeping the organisms alive, keeping them free from contamination by other organisms and keeping biochemical and physiological stability and it follows reproducibility of the results. The last of these requirements is the most difficult, for fungi are extremely variable organisms – strains obtained from different sources, while appearing morphologically identical, will not necessarily behave in the same way biochemically. What is worse, variation can occur within a given strain. The mycelium undergoes spontaneous changes – mutation. The mutation can be associated with a visible change, *e.g.* production or absence of pigment, or with a biochemical function. This can happen during repeated subculturing of an organism and can lead to the loss of the given feature. This tendency to change is universal among fungi, though some are more stable than others (*Turner, 1971*).

3. 5. Glycobiology

Carbohydrates are unique in the complexity of their structures. In contrast to nucleic acids and proteins, carbohydrates are linked together in various ways. First, one sugar residue can be bound to four of five different positions of hydroxyl groups of neighbouring sugar residues. Second, the linkage between two sugar residues can be of the axial or equatorial configuration (α- and β-linkages). Third, the carbohydrate structure allows branching. Besides this carbohydrates can exist in more forms, *e.g.* furanoses and pyranoses. These characteristics distinguish carbohydrates from the rest of the biological macromolecules, which contain almost exclusively linear structures. Because of this complexity, carbohydrates can provide almost unlimited variations in the structure. Thus, it is safe to say that carbohydrates have high information capacity, *e.g.* providing various recognition signals. On the other hand, it is also noticeable that the actual variation in carbohydrate structure is

rather limited and only a fraction of the theoretically possible structures are found in nature. Actual variation, therefore, appears to be borne out of necessity and it is likely that most cell type-specific carbohydrates are functional, although we have not yet succeeded in determining their functions in all of the cases. This limitation in variations is caused mainly by two factors. Firstly, carbohydrates are not primary gene products. They are synthesised by glycosyltransferases. Thus, it is necessary to provide genes for every new structure of carbohydrates. It is then reasonable that living cells have a limited number of variations in carbohydrates since necessity of gene diversification is involved. Secondly, carbohydrates are very often synthesised from a common precursor, what also limits the variation of the final products. In addition to this, carbohydrates can be also present without being attached to other molecules. However, the majority of carbohydrates present in cells are attached to proteins (proteoglycans and glycoproteins) or lipids (glycolipids). In proteoglycans, the carbohydrates are composed of repeats of disaccharide units and they are relatively large (>3000 Dalton). In glycoproteins, a repeating structure is usually absent except for *e.g.* poly-*N*-acetyllactosamines. Carbohydrates attached to proteins can be classified into two groups, *N*-glycans and *O*-glycans. In *O*-glycans, saccharides, mostly β-galactosyl-(1→3)- 2-acetamido-2-deoxy-α-D-galactopyranosyl, are attached to hydroxyl groups of amino acids, typically serine and threonine, by α-linkage. In N-glycans, the reducing terminal 2-acetamido-2-deoxy-D-glucopyranosyl is β-linked to the amide group of asparagine, thus forming an aspartylglycosylamine linkage.

Cell surface carbohydrates are major components of the mammalian cell surface and these carbohydrates are very often typical characteristics of cell type. Carbohydrate structures change dramatically during mammalian development. Specific sets of carbohydrates are expressed at different stages of differentiation and in many instances these carbohydrates are recognised by specific antibodies, thus providing differentiation antigens. In mature organisms, expression of distinct carbohydrates is eventually restricted to specific cell types, providing cell-type-specific carbohydrates. Aberrations in these cell surface carbohydrates are associated with various pathological conditions, including malignant transformations.

It is assumed that cell type-specific carbohydrates are probably involved in cell-cell interaction, in particular as molecules that are recognised by carbohydrate-binding proteins. The latter include lectins and some glycosyltransferases.

3. 5. 1 Glycosidic Residue in Biological Activity of „Small" Molecules

Numbers of biologically active compounds are glycosides. Sometimes, the glycosidic residue is crucial for their activity, in other cases glycosylation only improves pharmacokinetic parameters. Recent developments in molecular glycobiology brought better understanding to the aglycone *vs*. glycoside activities, and made it possible to develop new, more active or more effective glycodrugs based on these findings. The story of involvement of glycosyl moiety in the vancomycin activity is probably one of the most illustrative and also very topical as the glycosyl-derivatives of vancomycin quite recently proved to be very effective against multiple resistant (even vancomycin-resistant) bacteria (*Solenberg et al., 1997*).

Glycosides comprise several important classes of compounds such as hormones, sweeteners, alkaloids, flavonoids, antibiotics, *etc*.

3. 5. 2 Influence of Glycosylation

It is generally accepted that glycosides are more water-soluble than the respective aglycons. Attaching of the glycosidic moiety into the molecule increases its hydrophilicity. This effect influences pharmacokinetic properties of the respective compounds, *e.g*. circulation, elimination, and the concentrations in the body fluids.

Modified hydrophilicity, however, influences mainly the membrane transport. Some compounds enter the cells just because of their "solubility" in the membrane components. Glycosylation can, in some cases, restrict or inhibit cell uptake of the particular compounds. Glycosylation can strongly influence transport through such important barriers as the haemato-encephalitic barrier and block the entrance of many compounds into the brain tissue. Contrary, some glucosides can be transported actively into the brain tissue using the glucose-transport system. Another important interface in which glycosylation plays a crucial role is the placental barrier. Here many glucuronides are blocked to enter foetal tissue, thus preventing intoxication by metabolites of xenobiotics.

Interaction of some glycosidic moieties with receptors or lectins on the cell surfaces makes possible active uptake of the glycosides. A good example is the high affinity of β-galactosides to hepatocytes due to galectin-C occurring in high concentrations on cell surface (*Kempka et al., 1990*).

An important aspect for the prediction of glycoconjugate activities is also their susceptibility towards glycosidic cleavage at various sites of application. In the stomach and in the intestine most glycosides are hydrolysed, either by the acidic environment (stomach) or by the action of glycosidases (small intestine). There are, however, some glycosides - *e.g.* α-galactosides – that are not hydrolysed easily and such compounds are either unable to pass the haemato-intestinal barrier or penetrate unhydrolysed. Non-resorbed glycoconjugates can be cleaved later in the colon and metabolised by the intestinal microflora.

Some glycosides have specific individual biological activity that cannot be simply derived from the activity of the respective aglycone. The final activity is then given by the overall molecular structure. Comparison of the biological activities of the aglycon and the respective glycoside can elucidate the structure-activity correlations and can also demonstrate the advantage (or uselessness) of glycosylations of pharmacologically interesting molecules (*Křen and Martínková, 2001)*.

3. 5. 3 Role of Saccharides in Cell-Cell Interactions

New knowledge about the role of saccharides in cell-cell interactions, in immunology and in the initiation of some microbial infections lead to the practical application of saccharides. It was found that some saccharidic structures can be used as vaccines (synthetic) against some tumours and virus diseases, against unspecific inflammatory processes and for treatment of some immune diseases.

Saccharidic ligands interact with their receptors in a rather specific way; mostly by means of weak interactions (hydrogen-bonds), thus their saturating concentrations are relatively high (10^{-3} ~ 10^{-5} M). This is in 3 orders more than of many other – "strong" – ligands. Multiplying of these ligands leads to qualitative change of interaction, strengthening in several orders – so called multivalent effect. Polyvalent glycosidic structures of this type, *e.g.* glycodendrimers or glycomimetics ("glycodrugs") have substantial therapeutical potential. Further application of these phenomena is the use of glycoconjugates for "drug targeting" (*David et al., 2004*).

3. 6. Enzymes in Research

Application of enzymes in combination with synthetic methods is one of the leading trends in glyco-sciences at present. This frontier area of biochemistry, microbiology, chemistry, and biomedicine is a source of vast amount of new seminal information on the intercellular communication.

3. 6. 1 Enzymes in Synthetic Organic Chemistry

3. 6. 1. 1 Enzyme Capabilities

Enzymes catalyse most of the biological reactions *in vivo*. They also catalyse reactions involving both natural and unnatural substrates *in vitro* (*Wong and Whitesides, 1994*). As catalysts, enzymes have following major characteristics:

1. They accelerate the rate of reactions and can operate under mild conditions.
2. They can be highly selective for substrates and stereoselective in reactions they catalyse, selectivity can range from very narrow to very broad.
3. They may be subject of regulation; that is, the catalytic activity may be strongly influenced by the concentrations of substrates, products or other species present in solution.
4. They normally catalyse reactions under the same or similar conditions.
5. They are generally unstable.

3. 6. 1. 2 Progress in Enzyme Application

The characteristics of instability, high costs, and narrow substrate specificity have been considered to be the most serious drawbacks of enzymes for use as synthetic catalysts. Many techniques have been developed to improve the stability of enzymes and to facilitate their recovery (*Sun and Clark, 2001*). Advances in molecular and cell biology have created new tools for the manipulation of genetic material to construct genes for expression of desired proteins (*Goeddel, 1990; Steipe, 2004*). Recombinant DNA technology made possible the production of proteins and enzymes and the alteration of their properties. The area

of enzymatic catalysis was further stimulated by the discovery of catalytically active antibodies (*Lerner et al., 1991*).

3. 7. Glycoside Hydrolases (EC 3.2.1.-)

Glycoside hydrolases are a widespread group of enzymes, which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. The IUB-MB enzyme nomenclature of glycoside hydrolases is based on their substrate specificity and occasionally on their molecular mechanism; such a classification does not reflect (and was not intended to) the structural features of these enzymes. A classification of glycoside hydrolases in families based on amino acid sequence similarities has been proposed a few years ago (*Henrissat, 1991*) and it was updated (*Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997*). Because there is a direct relationship between sequence and folding similarities, such a classification:

(i) reflects the structural features of these enzymes better than their sole substrate specificity,
(ii) helps to reveal the evolutionary relationships between these enzymes, and
(iii) provides a convenient tool to derive mechanistic information (*Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Davies, 1997*)

The Carbohydrate-Active Enzymes database (CAZy) (http://afmb.cnrs-mrs.fr/CAZY/) provides a continuously updated list of the glycoside hydrolase families. Because the fold of proteins is better conserved than their sequences, some of the families can be grouped in 'clans'

(i) when new sequences are found to be related to more than one family,
(ii) when the sensitivity of sequence comparison methods is increased or
(iii) when structural determinations demonstrate the resemblance between members of different families (*Henrissat and Bairoch, 1996; Henrissat and Davies, 1997*).

In most cases, the hydrolysis of the glycosidic bond is performed by two catalytic residues of the enzyme: a general acid (proton donor) and a nucleophile/base (*Davies and Henrissat, 1995*). In some cases, the catalytic nucleophile is not borne by the enzyme, and is

replaced by the acetamido group at C-2 of the substrate (*Terwisscha et al., 1995*). A completely unrelated mechanism has been demonstrated recently for a family of glycosidases utilizing NAD^+ as a cofactor (*Rajan et al., 2004*). Depending on the spatial position of the catalytic residues, hydrolysis occurs *via* overall retention or overall inversion of the anomeric configuration, thus glycosidases are classified as either retaining (β-*N*-acetylhexosaminidases, α-galactosidases) or inverting (α-rhamnosidases) (*Withers, 2001*). Retaining glycoside hydrolases operate *via* a double displacement mechanism that leads to the retention of the configuration at the anomeric carbon of the sugar ring undergoing catalysis. These enzymes often display transglycosylating abilities. The catalytic machinery of these enzymes involves two catalytic carboxylates located on opposite sides of the sugar plane and that perform two separate chemical steps. In the first step (glycosylation), a carboxylic group provides general acid-catalysed leaving group departure simultaneously with a nucleophilic attack by the second carboxylate to form a glycosyl-enzyme intermediate. In the second step (deglycosylation), the first residue functions as a general base to activate the incoming nucleophile (a water molecule in the case of hydrolysis, and an alcohol in the case of transglycosylation), which hydrolyses the glycosyl-enzyme. The distance between the two carboxylates is approximately 5.5 Å. Many retaining glycoside hydrolases hydrolysing β-*N*-acetyl glucosaminic bonds utilise a double-displacement mechanism, in which the nucleophile is donated not by the enzyme, but by the C-2 *N*-acetamido group of the substrate itself (*Terwisscha et al., 1995*).

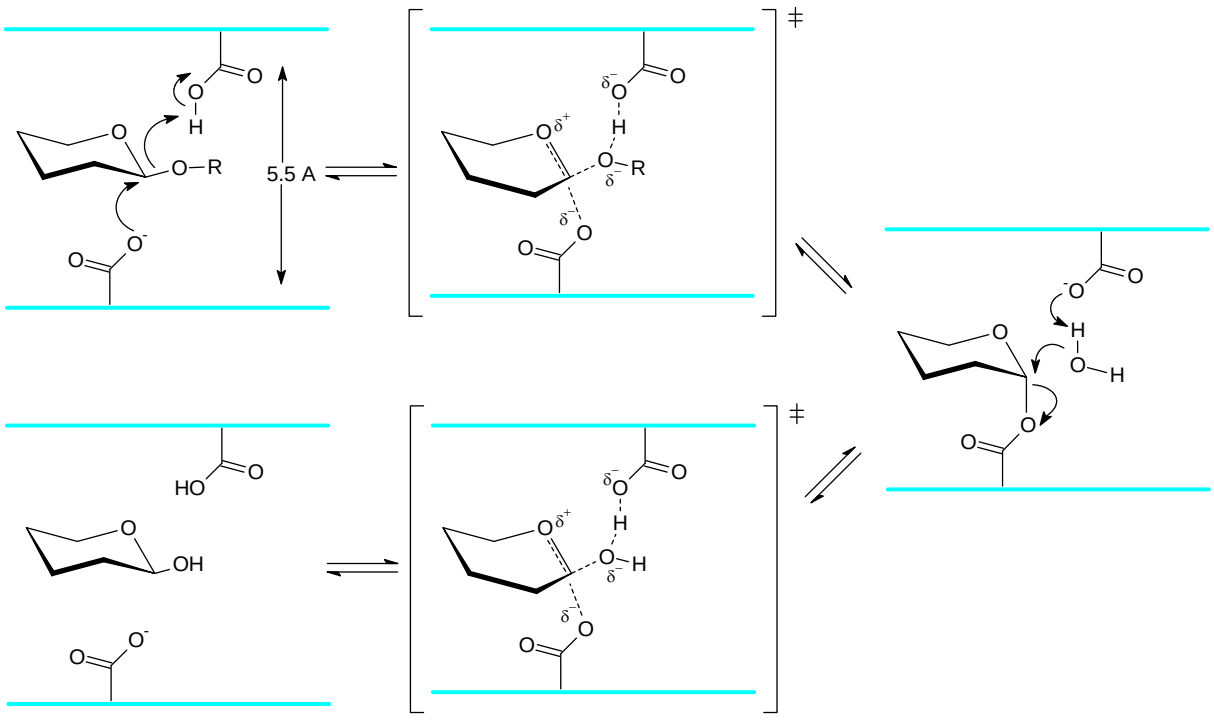


Fig 1: Mechanism of retaining glycosidases

Inverting glycoside hydrolases lead to an inversion of the anomeric configuration *via* a single nucleophilic displacement. Hydrolysis of a β-glycosidic bond thus creates a product with the α-configuration or *vice-versa*. The catalytic machinery of these enzymes involves two catalytic carboxylates in order to provide (i) general acid-catalysed leaving group departure and (ii) general base-assistance to nucleophilic attack by a water molecule from the opposite side of the sugar ring. The distance between the two carboxylates is less constrained as for the retaining enzymes and is in the range 6.5 - 9.5 Å.
(Sinnott, 1990; McCarter and Withers, 1994; Withers, 2001)

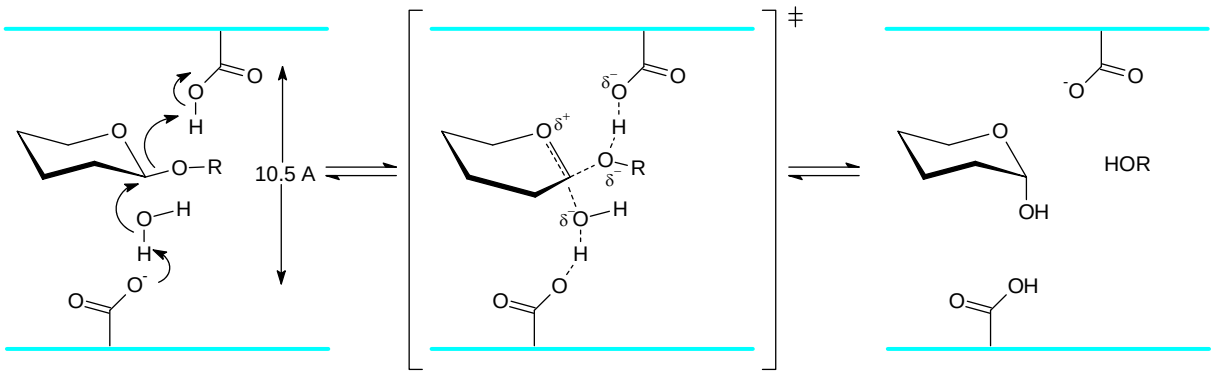


Fig 2: Mechanism of inverting glycosidases

The glycosidases, in general, can be divided into two groups: the exoglycosidases, which cleave glycosidic bonds at the non-reducing end of the oligosaccharide, and the endoglycosidases cleaving internal glycosidic bonds.

3. 7. 1 Application of Glycosidases

Glycosidases are very abundant and they are found almost in every organism. Some enzymes have been isolated and characterised, and they are commercially available as purified preparations. However, the number of these preparations is limited. For exploitation of these enzymes in practice it is required that their source is easily available, recoverable and that the enzymes are in a reproducible quality and quantity. Plant and animal tissues can be used as a starting material for purification procedures, but the rich source of a wide variety of glycosidases are microorganisms, particularly fungi. The advantage of microbial glycosidases in comparison with plant or animal ones is their rather simple isolation, especially if they are produced extracellularly, cultivation under reproducible laboratory conditions and possibility of scale up to industrial conditions. By a careful adjustment of cultivation conditions, and/or the use of suitable inducers an overproduction of a desired enzyme could be achieved (*Huňková et al., 1996 a; Huňková et al., 1999; Wallis et al., 2001*).

Glycosidases from fungi proved to be very useful in the preparation of many glycosidic structures, such as β-galactosides, β-glucosides, β-mannosides, β-*N*-acetylglucosaminides and β-*N*-acetylgalactosaminides, by transglycosylation or reversed glycosylation. Despite of the copious methods developed for the chemical synthesis of glycosides, the use of enzymes as catalysts is an attractive alternative, since the sugar coupling steps can be performed with high stereoselectivity and certain regioselectivity (*Huňková et al., 1999*). Another advantage of glycosidase-catalysed synthesis of glycosidic bonds is the lack of any requirement for protection-deprotection sequences (*Huňková et al., 1996 a*). Enzymatic glycosylation is important modification of biologically active substances. It allows conversion of lipophilic compounds to hydrophilic ones, thus it changes their pharmacological properties. The glycosylation using enzymes has an application particularly in the synthesis of thermodynamically not preferred glycosidic bonds, as, *e.g.* β-mannopyranosides, when the substrates are sensitive to chemical reaction conditions and where chemical glycosylation does not provide any product.

In addition, biochemical characteristics, *i.e.* production of particular glycosidases and their inducibility, can reveal new chemotaxonomic features for better characterisation and eventual taxonomic re-classification of the strains. So far, profiles of secondary metabolites and isoenzymes were used as an aid in the identification of many fungal genera, *e.g. Penicillium* (*Hansen et al., 2005; Fischer et al., 2006*; *Cruickshank and Pitt, 1987; Frisvad and Filtenborg, 1989; Paterson et al., 1989*), *Aspergillus* (*Fischer et al., 2006*; *Zohri and Ismail, 1994; Bridge and Hawksworth, 1984*), *Fusarium* (*Wasfy et al., 1987*), *Phoma* (*Monte et al., 1991*), *Monascus* (*Bridge and Hawksworth, 1985*), and *Beauveria* (*Mugnai et al., 1989*). Besides their production, enzyme inducibility and catabolic repression can provide an additional set of data reflecting regulatory systems and physiological typology of the strains. Biochemical and physiological differences can, therefore, help to identify species, which can be hardly distinguished morphologically.

We concentrated on exoglycosidases, mainly on β-*N*-acetylhexosaminidases, α-D-galactosidases, and α-L-rhamnosidases.

3. 7. 1. 1 β-*N*-Acetylhexosaminidases (EC 3.2.1.52)

β-*N*-Acetylhexosaminidase (2-acetamido-2-deoxy-β-D-hexopyranoside acetamidodeoxyhexohydrolase) catalyses hydrolysis of terminal non-reducing 2-acetamido-2-deoxy-β-D-glucopyranoside and 2-acetamido-2-deoxy-β-D-galactopyranoside residues with the release of 2-acetamido-2-deoxy-β-D-glucopyranose resp. 2-acetamido-2-deoxy-β-D-galactopyranose. It is widely distributed in nature, and purification procedures from bacterial (*Sakai et al., 1994*), fungal (*Yamamoto et al., 1986*; *Zhuravleva et al., 2004-marine fungus*), plant (*Jordan and Barber, 1995*), and animal (*Tomiya et al., 2006*) sources have been reported.

The hexosamines [2-acetamido-2-deoxy-glucopyranose **(4)** and 2-acetamido-2-deoxy-galactopyranose **(5)**] were found to be important ligands for the major activating receptor protein of rat natural killer cells (NKR-P1) (*Krist et al., 2001*). An important factor for the binding potency to the receptor is oligosaccharide chain length. Hexosamine oligosaccharide building blocks that can eventually be turned into multivalent neoglycoconjugates can be synthesised by the use of β-*N*-acetylhexosaminidases. *p*-Nitrophenyl glycosides are suitable building blocks for the synthesis of cluster glycosides and glycodendrimers and, moreover, the aromatic moiety improves the binding to the NK

receptors. Therefore, *p*-nitrophenyl β-chitobioside became one of targets of our laboratory as it can be expected to bind effectively to the NKR-P1 protein and can be clustered to multivalent conjugates (*Bezouška et al., 1997; Krist et al., 2001*).

3. 7. 1. 2 α-D-Galactosidases (EC 3.2.1.22)

α-D-Galactosidase (α-D-galactopyranoside galactohydrolase) catalyses hydrolysis of terminal non-reducing α-D-galactopyranoside residues with the release of α-D-galactopyranose.

α-D-Galactosidases from microbial sources – mostly fungal – are useful in various industrial applications, such as hydrolysis of raffinose-type oligosaccharides in sugar beet syrup, soya milk, cowpea flour and modification of galactomannans (gelling properties). α-D-Galactosidase preparations are used as digestive aid for people intolerant to oligosaccharides from legumes or some other vegetables (flatulence) (*LeBlanc et al., 2004*).

D-Galactopyranose (Gal, **1**) is a ubiquitous constituent of oligosaccharide chains of soluble and cell surface bound glycoconjugates with important biological functions. α-Gal moiety is an instrumental part of the globo-structures expressed on the cell surface, such as globotriose (α-Gal-(1→4)-β-Gal-(1→4)-Glc), which is the binding ligand of verotoxin (*E. coli* 0157 food poisonings), shiga toxin (*S. dysenteriae*) and adhesin (*Streptococcus suis*). Derivatives of these saccharides can be used as inhibitors of many deadly microbial infections. α-Gal-(1→3) terminated epitopes (typically *iso*globotriose α-Gal-(1→3)-β-Gal-(1→4)-Glc or so-called "Galili epitope" α-Gal-(1→3)-β-Gal-(1→4)-GlcNAc) are abundantly present on the cell surface of most mammals with the exception of humans and Old World primates. On the contrary, anti-Gal antibodies exist only in these primates. Xenotransplantation, *e.g.* pig-human are hampered by hyperacute rejection reaction caused by these antibodies. Derivatives of *iso*globotriose (multivalent or bound to a solid phase) can be used for removing the antiGal antibodies from the recipient's blood or block them *in situ* and thus eliminate the hyperacute rejection (*Buhler et al.*, *2003; Wiebe et al., 2006*). α-Gal-(1→3)-β-Gal is also the terminal part of agglutinogen of blood group B.

3. 7. 1. 3 α-L-Rhamnosidases (EC 3.2.1.40)

α-L-Rhamnosidase (α-L-rhamnoside rhamnohydrolase) catalyses hydrolysis of terminal non-reducing α-L-rhamnopyranoside residues with the release of α-L-rhamnopyranose.

Many microorganisms have been studied for their potential to produce glycosidases. However, little is known about microorganisms that produce α-L-rhamnosidases (*Manzanares, 2001*). The production of α-L-rhamnosidases (EC 3.2.1.40) by a number of mammalian tissues, plants, bacteria, and fungi was described. Bacterial α-L-rhamnosidases were isolated from *Sphingomonas* sp. (*Hashimoto and Murata, 1998*), *Bacteroides* JY-6 (*Jang and Kim, 1996*), and *Pseudomonas paucimobilis* (*Miake et al., 2000*), and the primary structures of the enzymes from *Clostridium stercorarium* (*Zverlov et al., 2000*) and *Bacillus* sp. GL1 (*Hashimoto et al., 2003*) were determined. Moreover, α-L-rhamnosidases of fungal origin were identified in different strains of *Penicillium* (*Young et al., 1989*) and *Aspergillus* species (*Gallego et al., 2001; Manzanares et al., 2001*) and, more recently, also in some *Mucor* and *Fusarium* strains (*Scaroni et al., 2002*). The gene sequences of two α-L-rhamnosidases (isoenzymes) produced by an *A. aculeatus* strain induced by hesperidin were recently determined (*Manzanares et al., 2001*).

L-Rhamnose (Rha, **2**) is widely distributed in plants and bacteria as a component of cell walls (*Perez et al., 2003*) or of various natural products (*Ikan, 1999*). Some rhamnosides are important bioactive compounds, *e.g.* cytotoxic saponins (*Yu et al., 2002; Bader et al., 1998*), antifungal plant glycoalkaloids (*Oda et al., 2002*), and bacterial virulence factors (*Deng et al., 2000*). Clear correlations between the presence of specific sugar residues and the biological activity of these molecules were shown in many cases (*Křen and Martínková, 2001*). In plants, L-rhamnose is also bound to several volatile compounds, *e.g.* aroma terpenol glycosides of wine (*Caldini et al., 1994; Spagna et al., 2000*), with a possible protective role against the toxicity of the free lipophilic aglycons. Finally, *Citrus* spp. accumulate large amounts of L-rhamnose-containing flavonoid glycosides (*e.g.* naringin and hesperidin), having different flavours and important antioxidant and anti-inflammatory activity (*Benavente Garcia et al., 1997*). Glycosides from rhizomes of *Ruscus aculeatus* have important pharmacological properties, in particular the anti-inflammatory activity. One of them, desglucoruscin (**3**), can be used for the treatment of chronic venous insufficiency (*Boyle et al., 2003*). Presently this compound is investigated for its cytostatic activity on leukaemia HL-60 cells (*Mimaki et al., 1998*). Ginseng saponins,

e.g. dammarane-type saponins, are extracted from the root of *Panax ginseng*. They are used in various formulations in oriental countries for more than 5000 years for medicinal purposes especially as a tonic, for treatment of cancer, diabetes, and heart problems (*Helms, 2004*). Ginsengosides are highly glycosylated and their activity often differs depending on the number of glycosyl units attached. They can be interconverted by trimming by the glycosidases and this is a way by which some more scarce ginsengosides are produced (*Ko et al., 2003; Kohda and Tanaka, 1975*). Asiaticoside (**6**), a saponin component isolated from *Centella asiatica* with an ursane-type triterpene structure carrying a trisaccharide unit, has anti-inflammatory properties by stimulating wound-healing processes *via* fibroblast proliferation and collagen and glycosaminoglycan synthesis (*Lu et al., 2004; Sampson et al., 2001*). Glycosidases, *i.e.* β-D-glucosidases and α-L-rhamnosidases, modify asiaticoside to more lipophilic substance, thus modulate its bioactivity. Rutin (**7**) (3-*O*-rhamnosyl-glucosyl-quercetin) widely occurs in many plants, fruits and vegetables. During digestion major part of the rutin is metabolised to its aglycone, quercetin (3,3′,4′,5,7-pentahydroxyflavone), which is a good antioxidant (*Duthie et al., 1999; Grinberg et al., 1994*). They are used in many countries as vasoprotectants. Another flavone glycoside quercitrin (**8**) (3-*O*-rhamnosyl-quercetin), isolated from the bark of the oak (quercus) as a bitter yellow substance, used as a pigment and called quercitron, is also hydrolysed to its aglycone quercetin. Quercitrin has also antioxidant and anti-inflammatory properties (*Comalada et al., 2005*). Several technological applications of fungal α-L-rhamnosidases in the food industry, such as the debittering of grapefruit juice by hydrolysis of the bitter component naringin (**9**) *(Soares and Hotchkiss, 1998; Soria and Ellenrieder, 2002*), the elimination of hesperidin (**10**) crystals from orange juice *(Terada et al., 1995*), and the enhancement of wine aromas by enzymatic hydrolysis of terpenylglycosides *(Caldini et al., 1994; Spagna et al., 2000*), have been investigated. Despite this industrial interest, only few crude α-L-rhamnosidase preparations are commercially available so far, specifically the so-called hesperidinase and naringinase. Moreover, all these preparations, presently obtained from genera *Aspergillus* and *Penicillium*, contain contaminating β-D-glucosidase activities that can limit their synthetic exploitation.

3. 8. 2-Acetamido-2-deoxy-mannopyranose (ManNAc, 11)

2-Acetamido-2-deoxy-mannopyranose (ManNAc, **11**) is ubiquitous in nature and it is present in a range of both simple and complex biopolymers.

ManNAc unit is an integral part of a number of bacterial capsular polysaccharides, *e.g*. trisaccharide repeating unit of *Streptococcus pneumoniae* (**Fig. 3**) and lipopolysaccharides, *e.g*. bacteria *Pseudomonas cepacia* O5, *Pseudomonas aeruginosa* X, and *Aeromonas salmonicida* involve ManNAc-β-(1→4)-L-Rha disaccharidic units and *Escherichia coli* O1A contents ManNAc-β-(1→2)-L-Rha units.

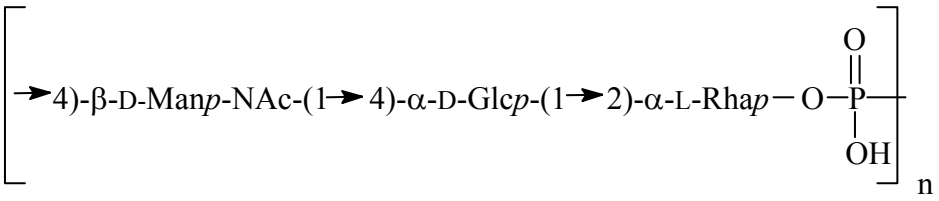


Fig. 3 The repeating unit of *Streptococcus pneumoniae* 19F polysaccharide

The capsular polysaccharides display type-specific immunogenic character (*Gridley and Osborn, 2000*). They are considered as the principle antigens in the majority of Gram positive and negative organisms. Disaccharide GlcNAc-β-(1→4)-ManNAc was identified as one of the strongest oligosaccharidic ligands of NK-cell activating receptor NKR-P1 (*Sedmera et al., 1998*). In the cell walls of gram-positive bacteria, various types of teichoic acids have been reported to be attached to muramic acid 6-phosphate residues of peptidoglycan through special linkage units, which commonly consist of a linkage disaccharide, ManNAc-β-(1→4)-GlcNAc or Glc-β-(1→4)-GlcNAc, and glycerol phosphate-containing parts.

ManNAc is a key precursor of *N*-acetylneuraminic acid (NANA or Neu5Ac), a major representative of a special class of amino sugars, the sialic acids. They are incorporated at the terminal positions of glycoproteins and glycolipids and are important for cellular recognition. In prokaryotic cells, sialic acid has also been found as a capsular polysaccharide constituent of a few genera of pathogenic bacteria, *e.g*. *Neisseria meningitides*, *Escherichida coli*, *Pasteurella haemolytica*, *Moraxella nonliquefaciens,* and several strains of *Salmonella*. Bacterial polysialic acids show biochemical and epitope resemblances to certain eukaryotic cell glycoconjugates, such as cell adhesion molecules. This similarity protects them from host bacteria killing.

4. MATERIALS AND METHODS

4. 1. Materials

4. 1. 1 Microorganisms

4. 1. 1. 1 Filamentous Fungi

Almost all filamentous fungi originated from the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague. *Aspergillus niger* K1 and *A. niger* K2 originated from the Culture Collection of the Institute of Microbiology (CCIM), Prague. The strains were stored as slants in a fridge (5 °C). Our library of filamentous fungi comprises more than 150 strains of several genera (*e.g. Aspergillus, Fusarium, Mucor, Penicillium and Trichoderma)*.

4. 1. 1. 2 Bacteria

Rhodococcus equi A1, *R. equi* A2, *R. equi* A4, *R. equi* A5, *R. equi* A8, *R. equi* A9, *R. equi* A10, *R. equi* A11, *R. equi* A12 and *R. equi* A14 originated from the University of Veterinary and Pharmaceutical Sciences Brno (VFU Brno), Institute of Microbiology and Immunology, Brno. *Acinetobacter sp.* CCM 2881 and *Pseudomonas sp.* CCM 1640 originated from the Czech Collection of Microorganisms (CCM), Faculty of Science, Masaryk University, Brno. *Rhodococcus rhodochrous* IFO 15564 is deposited in the Institute for Fermentation (IFO), Osaka, Japan. *Bacillus licheniformis* E3, *Bacillus subtilis*, *Bacillus subtilis* BSA 3, *Corynebacterium sp*. 3B, *Pseudomonas stutzeri* and *Rhodococcus erythropolis* A4 were isolated and identified by the Institute of Microbiology of the ASCR, and are stored there in. 10 soil isolates so far unidentified were deposited also in Institute of Microbiology of the ASCR. Bacteria were stored as slants at 5 °C.

4. 1. 2 Enzymes

The enzymes used were prepared in our laboratory if not mentioned otherwise. Filamentous fungi were grown in liquid media containing respective inducer, *see* **4**. **2. 4**.

- Naringinase and hesperidinase used in the screening for selective derhamnosylation of desglucoruscin were from Sigma.
- α-Galactosidases from *Aspergillus niger* and *Coffea arabica*, used in the screening of the stability of *p*-nitrophenyl 6-*O*-acetyl-α-D-galactopyranoside were purchased from Sigma.
- The following enzymes were used for enzymatic acylations of *N*-acetylhexosamines: lipase PS from *Burkholderia cepacia* (Amano) adsorbed on celite (Hyflo Super Cell celite, Fluka) (*Bovara et al. 1991*), lipase B from *Candida antarctica* immobilised on macroporous acrylic resin ((Novozym 435, Novo-Nordisk), lipase from porcine pancreas (Sigma), lipase from *Chromobacterium viscosum* and lipase CE-5 from *Humicola lanuginosa* (Amano), protease N and proleather (both from *Bacillus subtilis*, Amano), alcalase (Novo-Nordisk) and subtilisin (protease type VIII from *Bacillus licheniformis*, Subtilisin Carlsberg, Sigma).

4. 1. 3 Chemicals

- Rutin, asiaticoside, *p*-nitrophenol, *p*-nitrophenyl α-D-galactopyranoside (*p*NP-α-D-Gal), *p*-nitrophenyl β-D-galactopyranoside (*p*NP-β-D-Gal), *p*-nitrophenyl α-L-rhamnopyranoside (*p*NP-α-L-Rha), *p*-nitrophenyl β-D-glucopyranoside (*p*NP-β–D-Glc), Tergitol (type NP-40), cystamine dihydrochloride, cysteamine hydrochloride, protease inhibitor cocktail for bacterial cells , pyridine, acetone, vinyl acetate, *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO) and acetonitrile, were from Sigma-Aldrich.
- Leupeptin and pepstatin A were from Serva Electrophoresis GmbH, DE
- 2-Methylpropan-2-ol was from Lachema, CZ
- Lactose and D-galactose were purchased from Fluka, CH

- *p*-Nitrophenyl α-L-arabinopyranoside (*p*NP-α-L-Ara), *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*p*NP-β-D-GlcNAc), *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (*p*NP-β-D-GalNAc), L-rhamnose, hesperidin, naringin, 2-acetamido-2-deoxy-D-glucopyranoside (GlcNAc) and 2-acetamido-2-deoxy-D-galactopyranoside (GalNAc) were purchased from Senn Chemicals, CH.
- Quercitrin was from Extrasynthese, FR.
- Bradford reagent, albumin standard (Pierce) and Bio P-2 Gel (200 Da – 3 kDa) were from Bio-Rad, USA.
- 6-Deoxyglucose was from Koch-Light, UK
- ManNAc was prepared by epimerisation of GlcNAc in our laboratory (*Mahmoudian et al.,* 1997).
- *p*-Nitrophenyl 2-acetamido-2-deoxy-α-D-mannopyranoside (*p*NP-α-D-ManNAc) and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-mannopyranoside (*p*NP-β-D-ManNAc) were prepared in our laboratory by Dr. P. Krist (*Krist et al.*, 2003)
- Trifluoroethyl acetate and trichloroethyl butyrate were prepared according to the standard procedure (*Riva and Klibanov, 1988*).
- Ginsenoside Re, desglucoruscin and a standard of desrhamnodesglucoruscin were a gift from Prof. Danieli, Milano University, Italy.
- All the other chemicals were of the analytical grade.

4. 1. 4 Materials Used for Microbiological Procedures

- Agar with malt extract used for preparation of solid medium for keeping filamentous fungi was from Imuna (Šarišské Michaľany, SK).
- AGAR No. 1, yeast extract, pepton, malt extract, casein hydrolysate and beef extract were from OXOID Ltd., UK.
- Crude chitin hydrolysates were prepared in our laboratory by controlled acid-catalysed hydrolysis (*Bredehorst et al., 1996, T. Semeňuk, 2000*) of chitin (Sigma).

4. 1. 5 Other Materials

- Thin-layer chromatography (TLC) was carried out on Merck (DE) precoated 60 F_{254} plates.
- Flash column chromatography was performed on silica gel 60 M (40 - 63 μm,), Merck, DE
- Ionic exchanger Dowex 50WX2-200 (H^+) was from Aldrich.
- Toyopearl HW-40F (separation range 100 Da – 10 kDa) was obtained from Tosoh Corp., JP
- Amberlite XAD-4, BDH Chemicals Ltd., GB
- BioGel P-2 Fine (separation range 100 – 1800 Da), Bio-Rad, US
- Snail gut enzyme was prepared from locally collected snails *Helix pomatia*.

4. 1. 6 Buffers

- Citrate-phosphate buffer (0.05M, pH 3.5, 4.5, 5.0)
- McIlvaine buffer (0.1M citric acid and 0.2M Na_2HPO_4, pH 5.0)

4. 1. 7 Equipment

- Laboratory balance KERN 440-45, Gottl. KERN&Sohn GmbH, DE
- Analytical balance Precisa 80 A-200 M, Precisa Gravimetrics AG, CH
- pH meter GRYF 208 L, GRYF HB spol. s r.o., CZ
- Combined pH electrode THETA ’90 type HC114, 2 THETA ASE, s.r.o., CZ
- Combined pH microelectrode THETA ’90 type HC153, 2 THETA ASE, s.r.o., CZ
- Thermomixer compact 5350, Eppendorf AG, DE
- Refrigerated centrifuge UNIVERSAL 16 R, A. Hettich, DE
- Refrigerated centrifuge Beckman J2-21, Beckman Coulter, Inc, US
- Microbial safety cabinet HOTTE MSC.9 STD GAZ, Jouan S.A., FR
- Orbital incubator IOC400.XX2.C, SANYO Gallenkamp PLC, UK
- Spectrophotometer UV-1202 UV-VIS, SHIMADZU CORPORATION, JP
- Spectrophotometer Jasco V-530 UV/VIS, Jasco, Inc., US
- Lyophilizer Lyovac GT 2, Leybold AG, DE

- High-Performance Liquid Chromatograph:

  a) Lichrospher 100 NH_2 column (250 × 4 mm) and guard column (10 × 4 mm) – both (5 μm; Watrex, CZ) using a modular system (Spectra Physics, US) consisting of SP 8800 ternary gradient pump, SP 8880 autosampler and Spectra Focus scanning UV/VIS detector operated at 210 nm

  b) Lichrospher 100-5 NH_2 column (5 μm, 250 × 8 mm, Watrex, CZ) using a modular system (Spectra Physics, CA, USA) consisting of SP 8810 ternary gradient pump, Rheodyne injection port with a 100 μl sample loop, and Spectra Focus scanning UV/VIS detector operated at 200 nm

  c) Polymer IEX H^+-form column (8 μm, 250 × 8 mm, Watrex) using a system consisting of a solvent-delivery system 600 (Waters), a photo-diode array detector 996 (Waters) operated at 210 nm

  d) Jasco 880-PU pump equipped with a Jasco 870-UV detector and a Hewlett-Packard HP-3395 integrator, Jasco, Inc., US
- ultrasonic bath KLN K 10, KLN Ultraschall GmbH, DE
- ultrasonic homogenizer model CP 50 with probe model ASI, Cole-Parmer Instrument Co., US
- Biological thermostat BT 120, Laboratory instruments Prague, CZ
- Vacuum evaporator Rotavapor R-114, BÜCHI, CH
- Labsystems Twinreader® PLUS, typ 381, Labsystems, FI
- Varian INOVA-400 NMR spectrometer, US
- Bruker AC-300 NMR spectrometer, Bruker-Franzen, DE
- LCQ™ quadrupole ion-trap mass spectrometer, Finnigan MAT, CA equipped with a Nano-ESI source, Protana, DK
- Gas Chromatograph: capillary crosslinked methyl silicone gum column (HP-1, 25 m × 0.32 mm × 0.52 μm film tickness, Hewlett-Packard).
- *Perkin-Elmer* 241 polarimeter, PerkinElmer Life And Analytical Sciences, Inc., US
- Bruker BIFLEX II MALDI-TOF mass spectrometer, Bruker-Franzen, DE

4. 2. Methods

4. 2. 1 General Methods

4. 2. 1. 1 Analytical and Semi-Preparative HPLC

HPLC methods are described at respective procedures.

4. 2. 1. 2 Analytical GC

GC analyses were performed with a capillary crosslinked methyl silicone gum column (HP-1, 25 m × 0.32 mm × 0.52 μm film tickness, Hewlett-Packard).

Sugar derivatisation for GC analysis:

A sample of the enzymatic reaction solution (30 μl) was diluted with pyridine (30 μl) and reacted with 1,1,1,3,3,3-hexamethyldisilazane (30 μl) and CF_3COOH (3 μl). After 5 minutes, 5 μl of this final solution was injected into the GC. Conditions: for GlcNAc and its derivatives: oven temperature at 200 °C for 3 minutes, then from 200 °C to 300 °C with a heating rate of 5 °C/min.

4. 2. 1. 3 Thin-layer Chromatography (TLC)

Thin-layer chromatography (TLC) was carried out on Merck precoated 60 F_{254} plates, for the specific mobile phase see respective procedures (**4. 2. 7**, **4. 2. 8**, and **4. 2. 9**), detection was performed with molybdate reagent (Hanessian reagent) ($(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 42 g; $Ce(SO_4)_2$, 2 g; H_2SO_4 conc., 62 ml made up to 1 l of deionised water) or by spraying with 20% H_2SO_4 in EtOH, followed by charring at 150 °C. UV active compounds were detected under UV lamp (254 nm).

4. 2. 1. 4 Flash Column Chromatography

Flash column chromatography was performed on silica gel 60 (40 - 63 μm, Merck), columns used were of glass for high pressure chromatography. For the respective eluent see the certain case (**4. 2. 7** and **4. 2. 8**). Flow rate of mobile phase was accelerated by the nitrogen pressure.

4. 2. 1. 5 Nuclear Magnetic Resonance

NMR spectra were measured on a Varian INOVA-400 spectrometer (399.89 MHz for ^{1}H, 100.55 MHz for ^{13}C) and Bruker AC-300 (300 MHz and 75.2) MHz in DMSO, CD_3OD, $CDCl_3$ or D_2O (*see Appendix*) at 30 ^{o}C. The residual solvent signal was used as an internal reference (DMSO: δ_H 2.50, δ_C 39.6; CD_3OD: δ_H 3.33, δ_C 49.3); internal acetone was used in D_2O (δ_H 2.03, δ_C 30.5) and $CDCl_3$ (δ_H 7.265, δ_C 77.00) solutions. Reported assignments are based on COSY, TOCSY, HMQC, and HMBC experiments. 1D-TOCSY was used to obtain ^{1}H NMR parameters in the case of overlap. The anomeric configuration in the *manno*- series is based on diagnostic direct couplings $^{1}J_{(C\text{-}1,H\text{-}1)}$ obtained from coupled-HMBC. The acylation sites were determined by HMBC (protons at the acylated carbon and the α-protons of the acyl are coupled to the same carbonyl). Supporting arguments were provided by downfield acylation shifts both in ^{1}H and ^{13}C NMR spectra. With the exception of non-reducing sugars and *p*-NP glycosides, all compounds were mixtures of tautomers. However, as the ^{1}H NMR spectra were measured from fresh solutions, the relative proportions of individual components might not reflect the equilibrium composition (*Angyal, 1984 and 1991*).

4. 2. 1. 6 Electrospray Mass Spectrometry

Electrospray Mass Spectrometry (ESI-MS) spectra were recorded with a *LCQ*™ quadrupole ion-trap mass spectrometer (*Finnigan MAT*, San Jose, CA) equipped with a Nano-ESI source (*Protana*, Odense, Denmark). Due to the unavoidable presence

of Na^+ ions during sample preparation and the high alkali-cation affinity of oligosaccharides, all positively charged ions produced were ionized with Na^+.

4. 2. 2 Microorganism Storage

4. 2. 2. 1 Storage of Filamentous Fungi

Filamentous fungi were stored as slants on agar at 5 °C. In order to keep their properties unchanged, they were stored on two different agars and regularly refreshed using cross subculturing on a new fresh agar. To reduce spontaneous changes (mutation), which the mycelium can undergo during repeated subculturing they were re-innoculated with low frequence.

Agar 1 (pH 7): Commercial agar with malt extract (53 g) was dissolved in distilled water (1000 ml); for better mechanical properties more agar (AGAR No. 1, 5 g/l) was added. pH was approximately 7 without adjusting. After sterilisation the slants were stored in a fridge (5 °C).

Agar 2 (pH 5.5) [g/l]: Glycerol 125, malt extract 45, NaCl 15, NH_4NO_3 0.44, $MgSO_4 \cdot 7\ H_2O$ 0.06, $CuSO_4 \cdot 5\ H_2O$ 0.0015 and agar (AGAR No. 1) 25 were dissolved in distilled water and pH was adjusted to pH 5.5 with KOH (40% w/v). After sterilisation slants were stored in a fridge (4 °C).

4. 2. 2. 2 Storage of Bacteria

Bacteria were stored as slants on MPA agar at 5 °C and they were approximately monthly re-innoculated.

Agar MPA (pH 7.1) [g/l] : (*Yanasa et al. 1983*) Beef extract 3, pepton 10, NaCl 5 and agar (AGAR No. 1) 15 were dissolved in distilled water and pH was adjusted to pH 5.5 with KOH (40% w/v). After sterilisation slants were stored at 5 °C.

4. 2. 3 Preparation of Crude Chitin Hydrolysate – Controlled Acid-catalysed Chitin Hydrolysis

Finely ground chitin (50 g) was dissolved in HCl (37% w/v, 200 ml) and reacted in the ultrasound bath (10 min/30 °C, 110 min/40 °C). Brown mixture was then cooled in ice bath and slowly neutralised with cold NaOH (50 % w/v, 126 ml). Undissolved part was removed by centrifugation (15000 rpm, 15min). GlcNAc (**4**) and oligosaccharides $(GlcNAc)_{n=2-6}$ were soluble and remained in liquid phase, which was lyophilised.

Lyophilisates were incinerated in order to determine amount of active components in the mixture with NaCl.

Lyophilisates were analysed by HPLC to determine percentage of each active component [$(GlcNAc)_{n=1}$- $(GlcNAc)_{n=6}$].

N-Acetylhexosamine-containing oligosaccharides were separated either on: a) a column (250 x 4 mm) with a guard column (10 x 4 mm) – both packed Lichrospher 100 NH_2 (5 μm; Watrex, Czech Rep.) using a modular system (Spectra Physics, CA, USA) consisting of SP 8800 ternary gradient pump, SP 8880 autosampler and Spectra Focus scanning UV/VIS detector operated at 210 nm. Samples (2 mg/ml, inj. vol. 20 μl) were eluted with acetonitrile– water (70 : 30 v/v) at a flow rate of 1 ml/min at 40 °C

or b) a Polymer IEX H^+-form column (8 μm, 250 × 8 mm, Watrex) using a system consisting of a solvent-delivery system 600 (Waters), a photo-diode array detector 996 (Waters) operated at 210 nm. Samples (2 mg/ml, inj. vol 20 μl) were eluted with 9mM H_2SO_4 at a flow rate of 0.5 mg/ml at 35 °C.

4. 2. 4 Epimerisation of 2-Acetamido-2-deoxy-D-glucopyranoside (GlcNAc, 4)

GlcNAc (**4**, 100 g, 452 mmol) was dissolved in water (1000 ml), then $Ca(OH)_2$ (1 g) was added under stirring to afford pH 11.0. Mixture was incubated at 24 °C for 24 hours and filtered. The filtrate was neutralised by Dowex 50WX2-200 (H^+), which was removed by filtration. Neutral filtrate (sample No. 1 - mixture of ManNAc (25 %) and GlcNAc (75 %) – *after epimerisation*) was lyophilised. The lyophilisate was extracted with MeOH [2 x 500 ml, 2 hours, filtration (sample No. 2 and No. 3), lyophilisation]. Latter lyophilisate (*after enrichment by extraction*) was final product – mixture of ManNAc (80 %) and GlcNAc (20 %).

Each sample and final product were analysed by HPLC to determine the percentage of ManNAc.

HPLC analyses (Polymer IEX H^+-form column (8 μm, 250 × 8 mm, Watrex) using a system consisting of a solvent-delivery system 600 (Waters), a photo-diode array detector 996 (Waters) operated at 210 nm. Samples (2 mg/ml, inj. vol 20 μl) were eluted with 9mM H_2SO_4 at a flow rate of 0.5 ml/min at 35 °C.)

4. 2. 5 Enzyme Preparation

Filamentous fungi were grown in respective liquid media designed for the enzyme required. Almost all enzymes prepared by us were inducible, therefore, media contained specific inducer for the respective type of enzyme. Among one type of enzyme respective source was often determinant for the inducer used. Only few strains were able to produce required enzyme in sufficient amount constitutively.

Media for the production of β-*N*-acetylhexosaminidases: (*Huňková et al*., *1999*)

Medium 1 (pH 6.0) [g/l]: KH_2PO_4 3.0, $NH_4H_2PO_4$ 5.0, pepton 5.0, yeast extract 0.5 and inducer - crude chitin hydrolysates 2.0 - were dissolved in distilled water and pH was adjusted to pH 6.0 with KOH (40 % w/v). After sterilisation $MgSO_4 \cdot 7\ H_2O$ sterile solution (10 % w/v, 5 ml/l) was added.

Medium 2 (pH 6.0) [g/l]: KH_2PO_4 3.0, $NH_4H_2PO_4$ 5.0, $(NH_4)_2SO_4$ 2.0, yeast extract 0.5, GlcNAc 5.0 and NaCl 15.0 were dissolved in distilled water and pH was adjusted to pH 6.0 with KOH (40% w/v). After sterilisation $MgSO_4 \cdot 7\ H_2O$ sterile solution (10 % w/v, 5 ml/l) was added.

Medium for constitutive production of β-*N*-acetylhexosaminidase: The same as the medium 1, the inducer was replaced by casein hydrolysate (7.5 g).

Media for the production of α-D-galactosidases: (*Huňková et al*., *1999*)

Medium for α-D-galactosidase from *Talaromyces flavus* CCF 2686 (pH 6.0) [g/l]: KH_2PO_4 3.0, $NH_4H_2PO_4$ 5.0, pepton 5.0, yeast extract 0.5 and inducer 6-deoxyglucose (**9**) 1.0 were dissolved

in distilled water and pH was adjusted to pH 6.0 with KOH (40% w/v). After sterilisation $MgSO_4 \cdot 7\ H_2O$ sterile solution (10 % w/v, 5 ml/l) was added.

Medium for the α-D-galactosidase production by the other fungi (pH 6.0) [g/l]: KH_2PO_4 3.0, $NH_4H_2PO_4$ 5.0, pepton 5.0, yeast extract 0.5 and inducer raffinose 2.0 were dissolved in distilled water and was adjusted to pH 6.0 with KOH (40% w/v). After sterilisation $MgSO_4 \cdot 7\ H_2O$ sterile solution (10 % w/v, 5 ml/l) was added.

Medium for constitutive production of α-D-galactosidase: The same as the latter medium, the inducer was replaced by casein hydrolysate (7.5 g).

Medium for the production of α-L-rhamnosidases (pH 6.0) [g/l]: (*Manzanares et al*., *2001*)

KH_2PO_4 15.0, NH_4Cl 4.0, KCl 0.5, yeast extract 5.0, casein hydrolysate 1.0, trace metal solution (*Vishniac and Santer*, *1957*) (1 ml/l) and inducer (*see later*) 5.0 were dissolved in distilled water and pH was adjusted to pH 6.0 with KOH (40% w/v). After sterilisation $MgSO_4 \cdot 7\ H_2O$ sterile solution (10 % w/v, 5 ml/l) was added.

Trace metal solution (*Vishniac and Santer*, *1957*) [g/l]: EDTA 50.00, $ZnSO_4 \cdot 7\ H_2O$ 22.00, $CaCl_2$ 5.54, $MnCl_2 \cdot 4\ H_2O$ 5.06, $FeSO_4 \cdot 7\ H_2O$ 4.99, $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ 1.10, $CuSO_4 \cdot 5\ H_2O$ 1.57, $CoCl_2 \cdot 6\ H_2O$ 1.61 – were dissolved in distilled water and pH was adjusted to 6.0 with KOH (40% w/v).

Inducers used: L-rhamnose (**2**), naringin (**6**), hesperidin (**7**) or rutin (**10**)

Medium for constitutive production of α-L-rhamnosidases: the inducer was replaced by casein hydrolysate (5 g).

The grown slants were washed with sterile Tween 80 solution (0.1% v/v, 5 ml/slant) and shaken. Conical flasks (500 ml) with 100 ml of medium were inoculated with 0.5 ml of the spore suspension. Fungi were cultivated on a rotary shaker at 200 rpm and 28 ºC. The samples from respective cultures were collected by filtration and respective enzyme activity was measured. When the maximum activity of the respective enzyme was reached,

culture was filtered through the filter paper. Crude enzyme preparations were obtained as ammonium sulfate precipitates (80 % saturation). These precipitates were overlaid with saturated ammonium sulfate solution and stored at 4 °C. Under these conditions the enzyme preparations are sufficiently stable and suitable for preparative and screening purposes.

4. 2. 6 Enzyme Activity Determination

Activities of various glycosidases were detected and quantified using corresponding *p*-nitrophenyl glycosides as substrates. Typical reaction mixture consisted of enzyme solution (30 μl), citrate-phosphate buffer (10 μl, 0.05 M, pH 5.0) and respective *p*-nitrophenyl glycoside (10 μl, 10 mM in distilled water). The mixture was incubated on a shaker at 900 rpm and 35 °C for 10 minutes. The reaction was stopped by the addition of Na_2CO_3 (1 ml, 0.1 M). The absorbance was read at 420 nm against the blank in order to exclude spontaneous non-enzymatic hydrolysis of substrate. The blank consisted of the same components as reaction mixture, but respective *p*-nitrophenyl glycoside was added at the latest (after addition of Na_2CO_3, in order to avoid enzymatic reaction). The blank was incubated under the same conditions as reaction mixture. The enzyme activity was calculated from a calibration curve based on standard solution of *p*-nitrophenol.

One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the conditions stated above.

Specific activity was calculated as the enzyme activity related the amount of proteins.

Proteins determination was performed using Bradford reagent (*Bradford, 1976*). To 1 ml of the commercial reagent 100 μl of enzyme solution was added, shaken properly and after 5 minutes the absorbance was read at 595 nm against the blank. In the blank the enzyme solution was replaced by the same amount of distilled water. The concentration of proteins was calculated from a calibration curve based on standard solution of albumin.

4. 2. 7 Enzymatic Acylation of *p*-Nitrophenyl α-D-galactopyranoside and *p*-Nitrophenyl β-D-galactopyranoside

4. 2. 7. 1 Acetylation of *p*-Nitrophenyl α-D-galactopyranoside (*p*NP α-Gal, 13)

p-Nitrophenyl α-D-galactopyranoside (**13**) (1.42 g, 4.7 mmol) was dissolved in pyridine (37.5 ml), acetone (97.5 ml) and the acyl donor (vinylacetate; 15 ml) were added followed by supplementation of lipase PS from *Burkholderia cepacia* (150 mg) adsorbed on celite (600 mg) and the mixture was shaken at 24 °C for 9 h. The reaction was monitored by TLC (AcOEt : MeOH : H_2O = 10 : 1 : 0.3). The enzyme was filtered off through filter paper and the subsequent evaporation of the solution and flash column chromatography (eluent: AcOEt : MeOH = 20 : 1) gave the pure product *p*-nitrophenyl 6-*O*-acetyl-α-D-galactopyranoside (**14**) (1.54 g, 4.49 mmol, 95.5 % yield).
Spectral data – *see Appendix*

4. 2 .7. 2 Acetylation of *p*-Nitrophenyl β-D-galactopyranoside (*p*NP β-Gal, 15)

p-Nitrophenyl β-D-galactopyranoside (**15**) (1 g, 3.3 mmol) was dissolved in pyridine (30 ml), acetone (60 ml) and the acyl donor (vinylacetate; 10 ml) were added followed by supplementation of lipase PS from *B. cepacia* (115 mg) adsorbed on celite (385 mg) and the mixture was shaken at 45 °C for 3 d. The reaction was monitored by TLC (AcOEt : MeOH : H_2O = 10 : 1 : 0.3). The removal of the enzyme and the following crystallisation from pyridine gave the pure product *p*-nitrophenyl 6-*O*-acetyl-β-D-galactopyranoside (**16**) (838 mg, 2.44 mmol, 73.75 % yield).
Spectral data – *see Appendix*

4. 2. 8 Enzymatic Acylation of *N*-Acetylhexosamines and their Derivatives

4. 2. 8. 1 Acetylation of 2-Acetamido-2-deoxy-D-glucopyranose (GlcNAc, 4)

4. 2. 8. 1. 1 Optimisation of the Reaction Conditions

A series of small-scale reactions were performed to optimise reaction conditions. Various organic cosolvents and a set of enzymes were tested.

GlcNAc (**4**) (20 mg, 0.09 mmol) was dissolved in 200 μl DMSO. Respective organic cosolvent: *tert*-amyl alcohol, dioxane, acetonitrile, acetone or DMF (700 μl) as well as trifluoroethyl acetate (100 μl) were added, followed by the addition of the respective enzyme (mg): protease N 30; subtilisin 5; alcalase 20; proleather 30; Novozym 435 10; porcine pancreatic lipase 50; lipase PS on celite 50; lipase CE-5 30; *Chromobacterium viscosum* lipase 50. The suspensions were shaken at 45 °C and samples were analysed by GC (**4. 2. 1. 2**). Data reported in **Table 6**.

4. 2. 8. 1. 2 Acetylation of 2-Acetamido-2-deoxy-D-glucopyranose (4) in DMF

GlcNAc (**4**) (500 mg, 2.3 mmol) was dissolved in DMF (22.5 ml) and trifluoroethyl acetate (2.5 ml) followed by supplementation of protease N (750 mg). The reaction was shaken at 40 °C (250 rpm) and monitored by TLC (AcOEt : MeOH : H_2O = 10 : 2 : 0.4). After 6 days the enzyme was filtered off through a filter paper. Flash chromatography (eluent: AcOEt : MeOH : H_2O = 10 : 1 : 0.3) gave 2-acetamido-6-*O*-acetyl-2-deoxy-D-glucopyranose (**17**) (370 mg, 1.41 mmol, 62 % yield).
Spectral data – *see Appendix*

4. 2. 8. 2 Acetylation of 2-Acetamido-2-deoxy-D-galactopyranose (GalNAc, 5) in Acetonitrile-DMSO

GalNAc (**5**) (500 mg, 2.3 mmol) was dissolved in DMSO (4 ml) followed by the addition of acetonitrile (13 ml) and trifluoroethyl acetate (2 ml) and supplementation of subtilisin lyophilised with K_2HPO_4 (200 mg). The mixture was shaken at 45 °C for three

days monitoring the reaction by TLC (AcOEt : MeOH : H_2O = 10 : 2 : 0.4). The enzyme was filtered off through a filter paper, the solvent evaporated and the residue purified by flash chromatography (eluent: first AcOEt to remove residual DMSO, then AcOEt : MeOH : H_2O = 10 : 2 : 0.4) to afford products 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose (**18**) (315 mg, 1.2 mmol, 51 %) and 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactofuranose (**19**) (31 mg, 0.1 mmol, 5 %).
Spectral data – *see Appendix*

4. 2. 8. 3 Acetylation of 2-Acetamido-2-deoxy-D-mannopyranose (ManNAc, 11) in Acetonitrile-DMSO

ManNAc (**11**) (500 mg, 2.3 mmol) was dissolved in DMSO (4 ml), acetonitrile (13 ml) and trifluoroethyl acetate (2 ml) were added followed by supplementation of subtilisin lyophilised with K_2HPO_4 (200 mg) and the mixture was shaken at 45 °C for two days monitoring the reaction by TLC (AcOEt : MeOH : H_2O = 10 : 2 : 0.4). The enzyme was filtered off through a filter paper, the solvent evaporated and the residue purified by flash chromatography (eluent: first AcOEt to remove residual DMSO, then AcOEt : MeOH : H_2O = 10 : 1 : 0.5) to give products 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose (**20**) (345 mg, 1.3 mmol, 59 %) and 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-D-mannopyranose (**21**) (95 mg, 0.3 mmol, 13 %).
Spectral data – *see Appendix*

4. 2. 8. 4 Butanoylation of 2-Acetamido-2-deoxy-D-mannopyranose (11) in Acetonitrile-DMSO

ManNAc (**11**) (250 mg, 1.1 mmol) was dissolved in DMSO (2 ml), acetonitrile (6.5 ml) and trichloroethyl butyrate (1 ml) were added followed by supplementation of subtilisin lyophilised with K_2HPO_4 (100 mg) and the mixture was shaken at 45 °C. At various intervals, the course of the reaction was monitored by TLC (AcOEt : MeOH : H_2O = 10 : 2 : 0.4). When the desired conversion rate was reached (after 1 day), the enzyme was filtered off. Evaporation of the solution and flash column chromatography (eluent: first AcOEt for removing DMSO, followed by AcOEt : MeOH : H_2O = 10 : 0.5 : 0.2) gave pure products 2-acetamido-6-*O*-butyryl-2-

deoxy-D-mannopyranose (**22**) (257 mg, 0.9 mmol, 77.9 %) and 2-acetamido-3,6-di-*O*-butyryl-2-deoxy-D-mannopyranose (**23**) (46 mg, 0.1 mmol, 11.5 %).
Spectral data – *see Appendix*

4. 2. 8. 5 Acetylation of *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (*p*NP β-D-GalNAC, 24)

*p*NP β-D-GalNAC (**24**) (280 mg, 0.8 mmol) was dissolved in pyridine (21 ml), acetone (29 ml) and vinyl acetate (10 ml) were added followed by supplementation of Novozym 435 (200 mg) and the reaction was shaken (200 rpm) at 45 °C and monitored by TLC (AcOEt : MeOH : H_2O = 10 : 1 : 0.3). Enzyme addition was repeated after 2 days (100 mg) and after 4 days (50 mg). After 7 days, TLC analysis showed almost 100 % conversion to a single product. The enzyme was filtered off through filter paper and the product *p*-nitrophenyl 2-acetamido-6-*O*-acetyl-2-deoxy-β-D-galactopyranoside (**25**) was crystallised from pyridine in 53.3 % yield (166 mg, 0.4 mmol).
Spectral data – *see Appendix*

4. 2. 8. 6 Acetylation of 2-Acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose (20)

2-Acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose (**20**) (200 mg, 0.8 mmol) was dissolved in pyridine (1 ml), acetone (8 ml), trifluoroethyl acetate (2 ml) and Novozym 435 (200 mg) were added, the reaction mixture was shaken at 45 °C and monitored by TLC (AcOEt : MeOH : H_2O = 10 : 2 : 0.4 and $CHCl_3$: MeOH : H_2O = 9 : 1.5 : 0.1). After 7 days the enzyme was filtered off through filter paper. Evaporation of the solvent and flash chromatography (eluent : $CHCl_3$: MeOH : H_2O = 9 : 1.5 : 0.1) gave product 2-acetamido-1,6-di-*O*-acetyl-2-deoxy-α-D-mannopyranose (**26**) in 18.4 % yield (42 mg, 0.1 mmol).
Spectral data – *see Appendix*

4. 2. 8. 7 Butanoylation of 2-Acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranose (22)

2-Acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranoside (**22**) (150 mg, 0.5 mmol) was dissolved in acetone (3 ml), trichloroethyl butyrate (1 ml) and Novozym 435 (100 mg) were added and the mixture was shaken at 45 °C. The course of the reaction was monitored by TLC (AcOEt : MeOH : H_2O = 10 : 1 : 0.3) and after 4 days the enzyme was filtered off through filter paper. Evaporation of the solution and flash chromatography (eluent : AcOEt : MeOH : H_2O = 10 : 0.5 : 0.2) gave 37.4 mg (0.1 mmol) of product 2-acetamido-1,6-di-*O*-butyryl-2-deoxy-D-mannopyranose (**27**) (19.4 %).
Spectral data – *see Appendix*

4. 2. 9 Library of Glycosidases

Requirements:
Well defined source – taxonomically well defined strains
Common availability – strains originate from public collections available to other scientists
Reproducible quality – defined culture media and cultivation conditions
Easy production method – large number (types) of the enzymes
Simple method of screening – *e.g.* colorimetric micro-well methods

Enzyme properties:
The enzymes were available in satisfactory and stable quality and quantity.
They were stable for a few years (4 ºC).
The enzymes were of satisfactory purity using simple methods, they could be directly used for synthetic reactions in preparative scale.

Such library enabled to test always a large panel of enzymes (typically over 20 types) for a particular synthetic problem.

4. 2. 9. 1 Synthesis of 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (28) and 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (29)

2-Acetamido-2-deoxy-D-glucopyranose (**4**) (97 mg, 0.438 mmol) and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**24**) (30 mg, 0.088 mmol) were dissolved in citrate-phosphate buffer (0.5 ml, 0.05M, pH 4.5) and 20% (w/v) of $MgSO_4$ was added. The reaction mixture was incubated with β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 (8.5 U) at 37 °C. After 3.5 h the reaction was stopped by heating at 100 °C for 10 min, extracted with ethyl ether (2 × 5 ml) and lyophilised. The residue was loaded on a Toyopearl HW-40F column (2.6 × 80 cm, flow rate 25 ml/h) and eluted with water to give **28** (9.9 mg, 0.023 mmol, 26.5%) and **29** (7.2 mg, 0.017 mmol, 19%). NMR and MS data were identical with data published previously (*Singh et al., 1995*).

4. 2. 9. 2 Synthesis of 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-galactopyranose (30)

2-Acetamido-2-deoxy-D-galactopyranose (**5**) (97 mg, 0.438 mmol) and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**24**) (50 mg, 0.146 mmol) were dissolved in citrate-phosphate buffer (0.5 ml, 0.05M, pH 4.5) and 20% (w/v) of $MgSO_4$ was added. The reaction mixture was incubated with β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 (5.6 U) at 37 °C. After 3.5 h the reaction was stopped by heating at 100 °C for 10 min, extracted with ethyl ether (2 × 5 ml) and lyophilised. The residue was loaded on a Toyopearl HW-40F column (2.6 × 80 cm, flow rate 25 ml/h) and eluted with water to give **30** (54 mg, 0.127 mmol, 87%). NMR and MS data were identical with data published previously (*Defaye et al., 1989*).

4. 2. 9. 3 Condensation Reactions of 2-Acetamido-2-deoxy-D-glucopyranose (3) at an Analytical Scale

To test the synthetic ability of β-*N*-acetylhexosaminidases for β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**31**) formation by reverse hydrolysis, condensation reactions with 2-acetamido-2-deoxy-D-glucopyranose were screened. Screening system composed of 2-acetamido-2-deoxy-D-glucopyranose (**4**, 2–16 mg, 0.2–1.6M) in McIlvaine buffer (45 µl, pH 5.0) using respective β-*N*-acetylhexosaminidase (2 U) incubated for 8 days at 37 °C. Reactions were monitored by TLC, the formation of **31** (depending on the enzyme source) was observed only at a substrate **4** concentration of 0.6 - 1.2M. Optimum GlcNAc concentration (1.0M) was employed for preparative procedures. Respective blank reactions void of enzyme were run to exclude the possibility of a non-enzymatic GlcNAc condensation - no spontaneous autocondensation was observed.

4. 2. 9. 4 Condensation Reactions of 2-Acetamido-2-deoxy-D-glucopyranose at a Preparative Scale

Analogously as in the **Section 4. 2. 10. 3**, 2-acetamido-2-deoxy-D-glucopyranose (**4**, 1.0 M) was treated with a series of 20 fungal β-*N*-acetylhexosaminidases (3 U) from the genera of *Acremonium*, *Aspergillus*, *Penicillium*, *Talaromyces* and *Trichoderma* (*see 5.* Table x). Substrate (**4**, 114 mg, 0.516 mmol) was dissolved in McIlvaine buffer (500 µl, pH 5.0) and the respective β-*N*-acetylhexosaminidase was added. After 2 days another 5U of the respective enzyme were added. The reaction mixture, monitoredby TLC, was incubated as a whole for 8 days at 37 °C. Then the enzyme was deactivated by heating (10 min, 100 °C). The mixture was centrifuged and the supernatant was loaded on a Toyopearl HW-40F column (900 mm x 26 mm, flow rate 17 ml/h) and eluted with water. Fractions (ca. 2.5 ml) containing disacharides were collected and lyophilised. Lyophilisates (15 – 100 mg) were peracetylated (Ac_2O : Py = 1 : 1, 3 – 5 ml, 30 h, 24 °C) and separated by silica gel flash chromatography ($CHCl_3$: MeOH = 97 : 3).

4. 2. 9. 5 Synthesis of:
2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucopyranose (β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc, 31),
2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc, 32) and
2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (β-D-Glc*p*NAc-(1→6)-D-Glc*p*NAc, 33)

Substrate (**4**, 114 mg, 0.516 mmol) was dissolved in McIlvaine buffer (500 μl, pH 5.0) and β-*N*-acetylhexosaminidase from *Penicillium funiculosum* CCF 1994 (3 U) was added. After 2 days another 5U of the enzyme were added. The reaction mixture, monitoredby TLC, was incubated as a whole for 8 days at 37 °C. Then the enzyme was deactivated by heating (10 min, 100 °C). The mixture was centrifuged and the supernatant was loaded on a Toyopearl HW-40F column (900 mm x 26 mm, flow rate 17 ml/h) and eluted with water. Fractions (ca. 2.5 ml) containing disacharides were collected and lyophilised. Lyophilisates were peracetylated (Ac_2O/Py, 30 h, 24 °C) and separated by silica gel flash chromatography ($CHCl_3$: MeOH = 97 : 3) yielding **31a** (6.6 mg, 3.8 %), **32a** (3.0 mg, 1.7 %) and **33a** (17.5 mg, 10.0 %)**.** Spectral data of **29a** – *see Appendix*. NMR and MS data of **32a** (*Singh et al., 1995*) and **33a** (*Rajnochová et al., 1997*) were identical with data published previously.

4. 2. 9. 6 Synthesis of:
2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→1)-β-D-galactopyranoside (β-D-Glc*p*NAc-(1→1)-β-D-Gal*p*, 34) and
2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→1)-β-D-galactopyranoside (β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→1)-β-D-Gal*p*, 35)

D-Galactose (**1**, 201 mg, 1.120 mmol) as an acceptor and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**36**, 48 mg, 0.140 mmol) as a donor were dissolved in McIlvaine buffer (5.0 ml, pH 5.0). The reaction mixture was incubated with the β-*N*-acetylhexosaminidase from *A. flavofurcatis* CCF 3061 (6 U) at 37 °C.

After 4.5 h the reaction was stopped by heating (100 °C, 10 min). The liberated *p*-nitrophenol was extracted with diethyl ether (2 × 10 ml). The reaction mixture was lyophilised and separated on a Biogel P-2 column (120 cm × 2.5 cm, flow rate 20.5 ml/h, water). The fraction containing disaccharides (58 mg) was further separated by semi-preparative HPLC (Lichrospher® 100-5 NH_2 column, 5 μm, 250 x 8 mm, Watrex; elution system: CH_3CN-H_2O = 79 : 21 v/v for **34** and **32**; flow rate: 3 ml/min, UV detector: 200 nm; at 24 °C) yielding **34** (12 mg, 0.031 mmol, 22%) and β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc (**32**) (14 mg, 0.033 mmol, 23.5%) with retention times 32.63 and 21.72 min, respectively. The fraction containing trisaccharides **35** and β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc -(1→4)-D-Glc*p*NAc (**37**) (20 mg) was also separated by semi-preparative HPLC (*see above*, elution system: CH_3CN-H_2O = 75 : 25 v/v for **35** and **37**) affording compounds **35** (7.5 mg, 0.012 mmol, 9%) and **37** (10.6 mg, 0.018 mmol, 13%) with retention times 31.72 and 22.44 min, respectively.

4. 2. 9. 7 Synthesis of:
β-D-Galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-2-acetamido-2-deoxy-β-D-glucopyranoside (β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc, 38) and
β-D-Galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→1)-2-acetamido-2-deoxy-β-D-glucopyranoside (β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→1)-β-D-Glc*p*NAc, 39)

β-*N*-Acetylhexosaminidase from *A. flavofurcatis* CCF 3061 (11 U) was added to the solution of lactose **40** (194 mg, 0.567 mmol) and glycosyl donor **36** (24.3 mg, 0.071 mmol) in McIlvaine buffer (3.0 ml, pH 5.0) and the mixture was incubated at 37 °C. After 7 h the reaction was stopped by heating (100 °C, 10 min), *p*-nitrophenol was extracted with diethyl ether, the mixture was lyophilised and separated on a Biogel P-2 column (120 cm × 2.5 cm, flow rate 15.0 ml/h, water). The trisaccharide fraction (15.5 mg) was acetylated (Ac_2O/Py, 2 h, 60 °C). The anomeric hydroxyls of reducing sugars (other unidentified regioisomers) were selectively 1-deacetylated with piperidine in THF (5%, 10 ml) at 0 °C for 16 h. This selective deprotection of reducing sugars lowered their R_f on the silica gel chromatography ($CHCl_3$/MeOH = 10/0.6), which enabled to separate them from the non-reducing ones. Compound **38** was characterised as peracetate **38a** (7.0 mg, 7.2 μmol, 10 %). Compound **39a** behaved during C-1 deprotection with piperidine/THF

in a rather surprising way as the C-2 acetate on the α-glucopyranosyl moiety was selectively removed to afford decaacetate **39b** (as determined later with MS and NMR). However, β-isomer **38a** remained stable during this treatment. Attempts to peracetylate (Ac_2O/Py) **39b** furnished only mixtures of acetates. Acetylation of **39b** (or peracetylation of **39**) was not feasible probably due to steric hindrances of this position (steric conflict of two axial bonds at αC-1 and C-2). Peracetylation of β-isomer **38** proceeded smoothly and the compound was stable. Therefore, the compound **39** was characterised as its decaacetate **39b** (6.5 mg, 6.7 μmol, 9%)

4. 2. 9. 8 Activity of α-D-Galactosidase for *p*-Nitrophenyl 6-*O*-acetyl-α-D-galactopyranoside (14)

Each reaction mixture contained compound **14** (60 μl, 10 mM), acetonitrile [40 μl, 8% (v/v)], McIlvaine buffer (390 μl, pH 5.0) and the tested α-D-galactosidase (0.2 U). The mixtures were incubated for 6 hours at 37 °C. A sample (50 μl) was added to 1 ml of 0.1M Na_2CO_3 and the absorbance was measured at 420 nm to determine the amount of the released *p*-nitrophenol. Compound **14** was hydrolysed by none of the following α-galactosidases (from *Aspergillus flavipes* CCF 2026, *A. niger* (Sigma), *A. parasiticus* CCF 3058, *A. phoenicis* CCF 61, *A. sojae* CCF 3060, *A. tamarii* CCF 3085, *Circinella muscae* CCF 1568, *Coffea arabica* (Sigma), *Penicillium daleae* CCF 2365, *P. chrysogenum* CCF 1269, *P. melinii* CCF 2440, *P. multicolor* CCF 2244, *Talaromyces flavus* CCF 2324, *T. flavus* CCF 2686), which was also confirmed by TLC (propan-2-ol : H_2O : aqueous ammonia = 7 : 2 : 1).

4. 2. 9. 9 Influence of Organic Co-solvents on the Activity and Stability of the α-D-Galactosidase from *Talaromyces flavus*

The influence of different water-miscible co-solvents (acetone, acetonitrile, dimethylformamide (DMF), dimethylsulfoxide (DMSO), 1,4-dioxane, 2-methoxyethanol, tetrahydrofuran (THF), 2-methylpropan-2-ol and pyridine) on the activity of the α-D-galactosidase from *T. flavus* CCF 2686 was tested. The reaction mixture contained substrate solution (**13**, 100 μl, 10 mM), α-D-galactosidase from *T. flavus* CCF 2686 (0.03 U),

0 - 60 % (v/v) of the respective co-solvent. The solution was made up to the total volume of 500 µl with citrate-phosphate buffer (50 mM, pH 3.5). Blank reaction was performed in the absence of enzyme. The mixtures were incubated for 10 minutes at 37 °C and then 50 µl of sample was added to 1 ml of 0.1 M Na_2CO_3. The absorbance was measured at 420 nm against the corresponding blank. Relative activity in the presence of a co-solvent was compared with the values obtained in the respective reaction without co-solvent.

In order to determine the long-term stability of the enzyme in the presence of co-solvents, the α-D-galactosidase (0.03 U, 25 µl) was incubated at 37 °C in a mixture of citrate-phosphate buffer (111 µl, pH 3.5) and 2-methylpropan-2-ol [63.8 µl, 25.5 % (v/v)] or McIlvain buffer (94 µl, pH 3.5) and acetone [81 µl, 32.5 % (v/v)]. After 1 - 27 h, substrate solution (**13**, 10 mM, 50 µl**)** was added and the reaction was stopped within 10 minutes by transferring the sample (50 µl) to 1 ml of 0.1 M Na_2CO_3. The absorbance was measured as described previously.

4. 2. 9. 10 Synthesis of *p*-Nitrophenyl α-D-galactopyranosyl-(1→3)-6-O-acetyl-α-D-galactopyranoside (41) with 2-Methylpropan-2-ol as a Cosolvent

4. 2. 9. 10. 1 Analytical Procedure

Glycosyl donor **(*p*NP α-Gal, 13)** (5 mg, 0.02 mmol) and *p*-nitrophenyl 6-*O*-acetyl-α-D-galactopyranoside (**14**, 20 mg, 0.06 mmol) as an acceptor were suspended in citrate-phosphate buffer (1 ml, pH 3.5) and completely dissolved by the addition of 2-methylpropan-2-ol (350 µl, 3.6 mmol, final conc. 25.5 % v/v). After supplementing the α-galactosidase from *T. flavus* (2 U), the reaction mixture was shaken at 37 °C. The course of the reaction was monitored by TLC (propan-2-ol : H_2O : aqueous ammonia = 7 : 2 : 1, AcOEt : MeOH : H_2O = 8 : 1.5 : 0.3) and after 5 days the reaction was stopped by boiling for 10 minutes.

4. 2. 9. 10. 2 Semipreparative Procedure

Compound **13** (100 mg, 0.3 mmol) and acceptor **14** (400 mg, 1.2 mmol) were dissolved in citrate-phosphate buffer (8 ml, pH 3.5) and 2-methylpropan-2-ol (2.8 ml, 0.03 mol, 25.5 % v/v). The reaction was started by the addition of the α-galactosidase from *T. flavus* (8.7 U), after 4 and 8 hours each 3.3 U of the enzyme were added (total amount 15.3 U). The mixture was shaken at 37 °C for 27 hours and stopped by boiling. After the extraction with diethyl ether to remove *p*-nitrophenol released and after reducing volume *in vacuo*, the solution was loaded on a column with Amberlite XAD-4, which adsorbed all compounds carrying aromatic (lipophylic) moieties. After extensive washing with water to remove salts, proteins and unsubstituted carbohydrates (*e.g.* galactose), the column was eluted with MeOH. The eluate was concentrated to a small volume and loaded on a Sephadex LH-20 column (25 x 1950 mm). The column was eluted with 80% v/v MeOH (flow rate 13 ml/h) to give 5.7 mg of the unexpected product 2-methylpropyl α-D-galactopyranoside (**42**), *p*-nitrophenyl α-D-galactopyranosyl-(1→6)-α-D-galactopyranoside (**43**) as a by-product and a mixture of the disaccharides *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside (**41**) and *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-α-D-galactopyranoside (**44**). These products were further separated by HPLC (modular system Spectra Physics, San Jose, US, 250 × 4 mm and 250 × 8 mm for analytical and preparative HPLC respectively, stationary phase Lichrospher 100 NH_2, 5 μm, mobile phase acetonitrile : water = 79 : 21 for analytical and 80 : 20 for preparative HPLC, flow rate 0.6 ml/min for analytical and 2.0 ml/min for preparative HPLC, detection at 200 nm (analytical) and 209 nm (preparative)) to give 2.3 mg of **41** (1.4 %) and 5.2 mg of **44** (3.4 %).

4. 2. 9. 11 Synthesis of *p*-Nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside (41) with Acetone as Cosolvent

An analogous reaction was performed using acetone as a cosolvent to eliminate the unexpected by-product 2-methylpropyl α-D-galactopyranoside **42**. The reaction mixture was composed of glycosyl donor **13** (100 mg, 0.3 mmol), 6-*O*-acylated acceptor **14** (400 mg, 1.2 mmol), citrate-phosphate buffer (7.24 ml, pH 3.5) and acetone (3.56 ml, 32.5 % v/v). α-Galactosidase from *T. flavus* CCF 2686 (20 U) was gradually (5 U every 6 h) added

to the mixture shaken at 37 °C. The reaction was monitored by TLC and stopped after 24 hours. The mixture was filtered, evaporated, treated with Amberlite XAD-4 and the fraction containing **41** and **44** was isolated by gel chromatography on Sephadex LH-20 as mentioned above. The compound **41** was easily purified by flash chromatography (eluent: AcOEt : MeOH : H_2O = 8 : 1.5 : 0.3) to give 8.3 mg (4.9 %).

4. 2. 9. 12 Screening of α-L-Rhamnosidases Substrate Specificity

Substrate (*see bellow*) (2 mg) was dissolved in potassium phosphate buffer (1 ml, 50 mM, pH 6.0) or in the same buffer containing 20 % (v/v) tetrahydrofuran (THF). Respective α-L-rhamnosidase (about 3 U) (differ from the source and inducer) was added and the mixture was incubated on a rotary shaker at 250 rpm and at 35 °C for 48 hours. Derhamnosylation of the substrates was monitored by TLC (AcOEt : MeOH : H_2O = 8 : 3 : 0.5) by detecting the L-rhamnose (**2**) (R_f = 0.21) released from the substrates ((R_f substrates: rutin (**7**), 0.10; hesperidin (**10**), 0.29; naringin (**9**), 0.16; quercitrin (**8**), 0.29; ginsenoside Re (**45**), 0.12; asiaticoside (**6**), 0.10) with the molybdate reagent (*see* **4. 2. 1. 3**).

4. 2. 9. 13 Screening of α-L-Rhamnosidase Library for the Selective Derhamnosylation of Desglucoruscin (3)

The screening of the α-L-rhamnosidase library for the selective derhamnosylation of desglucoruscin was performed either with a 1 mg/ml desglucoruscin solution in 50 mM potassium phosphate buffer, pH 6.0, containing 20 % v/v organic cosolvent (THF, CH_3CN, DMF, MeOH and DMSO), or using a biphasic system made of a 1 mg desglucoruscin solution in 0.5 ml AcOEt (organic phase) and 0.5 ml of 50 mM potassium phosphate buffer, pH 6.0 (water phase). In both cases, a sample of each preparation of the enzyme library (30 µl, minimum 18 U) was added, and the mixtures were incubated at 30 °C and 250 rpm for 48 h. The reactions were monitored by TLC (AcOEt : MeOH : H_2O = 8: 2: 0.3). R_f of respective compounds: L-rhamnose (**2**), 0.12; desglucoruscin (**3**), 0.31; desrhamnodesglucoruscin (**46**), 0.47.

Cosolvents used were purified prior to the use; namely THF must have been freshly distilled from CaH_2 to remove all traces of peroxides that strongly deactivate the enzymes.

4. 2. 9. 14 Preparative Derhamnosylation of Desglucoruscin (3)

Desglucoruscin (**5**, 100 mg, 0.14 mmol) was dissolved in 60 ml of AcOEt and 30 U of α-L-rhamnosidase from *A. niger* CCIM K2 (inductor: L-rhamnose) dissolved in 30 ml of 50 mM potassium phosphate buffer, pH 6.0, were added. The reaction was shaken at 37 °C and 250 rpm and monitored daily by TLC (AcOEt : MeOH : H_2O = 8: 2: 0.3) and HPLC analyses (analytical column Lichrospher® RP-18, 5 μm, 125 x 4 mm, Merck; elution system: CH_3CN- H_2O = 35: 65 v/v; flow rate: 1 ml/min, UV detector: 205 nm; at 24 °C, retention times: desglucoruscin (**5**), 22 min; desrhamnodesglucoruscin (**46**), 27 min). After 4 days, the organic phase was recovered and the solvent was evaporated. The crude residue was purified by flash chromatography (eluent: AcOEt : MeOH : H_2O = 10 : 0.5 : 0.1) to give 29 mg (0.05 mmol) of desrhamnodesglucoruscin (**46**) (38 % yield).

4. 2. 9. 15 Determination of the Mechanism of Fungal α-L-Rhamnosidase (Inverting/Retaining Type)

By continuous NMR-^{1}H scanning of the α-L-rhamnosidase cleavage of *p*-nitrophenyl α-L-rhamnopyranoside first appearance of β-L-rhamnose was detected. Reactions were performed at 30 °C. Crude enzymes after precipitation at 80% saturation of ammonium sulfate were stored under the saturated ammonium sulfate solution. Before assays samples were centrifugated and saturated ammonium sulfate was removed. Enzymes were resuspended in D_2O.

4. 2. 10 Screening for α/β-*N*-Acetylmannosaminidase Activity

4. 2. 10. 1 Screening of Bacteria for α/β-*N*-Acetylmannosaminidase Production

4. 2. 10. 1. 1 Cultivation of Bacteria

Bacteria were grown in liquid medium (*Kuboki et al*., *1997*).

Medium (pH 7.2):

$MgSO_4 \cdot 7\ H_2O$ (0.5 g), yeast extract (1 g), KH_2PO_4 (4.1 g), K_2HPO_4 (1.22 g) - were dissolved in distilled water (900 ml) and was adjusted to pH 7.2 with KOH (40%). The solution was dispensed into Erlenmeyer flasks of 250 ml (45 ml/flask). After sterilisation a sterile solution of GlcNAc (15% w/v, 5 ml) was added. GlcNAc solution was sterilised by filtration through Seitz – filter No. 5. Final volume of cultivation medium was 50 ml in flask (250 ml). Final rate of GlcNAc was 0.75 %. A part of GlcNAc (0.25 %) was replaced by ManNAc when induction by ManNAC was tested.

.

The slant of respective bacterium was covered with sterile physiological solution (NaCl, 0.9% w/v, 2 ml/slant) and shaken properly. Erlenmeyer flasks (250 ml) with 50 ml of medium were inoculated with 1 ml of such prepared cell suspension. Final $OD_{610\ nm}$ in the flasks should have been minimally 0.05, what was fulfilled using this procedure. Bacteria were cultivated on a rotary shaker at 200 rpm and 28 ºC. For the following work with bacteria 24, 48, 72 and 96 hours old cultures were used.

4. 2. 10. 1. 2 Preparation of Samples for Screening α/β-*N*-Acetylmannosaminidase Activity

Activities of both α-*N*-acetylmannosaminidase and β-*N*-acetylmannosaminidase were measured in medium (extracellular activity) and in disintegrated cells (intracellular activity).

Content of each flask was centrifugated (20 min, 4 ºC, RPM 15 000). Supernatant was used for extracellular activity. Pellet was divided into eppendorf tubes (2 g/tube). Several buffers [Tris (50mM), Tris (50mM) + EDTA (1mM), Tris (50mM) + DTT (1mM), Tris (50mM) + EDTA (1mM) + DTT (1mM), buffer with protease inhibitors)] were used.

Tris (50mM)

0,6055 g of Tris/100 ml distilled water, adjusted to pH 7,5 with 0.1M HCl

Tris (50mM) + EDTA (1mM)

to 20 ml of Tris (50mM) prepared as described above 7.5 mg of EDTA were added

Tris (50mM) + DTT (1mM)

to 20 ml of Tris (50mM) prepared as described above 3.1 mg of DTT were added

Tris (50mM) + EDTA (1mM) + DTT (1mM)

to 20 ml of Tris (50mM) prepared as described above 7.5 mg of EDTA and 3.1 mg of DTT were added

Buffer with protease inhibitors

20 ml (protease inhibitor cocktail (Sigma) : buffer = 1 : 100):

NP 40	2 ml
glycerol	1 ml
cystamine	200 µl
cysteamine	200 µl
leupeptin	2 ml
pepstatin	2 ml
cocktail (Sigma)	200 µl
Tris (50 mM)	12,4 ml

Preparation of each components of buffer:

NP 40 (1 % in Tris): 10 ml of NP 40 (10%) + 90 ml of Tris (50mM) (stored under argon and in the dark)

cystamine: 0,2252 g/10 ml of distilled water H_2O

cysteamine: 0,5680 g/10 ml of distilled water H_2O

leupeptin: 10 µl (10mM)/10 ml of distilled water H_2O

pepstatin: 10 µl (10mM)/10 ml of distilled water H_2O

solution of protease inhibitor cocktail: inhibitors ordered in lyophilised stage, DMSO included apart
Lyophilisate (43 mg) was added to DMSO (200 µl) and shook (1 min). Then distilled water (800 µl) was added.

To microtube with pellet (2 g) respective buffer (1 ml) was added. Microtubes were stored on the ice. Content of each microtube was disintegrated using ultrasonic homogenizer with titanium probe (20 x 20 sec, cooling in ice ca 20 sec between each processing). Disrupted cells were centrifugated (10 min, 4 ºC, rpm 15 000). Supernatant was used for screening for intracellular activity of α-*N*-acetylmannosaminidase and β-*N*-acetylmannosaminidase.

4. 2. 10. 1. 3 Screening for α/β-*N*-Acetylmannosaminidase Activity

Multiwell plates were used for screening for desired activity. Respective reactants were added using multi-channel pipettes (Transferpette, Brand).

Activities of respective enzyme were detected and quantified using corresponding *p*-nitrophenyl glycosides as substrates. Typical reaction mixture consisted of enzyme solution (30 μl), McIlvain buffer (10 μl, 0.05 M, pH 5.0) and respective *p*-nitrophenyl glycoside (10 μl, 5 mM in distilled water). Plates were incubated in a thermostate at 35 °C for 60 minutes. The reaction was stopped by addition of Na_2CO_3 (150 μl, 0.1 M). The absorbance was read at 414 nm (Labsystems Twinreader® PLUS) against the blank in order to exclude spontaneous non-enzymatic hydrolysis of substrate. The blank consisted of the same components as reaction mixture, but respective *p*-nitrophenyl glycoside was added at the latest (after addition of Na_2CO_3, in order to avoid enzymatic reaction). The blank was incubated under the same conditions as reaction mixture. The enzyme activity was calculated from a calibration curve based on standard solution of *p*-nitrophenol.

One unit of the enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the conditions stated above.

4. 2. 10. 2 Screening for α/β-*N*-Acetylmannosaminidase Activity in a Snail Gut Juice

Activities of respective enzyme were detected and quantified using corresponding *p*-nitrophenyl glycosides as substrates. Snail gut juice was dissolved in distilled water (5 mg/100 μl). Typical reaction mixture consisted of enzyme solution (30 μl), citrate-phosphate buffer (10 μl, 0.05 M, pH 5.0) and respective *p*-nitrophenyl glycoside

(10 µl, 5 mM in distilled water). The mixture was incubated on a shaker at 900 rpm and 35 °C for 10 minutes. The reaction was stopped by addition of Na_2CO_3 (1 ml, 0.1 M). The absorbance was read at 420 nm against the blank in order to exclude spontaneous non-enzymatic hydrolysis of substrate. The blank consisted of the same components as reaction mixture, but respective *p*-nitrophenyl glycoside was added at the latest (after addition of Na_2CO_3, in order to avoid enzymatic reaction). This blank was incubated under the same conditions as reaction mixture.

5. RESULTS AND DISCUSSION

5. 1. Library of Glycosidases – General Strategy

For the screening for a particular biotransformation or a synthetic problem a library of enzymes is usually required. The properties of the library should remain unchanged so that the enzymes prepared repeatedly must be of the constant quality. This can be achieved under certain requirements. Firstly, taxonomically well defined strains originated from public collections available to other scientists must be used for production of the enzymes. Then defined culture media and cultivation conditions must be used. Using easy production method a large number of the enzymes can be produced and screened by the simple method, *e.g.* colorimetric micro-well procedure. We developed cheap screening method using micro-well methodology able to test a large panel of the enzymes in the short time.

Requirements:

Well defined source – taxonomically well defined strains

Common availability – strains originate from public collections available to public

Reproducible quality – defined culture media and cultivation conditions

Easy production method – large number (types) of the enzymes

Simple method of screening – *e.g.* colorimetric micro well-methods

Enzyme properties:

The enzymes are available in satisfactory and stable quality and quantity.

They are stable for a few years (4 ºC).

The enzymes are of satisfactory purity using simple methods, they can be directly used for synthetic reactions in a preparative scale.

5. 2. Fungal Glycosidases and their Induction

The production of glycosidases with suitable properties for specific applications can be achieved by exploring the natural diversity of fungal strains. When taxonomically defined microbial strains from public collections are used, grown in defined culture media and under

defined cultivation conditions, the enzymatic preparations produced are generally available. Moreover, the production of the desired glycosidase activity can be stimulated by respective cultivation conditions, as *e.g.* by various inducers. Screening protocols should be easy, fast, and reproducible, typically based on the hydrolysis of the corresponding *p*-nitrophenyl glycosides in colorimetric assays (*Huňková et al., 1999*).

Basal level of fungal extracellular glycosidases is often very low. However, some fungal glycosidases are inducible.

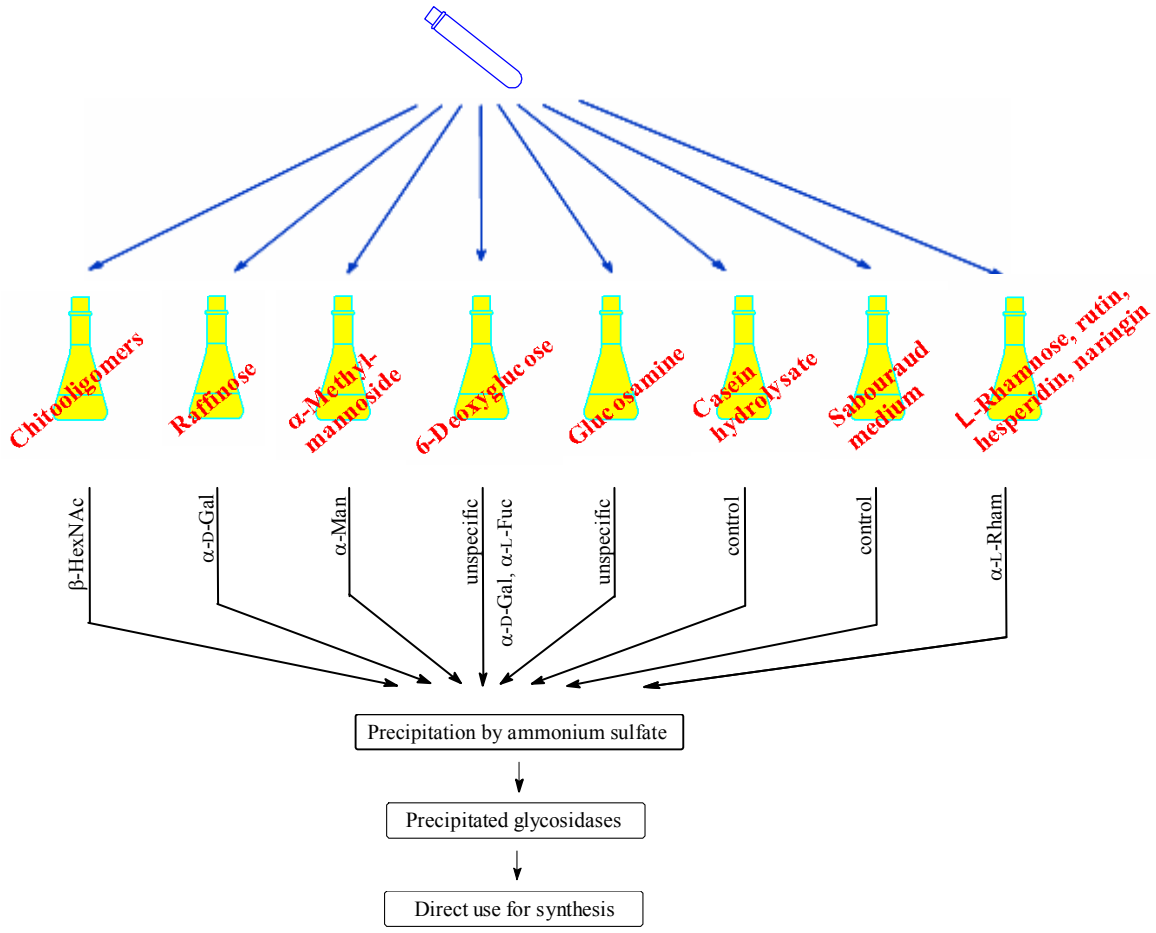


Fig. 4: Screening of glycosidases using inducers

Some glycosidases could be induced by one ore more inducers (*Huňková et al., 1996 b; Huňková et al., 1999*). For the tests available inducer was chosen (cheap, easy synthesis) or

with the highest specific activity. On the other hand respective source of a given enzyme (respective strain) often determines the inducer used. That means that the same enzyme could be induced by various inducers depending on species of fungi used for producing. Sometimes, general inducer for certain type of enzyme did not work for certain species. Good example was the α-D-galactosidase from *Talaromyces flavus* CCF 2686; generally, raffinose was a good inducer for α-D-galactosidases, however, it did not induce production of this enzyme by *T. flavus* CCF 2686. Among large panel of substances tested only 6-deoxyglucose (**12**) acted as its inducer.

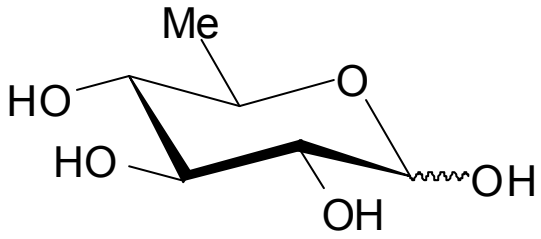


Fig. 5: 6-deoxy-D-glucopyranose (quinovose) (**12**)

5. 2. 1 Induction of β-*N*-Acetylhexosaminidases

Optimal inducer of β-*N*-acetylhexosaminidases (EC 3.2.1.52) should be a water soluble oligomer consisting of β-GlcNAc or β-GalNAc moieties. Chitooligomers containing 2-6 glycosyl units fulfilled these demands. Chitin was not suitable as it was water insoluble and it would, presumably, induce chitinases. Effects of ammonium, glucose, chitin, and chitooligomers on transcription of specific genes and secretion of fungal cell wall-degrading enzymes (chitinolytic, *e. g.* chitinases and *N*-acetylhexosaminidases, and glucanolytic) was studied a few years ago (*Donzelli and Harman, 2001*).

Mixture of chitooligomers was prepared by a controlled hydrolysis of chitin with HCl. Since removal of NaCl from the resulting mixture after neutralisation with NaOH requires special electrodialysis a preparative production of the enzyme from more easily accessible crude chitin hydrolysate containing only 22 – 25 % of effective matter (NaCl makes the rest) was tested. Desalted hydrolysates yielded lower enzyme activities making thus the use of crude chitin hydrolysates for enzyme induction even more favourable. As chitin

hydrolysates contain a considerable amount (ca 25 % related to $(GlcNAc)_{2-6}$) of free *N*-acetylglucosamine the influence of this substance on the enzyme production was tested as well. We found that GlcNAc itself induced β-*N*-acetylhexosaminidases, although to a lower extent than the crude chitin hydrolysates. These enzyme preparations had higher specific activity, which was also exploited in our regulatory study of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 (*Plíhal et al., 2007*). Addition of GlcNAc to the crude chitin hydrolysates had no significant effect upon the induction of the enzymes. No induction effect was observed also with chitin itself. Enhancement of the *N*-acetylhexosaminidase induction by NaCl was obviously unspecific as it was observable also in the controls supplemented with NaCl. This could be caused by the osmotic effect of the salt added.

A library comprising of more than one hundred β-*N*-acetylhexosaminidases from various fungal strains (*e.g.* genera *Aspergillus, Penicillium, Mucor, Fusarium, Talaromyces, Trichoderma, Mortierella, and Acremonium*) was developed. This library was then used in our experiments – *vide infra.*

5. 2. 2 Induction of α-D-Galactosidases

α-D-Galactosidases (EC 3.2.1.22) produced by almost all fungal strains from our collection could be induced by trisaccharide raffinose or disaccharide melibiose giving similar induction rates (*Huňková et al., 1996 b)*). Cheaper raffinose was chosen for further experiments (*Huňková et al., 1999*). A library of fungal α-D-galactosidases (*e.g.* from genera *Aspergillus, Penicillium, Talaromyces, Trichoderma, Micromucor, and Circinella*) was prepared and tested for its properties and application in synthetic reactions.

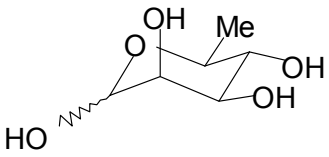
α-D-Galactosidase from *Talaromyces flavus* CCF 2686 strain was, however, unique, not only because of an unusual inducer, but also for its properties. It was induced solely by 6-deoxyglucose. Raffinose (as well as melibiose) was absolutely ineffective in this case. This was a big drawback, because 6-deoxyglucose was very expensive and its synthesis was rather laborious.

5. 2. 3 Induction of α-L-Rhamnosidases

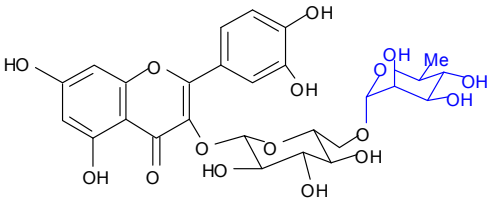
Although fungal α-L-rhamnosidases (EC 3.2.1.40) are already employed in the food industry, investigations on the occurrence of this enzymatic activity have been mainly limited to the members of the Aspergillus and Penicillium genus (*Gallego et al., 2001; Manzares et al., 1997, 2000; Young et al., 1989*) and they have been only recently extended to some Mucor and Fusarium strains (*Scaroni et al., 2002*). In most cases, the production of α-L-rhamnosidase activity has been achieved using L-rhamnose as an inducer. To the best of our knowledge, the effects of other potential inducers on the production level of this enzymatic activity as well as on the properties of the produced enzymes have not been investigated.

Despite the fact that fungi usually show low constitutive levels of glycosidase production (*Huňková et al., 1999*), none of the strains tested was able to produce α-L-rhamnosidases in the absence of inducers, while all the fungi used in this study produced this enzymatic activity in the presence of the respective inducers, showing that new fungal species and different induction conditions could be successfully combined for the rapid obtainment of wide glycosidase libraries. Moreover, some of the α-L-rhamnosidase positive strains (*Acremonium persicinum*, *Circinella muscae*, *Emericella nidulans*, *Eurotium amstelodami*, *Mortierella alpina*, *Rhizopus arrhizus*, *Talaromyces flavus*, and *Trichoderma harzianum*) belong to species or genus where the occurrence of α-L-rhamnosidases has never been detected.

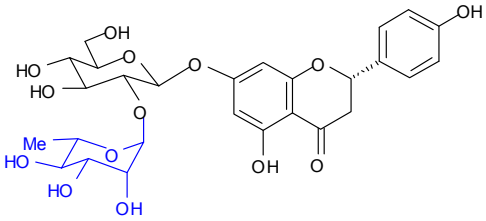
Sixteen different fungal strains were screened for their ability to produce α-L-rhamnosidases under different cultivation conditions in the presence of potential enzyme inducers. Several inducers: L-rhamnose (**2**) and L-rhamnose containing flavonoid glycosides rutin (**7**), naringin (**9**), and hesperidin (**10**) were tested.



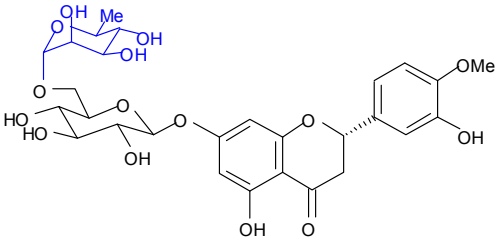
L-rhamnose (**2**)



rutin (**7**)



naringin (**9**)



hesperidin (**10**)

Fig. 6: Inducers of α-L-rhamnosidases

A control cultivation of each strain for the constitutive production of these enzymes was performed by replacing the inducer with a corresponding amount of casein hydrolysate, which was not expected to induce specifically any glycosidase activity. Our experiments showed that several fungi, *e.g.* the *Acremonium persicinum, Mortierella alpina, Mucor circinelloides, Rhizopus arrhizus,* and *Trichoderma harzianum* strains, were not induced by L-rhamnose. Satisfactory induction was achieved using rutin or naringin as inducers. α-L-Rhamnosidases from the majority of species tested were induced by more inducers, even though one of them was always superior. Enzymes with good activity were harvested by ammonium sulfate precipitation and tested for their properties, *e.g.* substrate specificity and stability towards organic cosolvents.

Table 1. Screening of extracellular α-L-rhamnosidase production by various fungal strains

Strain	Inductor			
	L-Rhamnose	**Rutin**	**Hesperidin**	**Naringin**
Acremonium persicinum CCF 1850	-	+	+	+
Aspergillus aculeatus CCF 108	++	+	++	++
Aspergillus aculeatus CCF 3134	++	+	+++	+
Aspergillus aculeatus CCF 3138	+	-	++	+
Aspergillus niger CCIM K2	+	+	+	+
Aspergillus terreus CCF 3059	+++	+++	++	++
Circinella muscae CCF 2417	+	++	-	-
Emericella nidulans CCF 2912	+++	++	+++	++
Eurotium amstelodami CCF 2723	+	+	-	+
Fusarium oxysporum CCF 906	+	++	+	+
Mortierella alpina CCF 2514	-	+	-	+
Mucor circinelloides griseo-cyanus CCIM	-	+	-	+
Penicillium oxalicum CCF 2430	+	-	-	+
Rhizopus arrhizus CCF 100	-	+	-	+
Talaromyces flavus CCF 2686	+	+	-	+
Trichoderma harzianum CCF 2687	-	+	-	+

Activity in the fermentation medium was determined using *p*-nitrophenyl α-L-rhamnopyranoside as a substrate. (-), no induction; (+), less than 0.1 U/ml; (++), 0.1 - 1 U/ml; (+++), more than 1 U/ml.

5. 2. 4 Induction of β-*N*-Acetylmannosaminidase

β-*N*-Acetylmannosaminidase has not been described so far. Due to the occurrence of saccharidic chains with β-ManNAc moieties in biological structures it is supposed that there should exist an enzyme able to hydrolyse these linkages. For screening of such enzyme, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-mannopyranoside as a substrate was employed. This substrate is not available commercially, therefore, it was synthesized in our laboratory (*Krist et al., 2003*) by an original multi-step synthesis in a sufficient amount for the screening of about 30 species of bacteria (*e.g. Acinetobacter sp.*, *Pseudomonas sp., Rhodococcus sp., Bacillus sp., and Corynebacterium sp*.) and for the screening for the enzyme

in snail gut juice, which is known to be rich in various hydrolytic enzymes. *p*-Nitrophenyl 2-acetamido-2-deoxy-α-D-mannopyranoside was synthesized as well (*Krist et al., 2003*), therefore, we performed screening for both α- and β-*N*-acetylmannosaminidase activities.

ManNAc is ubiquitous in nature and it is present in a range of both simple and complex biopolymers. ManNAc is a key precursor of *N*-acetylneuraminic acids, which are important for cellular recognition. β-ManNAc unit is an integral part of a number of bacterial capsular polysaccharides and lipopolysaccharides, in the cell walls of gram-positive bacteria, various types of teichoic acids have been reported to be attached to muramic acid 6-phosphate residues of peptidoglycan through special linkage units, which commonly consist of a linkage disaccharide, ManNAc-β-(1→4)-GlcNAc or Glc-β-(1→4)-GlcNAc, and glycerol phosphate-containing parts. With regard to this fact, we supposed, that the enzyme is produced during the lytic period of the growth of bacteria, when the cell components are decomposed. Due to this reason in very first experiments bacteria were cultivated for 48 h, because of the doubling time of bacteria. For extensive screening bacteria were cultivated for various time periods (24, 48, 72 and 96 h).

Bacteria were grown in a liquid medium (*Kuboki et al*., *1997*). Induction of tentative β-*N*-acetylmannosaminidase by ManNAc – analogously as in the case of β-*N*-acetylhexosaminidase – was attempted. However, no induction effect or enhancement of its activity was observed.

Activity of this enzyme was screened both in the medium (extracellular enzyme) and in disintegrated cells (intracellular enzyme) using protease inhibitors (protease inhibitor cocktail for bacterial cells, Sigma). In this case method using multi-well plates was useful. In the cultivation media neither α-*N*-acetylmannosaminidase nor β-*N*-acetylmannosaminidase activity was found. However, very low intracellular activity of both, α-*N*-acetylmannosaminidase and β-*N*-acetylmannosaminidase was found. The highest activity was found in the cells of *Rhodococcus equi* A5, therefore, this bacterium was chosen for further investigation. The highest α-*N*-acetylmannosaminidase activity (0.006 U/ml) was found in the 72h-old culture. The highest β-*N*-acetylmannosaminidase activity (0.008 U/ml) was found in the same cultivation time.

Five various disintegrating buffers were used: Tris, Tris + EDTA, Tris + DTT, Tris + EDTA + DTT and Tris with protease inhibitors. Best results were obtained using buffer with protease inhibitors and second best buffer was Tris + EDTA + DTT. Samples at 24, 48,

72 and 96 h were assayed. The highest activity both, α- and β-*N*-acetylmannosaminidase, was found after 72 h of growth, even though both activities already appeared after 48 h of growth and they remained detectable till fourth day of growth. Detection of these activities was repeated, however, the production of this enzyme was not increased by any means. Potential inducer can be the disaccharide ManNAc-β-(1→4)-Gly that, however, is not available. Moreover, β-*N*-acetylmannosaminidase could not be obtained by cloning, because this enzyme has not been found yet in any other organism and, therefore, the gene sequence of this enzyme is not known.

Both α-*N*-acetylmannosaminidase and β-*N*-acetylmannosaminidase activity were screened in snail gut juice, which is known to be rich of various hydrolytic enzymes. However, neither α-*N*-acetylmannosaminidase nor β-*N*-acetylmannosaminidase activity was found there.

In conclusion, only a few bacteria species produced respective activities intracellularly. The highest activities were found in *Rhodococcus equi* A5. This bacterium was chosen for further investigation. Nevertheless, the activity is very low, despite attempted ManNAc induction.

5. 3. Screening of Fungal Extracellular Glycosidases and their Applications

The glycosidases were produced by a simple, quick and robust production method and they were of a reproducible quality. Fungi were grown in a liquid medium of the defined composition and under standard cultivation conditions. Enzymes were isolated from the culture supernatant by precipitation by ammonium sulfate (typically by 20 - 80% saturation) at the maximum activity of respective glycosidase, typically after 4-14 days of growth. Precipitated enzymes were centrifuged and stored under saturated $(NH_4)_2SO_4$ at 4 °C. The precipitates were very stable (under described conditions even for a few years) and they were suitable for most synthetic applications or for further purification. The crude preparations had often comparable or even higher specific activity than some commercial purified enzymes [*e.g.* β-*N*-acetylhexosaminidase from *A. niger* (Sigma)].

5. 3. 1 β-*N*-Acetylhexosaminidases (EC 3.2.1.52)

5. 3. 1. 1 β-GalNAc-ase/β-GlcNAc-ase Activity Ratio of β-*N*-Acetylhexosaminidases

After numerous experiments aiming to separate and identify both activities (β-GalNAc-ase and β-GlcNAc-ase) (*Main et al., 1979*), it was concluded that β-*N*-acetylhexosaminidase is a single enzyme having affinity for both substrates (so called "wobbling" affinity). We demonstrated here the possibility of fine tuning the β-GalNAc-ase/β-GlcNAc-ase activity ratio by a rational enzyme source selection together with physiological (cultivation) and environmental modifications. The availability of enzymes with more distinct activities - either β-GalNAc-ase or β-GlcNAc-ase - is very important in their use for synthetic purposes. A fungal source with the high β-GalNAc-ase activity was located and the β-GalNAc-ase/β-GlcNAc-ase ratio improved by the cultivation conditions and by manipulation of the medium. Chitooligomers (see above) were found to be optimal inducers of these inducible fungal enzymes (*Huňková et al., 1996 a); Huňková et al., 1999*).

A substantial increase of β-GalNAc-ase activity was observed between the 12^{th} and 13^{th} day of cultivation, when the β-GalNAc-ase/β-GlcNAc-ase ratio reached 2.3 - 2.8. After precipitation of the enzyme by ammonium sulfate (80 % saturation) from the cultivation medium the ratio decreased to 1.2 - 1.5. These differences may be caused by possible changes in refolding or in the hydration envelope of the protein.

Different pH optima for β-GalNAc-ase (pH optimum 4.5) and β-GlcNAc-ase (pH optimum 5.0) were found. The highest β-GalNAc-ase/β-GlcNAc-ase ratio was at the pH value 4.5. These data were important for the design of the reaction conditions when using β-GalNAc-ase for the synthetic purposes.

Fifty-five fungal strains having high β-*N*-acetylhexosaminidase activity were screened for their β-GalNAc-ase/β-GlcNAc-ase ratio (**Table 2**). Generally, the β-GalNAc-ase/β-GlcNAc-ase ratio in most of β-*N*-acetylhexosaminidases is under 0.5. Higher activity of β-GalNAc-ase was found only in very few enzymes tested, the best one being from *Penicillium oxalicum* CCF 2430.

Table 2: β-GalNAc-ase/β-GlcNAc-ase ratio of various fungal β-*N*-acetylhexosaminidases

Strain [a]	β-GalNAc-ase/β-GlcNAc-ase ratio
Aspergillus oryzae CCF 147	0.26
A. oryzae CCF 172	0.34
A. oryzae CCF 1066	0.56
A. oryzae CCF 1602	0.26
A. oryzae CCF 3062	0.43
A. flavus CCF 146	0.23
A. flavus CCF 642	0.54
A. flavus CCF 1129	0.66
A. flavus CCF 3056	0.57
A. parasiticus CCF 141	0.25
A. parasiticus CCF 1298	0.59
A. parasiticus CCF 3058	0.32
A. sojae CCF 3060	0.51
A. flavofurcatus CCF 107	0.22
A. flavofurcatus CCF 3061	0.35
A. tamarii CCF 1665	0.30
A. terreus CCF 55	0.50
***A. terreus* CCF 57** [b]	**1.00**
A. terreus CCF 3059	0.46
***A. terreus* CCF 2539**	**1.01**
A. flavipes CCF 76	0.54
A. flavipes CCIM USA	0.68
***A. flavipes* CCF 2026**	**1.00**
A. flavipes CCF 3067	0.90
A. niveus CCF 544	0.75
A. niveus CCF 3057	0.15
A. niger CCIM K1	0.40
A. niger CCIM K2	0.42
A. phoenicis CCF 61	0.49
A. versicolor CCF 2491	0.09
Penicillium spinulosum CCF 2159	0.92
P. melinii CCF 2440	0.12
P. brasilianum CCF 2155	0.75
***P. funiculosum* CCF 1994**	**1.20**
P. funiculosum CCF 2325	0.76
***P. purpurogenum var. rubrisclerotium* CCF 2984**	**1.22**
***P. purpurogenum var. rubrisclerotium* CCF 2985**	**1.15**
P. pittii CCF 2277	0.63
***P. oxalicum* CCF 1667**	**2.00**
***P. oxalicum* CCF 1959**	**1.60**
***P. oxalicum* CCF 2315**	**1.40**
***P. oxalicum* CCF 2430**	**2.30**
***P. oxalicum* CCF 3009**	**1.40**
***P. oxalicum* CCF 2062**	**1.50**
Fusarium oxysporum CCF 377	0.18

CCF Culture Collection of Fungi, Department of Botany, Charles University, Prague
CCIM Culture Collection of the Institute of Microbiology, Prague

[a] More strains (5–10) from each species were tested, however only the most representative examples are given in this table.

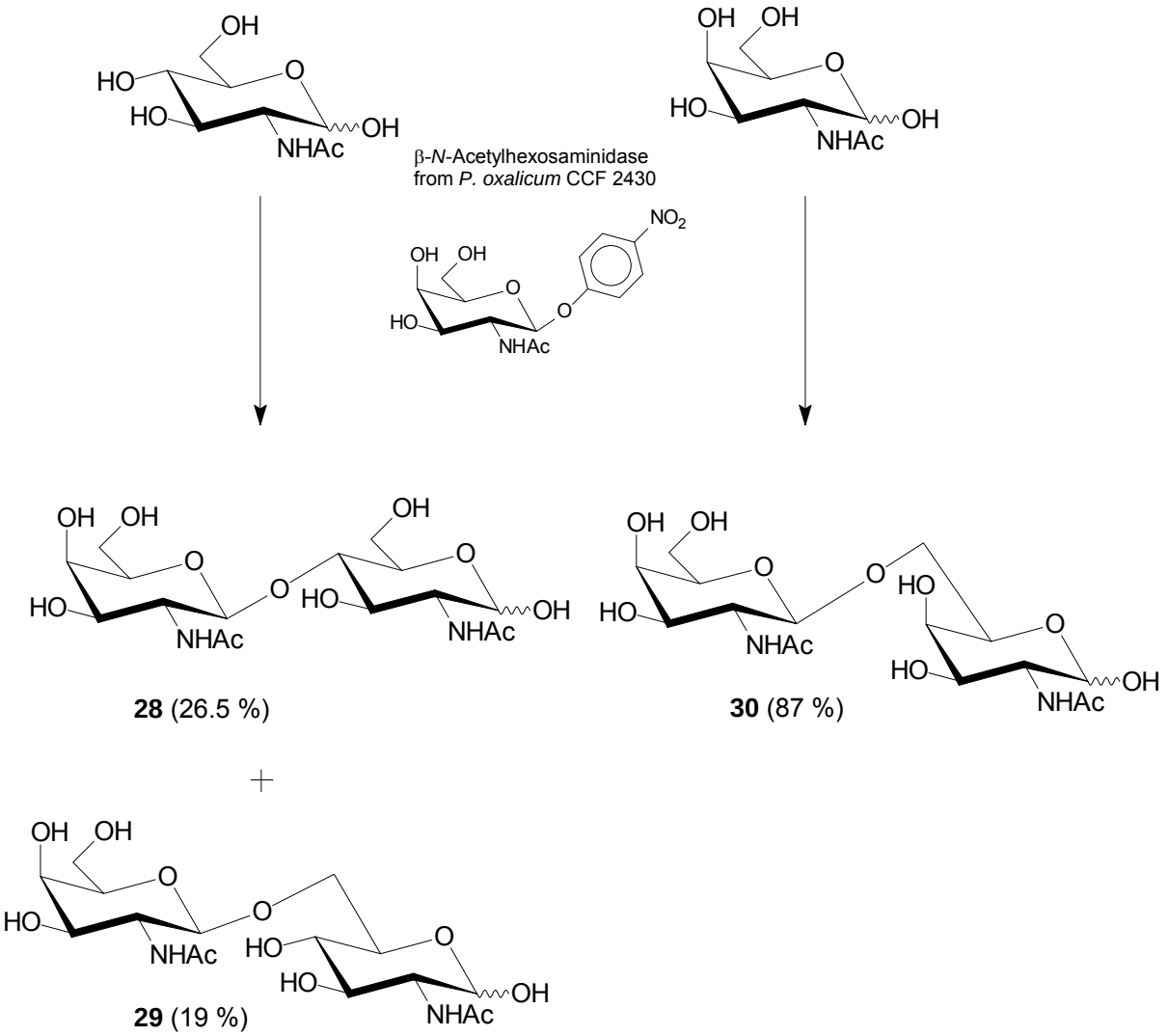
[b] Bold entries: β-GalNAc-ase/β-GlcNAc-ase ratio ≥1.0

Due to previous observation that the addition of some inorganic salts or organic solvents could positively stimulate the β-*N*-acetylhexosaminidase activity (*Kubisch et al., 1999; Rajnochová et al., 1997*), we also studied the influence of selected inorganic salts, *e.g.* $(NH_4)_2SO_4$, $MgSO_4$ and LiCl [in higher concentrations influencing water activity and/or enzyme hydration (*Rajnochová et al., 1997*)] on β-GlcNAc-ase and β-GalNAc-ase (and their ratio) activity of β-*N*–acetylhexosaminidase from *P. oxalicum* CCF 2430. $(NH_4)_2SO_4$, $MgSO_4$ and LiCl suppressed only β–GlcNAc-ase activity. The maximum ratio of 3.35 we observed in the presence of 20 % (w/v) of $MgSO_4$. Acetonitrile, which was used as cosolvent to solubilize the rather insoluble *p*NP-β-GalNAc (*Kubisch et al., 1999*), strongly inhibited β-GalNAc-ase activity (10 % of acetonitrile (v/v) – more than 60 % of the activity was lost). We also studied the influence of aminosugars (GlcN·HCl, GlcNAc and GalNAc) on the β *N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 (**Table 3**). All tested aminosugars decreased the β-GalNAc-ase/β-GlcNAc-ase ratio. These compounds are known feedback inhibitors of this enzyme. This was an important finding because during enzymatic reactions (synthesis, enzyme determination) these sugars (GlcNAc or GalNAc) were liberated into the reaction medium, thus changing these parameters.

Table 3: Influence of aminosugars (GlcN·HCl, GlcNAc and GalNAc) on β-*N*-acetylhexosaminidase activities

	β-GalNAc-ase activity (%)	β-GlcNAc-ase activity (%)	β-GalNAc-ase/ β-GlcNAc-ase ratio (%)
GlcN·HCl (mmol/l)			
0 (control)	100	66	1.5
5	78	63	1.2
10	71	53	1.3
15	46	44	1.0
GlcNAc (mmol/l)			
5	57	45	1.3
10	60	45	1.3
15	47	40	1.2
GalNAc (mmol/l)			
10	81	61	1.3
15	77	75	1.0
50	75	68	1.1
100	42	58	0.7

We used GlcNAc (**4**) as an acceptor for β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 (**Scheme 1**). The reaction was performed in 20% (w) $MgSO_4$ to increase the β-GalNAc-ase/ β-GlcNAc-ase ratio. The reaction gave 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**28**, 26.5 %) and 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (**29**, 19 %). Even though these regioisomers had the same molecular weight, the compounds could be separated simply by gel chromatography on Toyopearl HW40F. This fact could be explained by different exclusion volumes (shape, hydration envelope) of both disaccharides. The results showed that this enzyme represented a β-*N*-acetylhexosaminidase with a very high β-GalNAc-ase/β-GlcNAc-ase ratio. To the best of our knowledge this enzyme has not been used yet for transglycosylation reactions. We used GalNAc (**5**) as an acceptor under the same reaction conditions (**Scheme 1**). In this case only a single isomer 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-galactopyranose (**30**) was formed in an excellent yield of 87 %.



Scheme 1

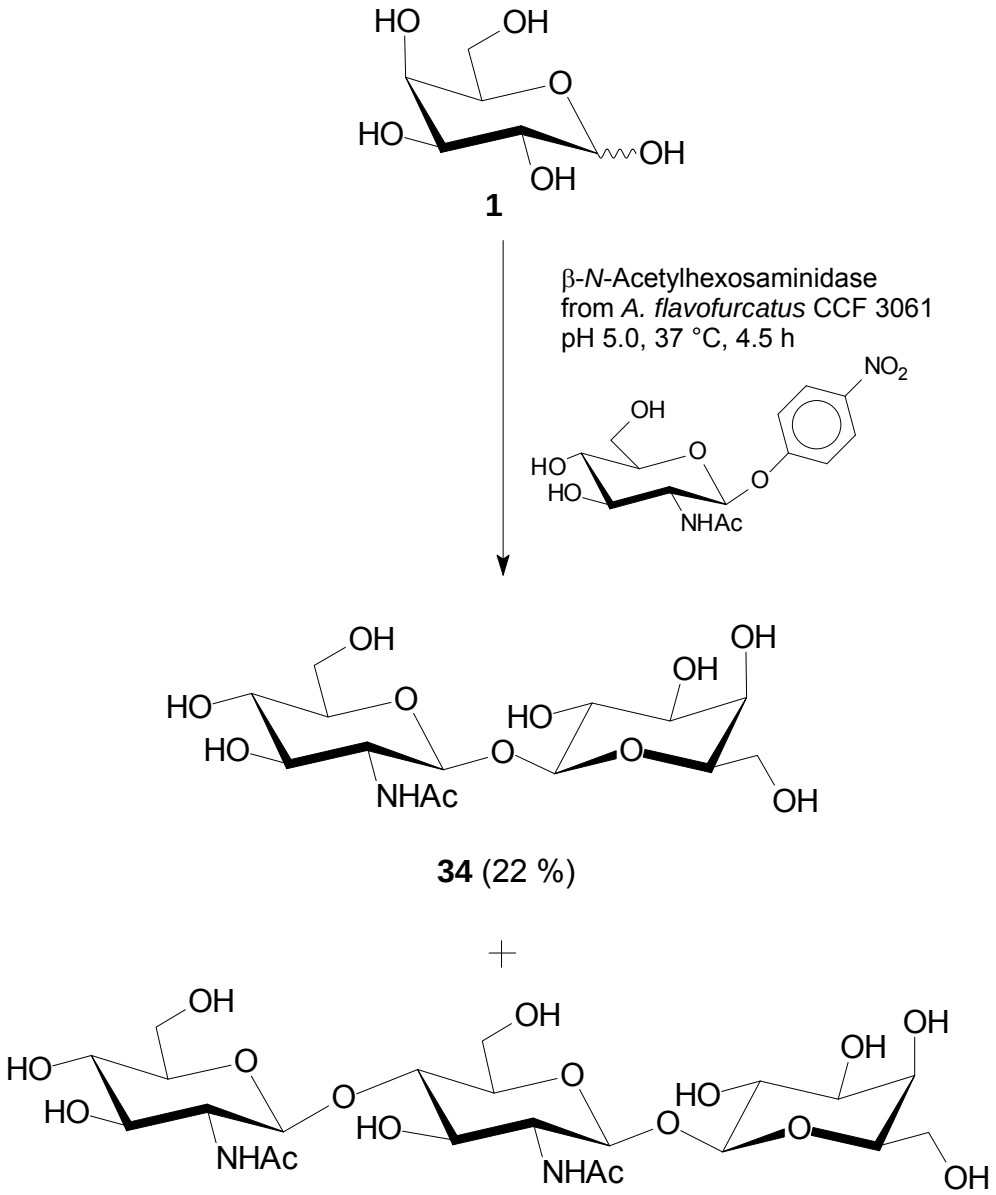
5. 3. 1. 2 Synthesis of Non-reducing Saccharides by Transglycosylation Reaction Catalysed by Fungal β-*N*-Acetylhexosaminidases

The ability of glycosidases to form non-reducing sugars is very rare. We have previously prepared β-D-Glc*p*NAc-(1→1)-β-D-Man*p* with the β-*N*-acetylhexosaminidase from *A. oryzae*, which was probably the first documented case of non-reducing disaccharide synthesis with this glycosidase type (*Křen et al., 1998*). Non-reducing galactooligosaccharide α-D-Gal*p*-(1→1)-α-D-Glc*p* was previously prepared by the α-galactosidase from *Candida guilliermondii* H-404 (*Hashimoto et al., 1995*). Another non-reducing disaccharide containing Gal-structures was 6′-sulfo β-D-Gal*p*-(1→1)-α-D-Glc*p*, recently

synthesised by the β-galactosidase from *Bacillus circulans* (*Murata et al., 2001*). Other two cases of enzymatic glycosylation at the anomeric hydroxyl of the glycosidic acceptor have been described, however, with glycosyltransferases: α-glucosylation of lactose (*Shibuya et al., 1993*) and β-galactosylation of xylose (*Wiemann et al., 1994*).

We screened the transglycosylation activity of forty-three extracellular fungal β-*N*-acetylhexosaminidases for their ability to transfer the β-GlcNAc moiety onto *galacto*-type acceptors, *e.g.* D-galactose (**1**), 2-acetamido-2-deoxy-D-galactopyranose (**5**) and lactose (**40**). The β-*N*-acetylhexosaminidases from *Aspergillus flavofurcatus* CCF 3061, *A. tamarii* CCF 1665 and *A. oryzae* CCF 1066 gave the best yields and thus they were selected for semi-preparative procedures.

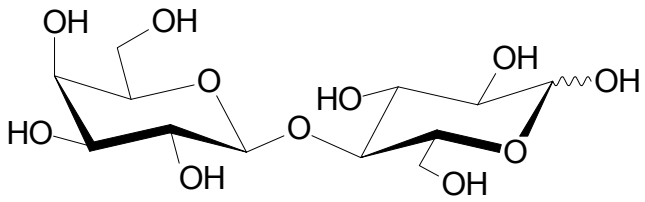
Under the catalysis of the β-*N*-acetylhexosaminidase from the *A. flavofurcatus* CCF 3061 with D-galactose and lactose as acceptors we synthesised a unique, non-reducing disaccharide β-D-Glc*p*NAc-(1→1)-β-D-Gal*p* (**34**) and trisaccharides β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→1)-β-D-Gal*p* (**35**), β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc (**38**) and β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→1)-β-D-Glc*p*NAc (**39**). Using D-galactose as the acceptor we obtained two main products **34** and **35** (**Scheme 2**). Moreover two side products, *i.e. N,N'*-diacetylchitobiose (**32**) and *N,N',N''*-triacetylchitotriose (**37**) were obtained as determined by HPLC co-chromatography with the authentic standards. The reaction mixture was lyophilised and separated on a Biogel P-2 column. The fractions containing disaccharides **35** and **37** and trisaccharides **35** and **37**, respectively, was further separated by semi-preparative HPLC.



Scheme 2

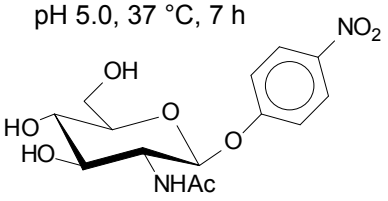
Using lactose as the acceptor non-reducing lacto-oligosaccharides β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc (**38**) and β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→1)-β-D-Glc*p*NAc (**39**) (**Scheme 3**) were formed together with side product **37.** The reaction mixture was lyophilised and separated on a Biogel P-2 column. Method of selective anomeric deacetylation was used to separate these trisaccharides. This method leads to removal of acetyls only on anomeric

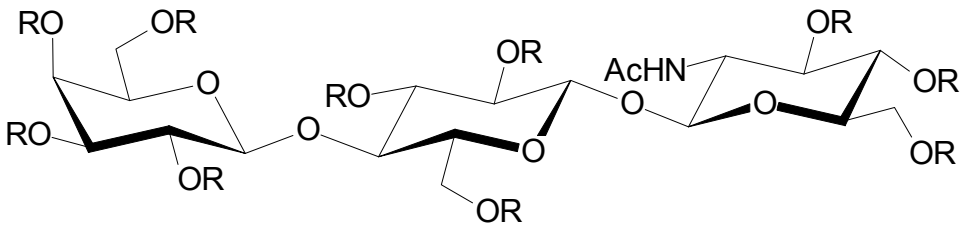
hydroxyls and thus to the change of polarity of reducing saccharides, while the polarity of non-reducing trisaccharides remains unchanged. The trisaccharide fraction was acetylated. The anomeric hydroxyls of reducing sugars were selectively 1-deacetylated with piperidine in THF. This selective deprotection of reducing sugars lowered their R_f on the silica gel chromatography, which enabled to separate them from the non-reducing ones. Thanks to this method separation of non-reducing trisaccharides from reducing one was achieved. Compound **38** was characterised as peracetate **38a.** Compound **39a** behaved during C-1 deprotection with piperidine/THF in a rather surprising way as the C-2 acetate on the α-glucopyranosyl moiety was selectively removed to afford decaacetate **39b** (as determined later with MS and NMR). However, β-isomer **38a** remained stable during this treatment. Attempts to peracetylate (Ac_2O/Py) **39b** furnished only mixtures of acetates. Acetylation of **39b** (or peracetylation of **39**) was not feasible probably due to steric hindrances of this position (steric conflict of two axial bonds at αC-1 and C-2). Peracetylation of β-isomer **38** proceeded smoothly and the compound was stable. Therefore, the compound **39** was characterised as its decaacetate **39b**.



40

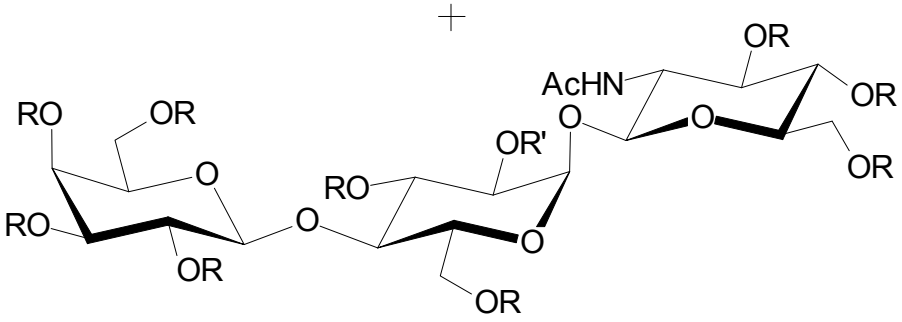
β-*N*-Acetylhexosaminidase
from *A. flavofurcatus* CCF 3061
pH 5.0, 37 °C, 7 h





38 (10 %)

+



39 (9 %)

38, **39**	R = R' = H
38a, **39a**	R = R' = Ac
39b	R = Ac, R' = H

Scheme 3

The use of GalNAc (**5**) as an acceptor with the β-*N*-acetylhexosaminidases from *A. flavofurcatus* CCF 3061, *A. oryzae* CCF 1066 and *A. tamarii* CCF 1665 afforded only β-D-Glc*p*NAc-(1→6)-D-Gal*p*NAc (**47**).

5. 3. 1. 3 Reverse Hydrolysis Catalysed by Fungal β-*N*-Acetylhexosaminidases

β-*N*-Acetylhexosaminidases are also able to synthesize glycosidic bonds by another mechanism, *e.g.* by condensation (reverse hydrolysis). Reports on the reverse hydrolysis with this type of enzymes are rather scarce (*Rajnochová et al., 1997; Wongvithoonyaporn et al., 1998*) especially because of low yields (< 1%) and due to a strong feedback inhibition of β-*N*-acetylhexosaminidase by 2-acetamido-2-deoxy-D-glucopyranose (the β-*N*-acetyl-hexosaminidase from *A. oryzae*, K_i = 1.6 mM).

Twenty extracellular β-*N*-acetylhexosaminidases of fungal origin were screened for the regioselectivity by the reverse hydrolysis with GlcNAc (**4**) as a substrate. Almost all enzymes exhibited a rather high selectivity for β(1→6) bond formation (**Table 4**) at a GlcNAc concentration of 0.3 - 1.4 M. The β-*N*-acetylhexosaminidase from *A. flavofurcatus* CCF 3061 displayed a selective preference for the β(1→4) product formation (**32**) from all β-*N*-acetylhexosaminidases tested.

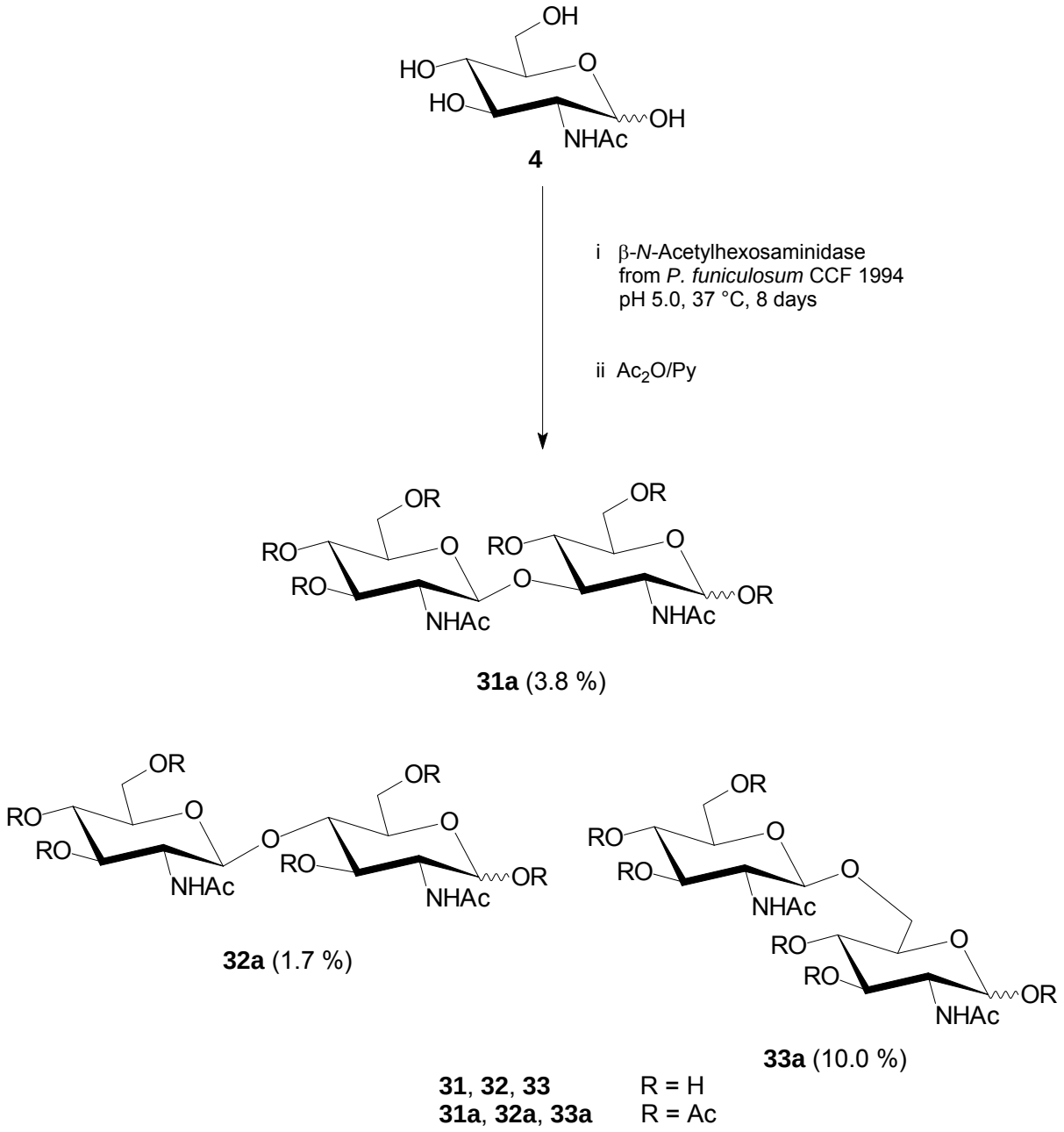
Table 4: Regioselectivity of fungal β-*N*-acetylhexosaminidase-production of *N*-acetylglucosaminobioses

Source of enzyme	Condensation products[a] (isolated yields %)		
	β(1→3)[b]	**β(1→4)[c]**	**β(1→6)[d]**
Acremonium persicinum CCF 1850	–	–	7.4
Aspergillus flavipes CCF 2026	–	–	3.2
A. flavofurcatus CCF 3061	–	8.4	–
A. flavus CCF 1129	–	–	4.0
A. fumigatus CCF 1059	–	3.2	5.1
A. niveus CCF 3057	–	–	–
A. oryzae CCF 147	–	–	2.3
A. oryzae CCF 1066	–	–	2.1
A. sojae CCF 3060	–	2.2	1.2
A. tamarii CCF 1665	1.6	–	5.2
A. terreus CCF 2539	–	–	2.2
Penicillium brasilianum CCF 2155	–	–	–
***P. funiculosum* CCF 1994**[e]	**3.8**	1.7	10.0
P. funiculosum CCF 2325	2.0	1.5	7.3
P. chrysogenum CCF 1269	< 1.5	1.6	3.1
P. multicolor CCF 2244	–	–	< 1.5
P. oxalicum CCF 2315	–	–	2.4
P. pittii CCF 2277	–	2.0	2.8
Talaromyces flavus CCF 2686	–	–	–
Trichoderma harzianum CCF 2687	< 1.5	3.3	4.6

[a]Yields (%) of condensation reactions with GlcNAc (**4**, 1.0M)), the respective β-*N*-acetylhexosaminidase (8 U), McIlvaine buffer (pH 5.0), 37 °C, 8 days – the yields of the respective peracetates **31a, 32a, 33a** after reaction mixture peracetylation and separation.
[b] β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**31a**)
[c] β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc (**32a**)
[d] β-D-Glc*p*NAc-(1→6)-D-Glc*p*NAc (**33a**)
[e] Bold entry: The maximum yield of product β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**31a**)

The three β-*N*-acetylhexosaminidases from *A. tamarii* CCF 1665, *P. funiculosum* CCF 2325 and *P. funiculosum* CCF 1994 (**Scheme 4**) produced relatively a high proportion of a rare chitobiose isomer β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**31**, yields approx. 5 %). To our knowledge, the formation of β(1→3) linkage by β-*N*-acetylhexosaminidases

has not been described yet. Recently, the compound **31** has been prepared by chemical synthesis (*Rohlenová et al., 2004*).



Scheme 4

We examined the β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994 as the enzyme with the most pronounced formation of **31** regarding the following criteria: the influence of reaction time and GlcNAc concentration on the respective isomer (**31**, **32**, **and 33**)

production. In the course of the first three days of the reaction (from the total reaction time 8 days, when we obtained maximum yield of isomer **31**), exclusively β-D-Glc*p*NAc-(1→6)-D-Glc*p*NAc (**33**) was formed. During next five days another isomers β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc (**32**) (from the fourth day) and β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**8**) (from the fifth day) were produced (**Fig. 7**).

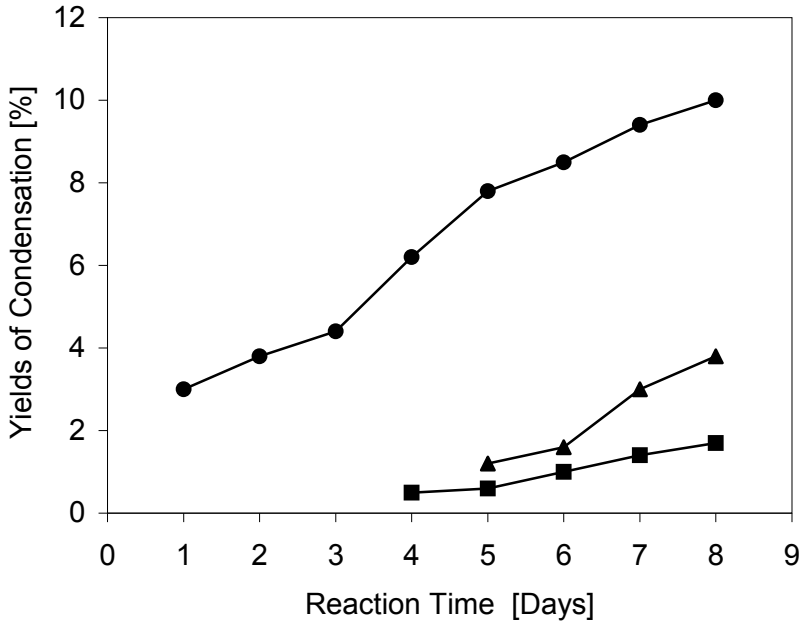


Fig. 7: Time profile of the formation of chitobiose isomers in the reaction catalysed by the β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994
(▲) β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**31**), (■) β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc (**32**), (●) β-D-Glc*p*NAc-(1→6)-D-Glc*p*NAc (**33**)

Doubling GlcNAc concentration (0.6 M to 1.0 M) (**Fig. 8**), the formation of **31** was tripled (1.3 to 4.0 %). A further increase of the substrate concentration caused the decrease of the product formation, probably due to the enzyme inhibition by substrate.

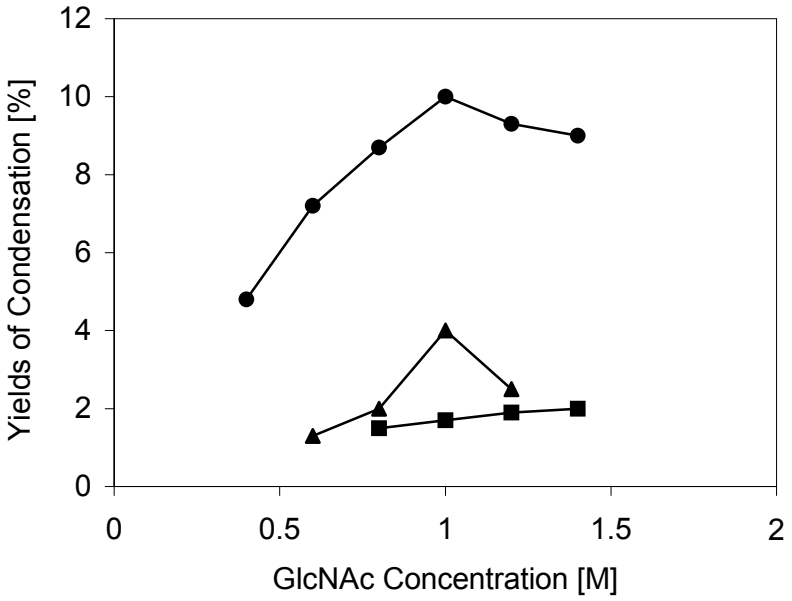


Fig. 8: Influence of GlcNAc concentration on the isomer yields in reaction catalysed by the β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994
(▲) β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**31**), (■) β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc (**32**), (●) β-D-Glc*p*NAc-(1→6)-D-Glc*p*NAc (**33**)

We have previously observed that the addition of some inorganic salts or organic solvents could stimulate the β-*N*-acetylhexosaminidase activity and shift the equilibrium in favour of product formation. Therefore, we studied the influence of inorganic salts [$(NH_4)_2SO_4$ and $MgSO_4$] on the product formation (**Table 5**). They stimulated strongly the β(1→6) isomer production. The isomer **31** was formed only at low $(NH_4)_2SO_4$ concentration (0.1 - 0.2M). The formation of product **32** was totally suppressed by the inorganic salt addition.

Table 5: Influence of salt addition on the isomer production in the reaction catalysed by the β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994

Salt added	Concentration [M]	Yields[a] [%]	
		β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc	β-D-Glc*p*NAc-(1→6)-D-Glc*p*NAc
None (control)	–	3.8	10.0
$(NH_4)_2SO_4$	0.1	4.3	11.2
$(NH_4)_2SO_4$	0.5	–	12.5
$(NH_4)_2SO_4$	1.0	–	14.0
$MgSO_4$	0.1	–	10.5
$MgSO_4$	0.5	–	11.7
$MgSO_4$	1.0	–	13.2

[a] Conversion of GlcNAc (**4**, 1.0 M) into *N*-acetylglucosaminobioses (two isomers – **31**, **33**), the respective salt [$(NH_4)_2SO_4$, $MgSO_4$] concentration, the β-*N*-acetylhexosaminidase (8 U), McIlvaine buffer (pH 5.0), 37 °C, 8 days. The yields of the respective peracetates **31a** and **33a** after reaction mixture peracetylation and separation.

Although products **31**, **32** and **33** were enzymatically synthesised in a single step and the starting material GlcNAc (**4**) can easily be recovered from the reaction mixture after gel filtration, the separation of respective isomers was not trivial. The course of the GlcNAc condensation could not be monitored by HPLC due to the similar retention time values of isomers (19.26 min for **31** and 19.76 min for **33**). However, the formation of β(1-3), β(1-4), β(1-6) linkages (depending on the enzyme source) could be monitored by TLC giving a clear resolution (R_f 0.58 for **31**, R_f 0.54 for **32** and R_f 0.67 for **33** with mobile phase propan-2-ol : H_2O : aqueous ammonia = 7 : 2 : 1). The compounds could be separated by silica gel chromatography in their peracetylated forms.

5. 3. 2 α-D-Galactosidases (EC 3.2.1.22)

Enzymatic glycosylations catalyzed by glycosidases are absolutely stereoselective but they often suffer from poor regioselectivity (*Weignerová et al., 1999a; Křen and Thiem, 1997*). This is especially problematic when using saccharide acceptors, which carry more reactive OH's groups. Generally, primary OH's groups are better acceptors than the secondary ones and,

as a consequence, a regioisomer mixture is often formed, frequently containing 1→6 (by)products. This occurs even when the used glycosidase possesses a significant regioselectivity for one of the secondary OH groups (*Weignerová et al., 2001*). As a consequence, the selective protection of the primary hydroxyls (*e.g.* 6-OH in hexopyranoses) in the acceptor saccharide was suggested. However, the protective group should not be too lipophylic and too bulky in order to keep the substrate water soluble and (sterically) acceptable for the enzyme. This requirement rules out the most currently used protective groups for primary alcoholic groups, *e.g.* the triphenylmethyl (trityl) or the *tert*-butyldimethylsilyl (TBDMS) moieties. Enzymatic regioselective esterification catalyzed by hydrolases (lipases or proteases) in organic solvents is an established process for obtaining 6-OH acylated monosaccharides (typically acetyl-, propionyl-, butyryl-) in considerably high yields (*Carrea and Riva, 2000; Riva and Roda, 2000*). Glycosidases can tolerate these modified substrates. Nevertheless, it has to be considered that any further substitution (acylation) on less soluble sugars, *i.e. galacto*-derivatives diminishes their solubility, thus making necessary the use of cosolvents.

Enzymatic transglycosylation of partially acylated saccharide acceptors is a rather novel approach that could be applied to solve synthetic problems in carbohydrate chemistry. We have used this approach on an enzymatic synthesis of *iso*globotriose α-Gal-(1→3)-β-Gal-(1→4)-Glc (*Weignerová et. al., 1999b*), which is an important epitope in pig-man xenotransplantations. Hereunder, we demonstrate a further extension of this approach consisting in a combination with the use of various organic cosolvents.

5. 3. 2. 1 Synthesis of Acylated Substrates by Regioselective Enzymatic Transesterification Used for Reactions Catalysed by α-D-Galactosidases

5. 3. 2. 1. 1 Enzymatic Acylation of *N*-Acetylhexosamines

One of the main problems related to the enzymatic acylation of free mono- and disaccharides is the low solubility of these compounds in the organic solvents that are more suitable to preserve the catalytic activity of lipases and proteases. The use of polar solvents, like pyridine or DMF, substantially decreases the number of enzymes that can be employed. For instance, only the protease subtilisin, commercially available under different trade names,

is significantly active in DMF. The data on the effect of DMSO, another very good solvent for carbohydrates, are more contradictory. DMSO can dissolve proteins and thus it causes a significant enzyme denaturation and a consequent loss of activity. On the other hand, there are recent reports that describe the acylation of sugars in a mixture of organic solvents containing a significant percentage of DMSO (Pedersen et al., 2002; Ferreira et al., 2002).

Our initial goal was the optimisation of the reaction conditions to achieve the selective acylation of the *N*-acetylhexosamines, such as 2-acetamido-2-deoxy-D-glucopyranose (**4**), 2-acetamido-2-deoxy-D-galactopyranose (**5**) and 2-acetamido-2-deoxy-D-mannopyranose (**11**). We chose the cheapest of these sugars as a reference compound and evaluated the performances of different enzymes under various reaction conditions. As a "reference" reaction we chose the acetylation of **4** in DMF catalysed by protease N (a crude preparation of subtilisin). As expected, the conversion was satisfactory and the product, isolated in 62 % yield, was found to be 2-acetamido-6-*O*-acetyl-2-deoxy-D-glucopyranose (**17**) according to ^{1}H and ^{13}C-NMR (*see Appendix*). The same reaction was repeated using different polar solvents containing 20 % v/v DMSO. As shown in **Figure 9**, compared with pure DMF, reaction outcomes in these solvent mixtures were comparable or even better, the only exception being just the reaction DMF containing 20 % DMSO, in which protease N was completely inactive.

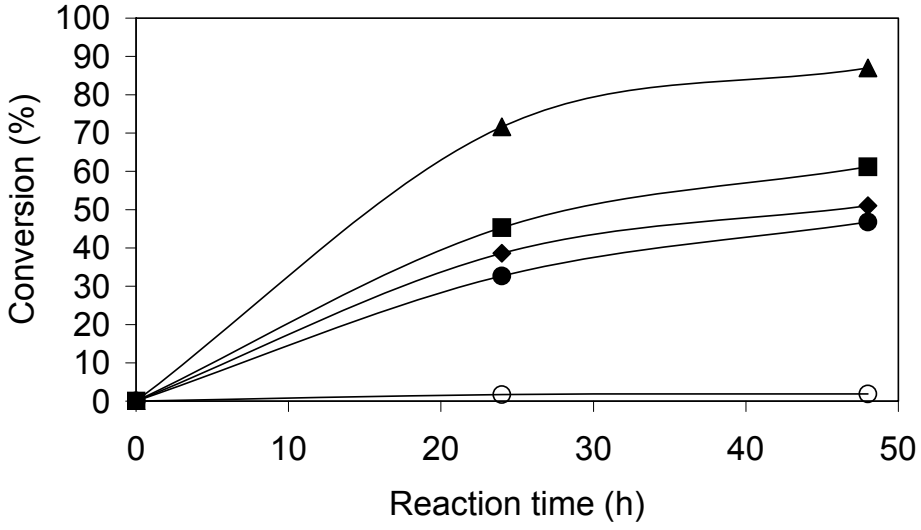


Fig. 9: Acetylation of GlcNAc catalysed by protease N in organic solvents *[tert*-amyl alcohol (♦), dioxane (■), acetonitrile (▲), acetone (●), DMF (o)] containing 20 % v/v DMSO.

The acetylation of 2-acetamido-2-deoxy-D-glucopyranose (**4)** was repeated in three best solvent mixtures using different commercially available protease and lipase preparations, known to be able to catalyse sugar esterifications. The results, summarised in **Table 6**, clearly indicate that proteases – and particularly subtilisin – were more suitable for this biotransformation than lipases and that the use of a mixture acetonitrile : DMSO (80 : 20 v/v) gave better results with most of the enzymes tested. By increasing the amount of DMSO, the protease activities dramatically decreased, while lipases were inactivated and the acylation did not proceed at all.

Tab 6: Conversion (%)[a)] of GlcNAc (**3**) during the regioselective acetylation catalysed by proteases and lipases suspended in various organic solvents mixtures

Enzyme	**Conversion [%]; Solvent : DMSO (8 : 2 v/v)**		
	***t*-Amyl alcohol**	**Acetonitrile**	**Dioxane**
Proteases:			
Protease N	51	87	61
Subtilisin	95	67	48
Proleather	72	73	7
Alcalase	12	17	3
Lipases:			
Novozym 435	51	72	11
Porcine pancreatic lipase	6	8	1
Lipase PS	48	7	3
Chromobacterium viscosum lipase	13	15	1
Humicola lanuginosa lipase	1	1	1

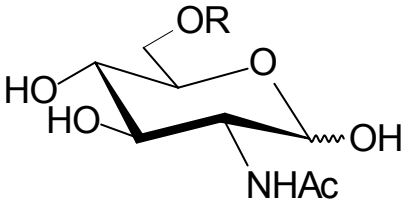
[a)] Degrees of conversion were evaluated after 48 h by GC

The best reaction conditions (*see above*) were applied to the acylation of compounds 2-acetamido-2-deoxy-D-galactopyranose **(5)** and 2-acetamido-2-deoxy-D-mannopyranose (**11)**. In the case of GalNAc, two monoesters, 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose (**18**), the expected main product and 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactofuranose (**19**), by-product, were isolated in 51 and 5 % yields, respectively, and characterised by NMR. The acetylation of ManNAc also gave two products, **20** and **21**, in 59 and 13 % yields, respectively. The main product **20** was the expected 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose, as unambiguously determined by NMR. The by-product **21** was 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-D-mannopyranose, also determined by NMR. The acylation of 2-acetamido-2-deoxy-D-mannopyranose **(11)** was repeated using trifluoroethyl butanoate as an activated ester and the corresponding butanoyl derivatives 2-acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranose **(22**) and 2-acetamido-3,6-di-*O*-butyryl-2-deoxy-D-mannopyranose **(23**) were recovered in good yields.

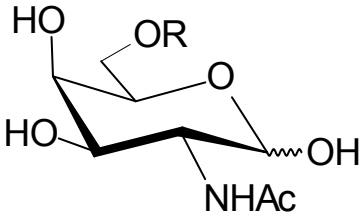
5. 3. 2. 1. 2 Enzymatic Acylation of *N*-Acetylhexosamine Derivatives

The solubility of the mono-acetylated aminosugars 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose (**18**) and 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose **(20**) was higher in organic solvents that are more suitable to preserve lipases activity. In order to evaluate the regioselectivity of these enzymes towards the secondary free OH's at C-3 and C-4 of **18** and **20**, these compounds were dissolved in the mixture of acetone : pyridine (8 : 1 v/v) containing trifluoroethyl acetate. The samples of lipases reported in **Table 6** were then added to the reaction mixtures. The formation of a defined new product was observed only with 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose (**20**) using *Candida antarctica* lipase B (Novozym 435). Compound containing three acetyls 2-acetamido-1,6-di-*O*-acetyl-2-deoxy-α-D-mannopyranose (**26**) was isolated in 18 % yield. Similarly, Novozym 435-catalysed the butanoylation of 2-acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranose (**22**)**,** which gave 2-acetamido-1,6-di-*O*-butanoyl-2-deoxy-α-D-mannopyranose (**27**) in 19 % yield. Finally, for further synthetic applications, we reacted *p*-nitrophenyl 2-acetamido-2-deoxy-*β*-D-galactopyranoside (**24**) with Novozym 435 in a mixture of pyridine-acetone-vinyl acetate, in this case an almost quantitative conversion to the 6-*O*-acetate **25** was observed.

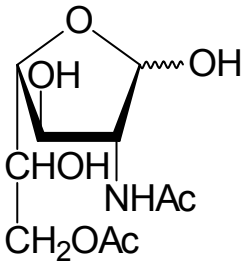
We have shown that a careful choice of the reaction conditions (solvent, enzyme, acylating agent) can allow the efficient regioselective acylation of poorly soluble carbohydrates like *N*-acetylhexosamines and their derivatives.



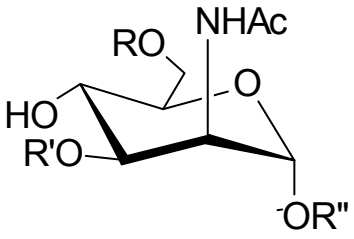
4: R = H
17: R = Ac



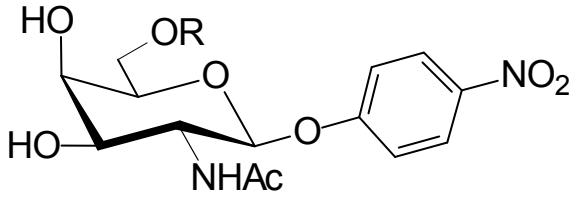
5: R = H
18: R = Ac



19



11: R = R' = R'' = H
20: R = Ac; R' = R'' = H
21: R = R' = Ac; R'' = H
22: R = Bu; R' = R'' = H
23: R = R' = Bu; R'' = H
26: R = R'' = Ac; R' = H
27: R = R'' = Bu; R' = H



24: R = H
25: R = Ac

Fig. 10: Substrates and products of acylation

5. 3. 2 Application of Selectively Acylated Substrates

Using a modification of the usual enzymatic protocol, two selectively modified substrates, *p*-nitrophenyl 6-*O*-acetyl-α-D-galactopyranoside (**14**) and *p*-nitrophenyl 6-*O*-acetyl-β-D-galactopyranoside (**16**), were obtained in good yields (95.5 % for **14**, 73.8 % for **16**) in a regioselective enzymatic transesterification catalyzed by lipase PS in acetone-pyridine with vinyl acetate as an acyl donor.

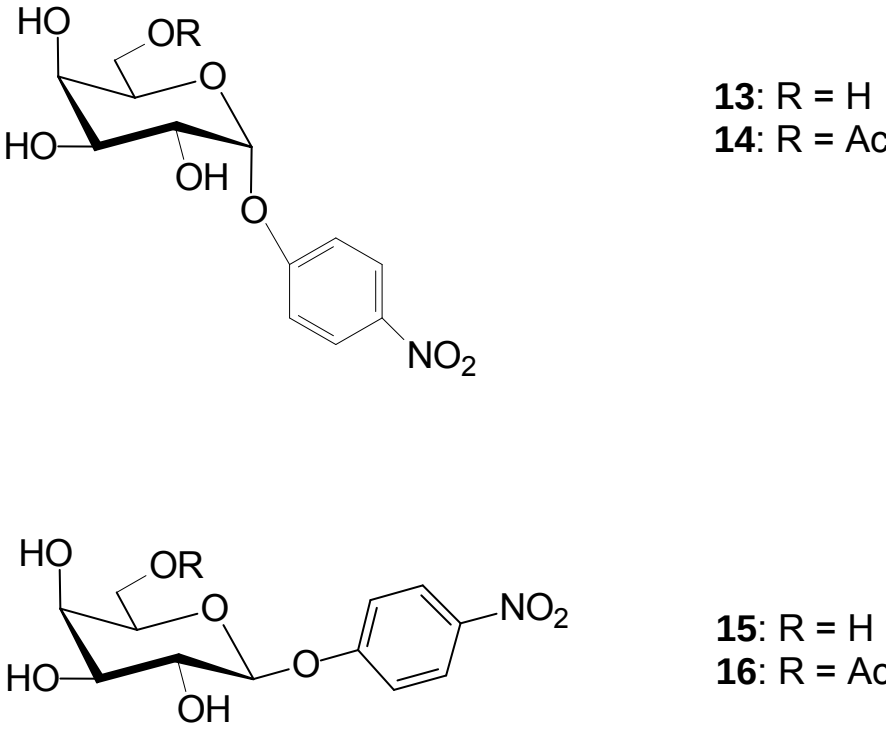


Fig. 11: *p*NP substrates and products of their acylation

A preliminary screening showed that **14** was not hydrolyzed by a series (14 enzymes) of α-galactosidases, ruling out the possibility of using them as modified sugar donors. The hydrolysis of *p*-nitrophenyl β-D-galactopyranoside modified at 6-OH position (*p*-nitrophenyl 6-deoxy-6-methyl-(*R*) and (*S*)-sulfinyl-β-D-galactopyranoside, *p*-nitrophenyl 7–deoxy-D- and L-*glycero*-β-D-*galacto*-heptopyranoside and *p*-nitrophenyl 6,7-anhydro-D- and L-*glycero*-β-D-*galacto*-heptopyranoside) was described (*Grabowska et al., 1998*), but we have not observed this reaction in our case. These data also confirm the peculiarity of the results previously obtained with β-*N*-acetylhexosaminidases (*Hušáková et al., 2001*), which were

able to accept 6-*O*-acylated substrate analogous. On the other hand, this result was also a clear indication that, in principle, we could use **14** (as well as **16**) as a sugar acceptor for transglycosylation reaction without risking that their sensitive glycosidic bond would be hydrolysed. Unfortunately, compound **16** suffered from a very poor solubility in water even in the presence of organic cosolvents, which prevented its use in enzymatic glycosylations.

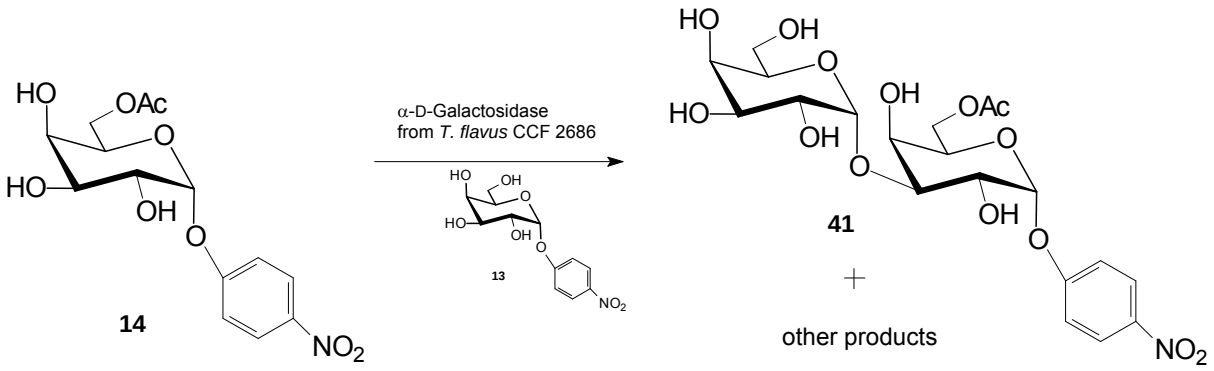
We decided to investigate the performance of the α-galactosidase from *Talaromyces flavus* CCF 2686, as we have found that this enzyme accepts a large variety of acceptors and it was found to be particularly suitable for synthetic applications.

Solubilities of **14** and **16** were quite low in the aqueous medium and, as a result, we were forced to use a significant amount of water-miscible organic solvents to reach a sufficient concentration (acetone, acetonitrile, *N*,*N*-dimethylformamide, dimethylsulfoxide, dioxane, 2-methoxyethanol, pyridine, 2-methylpropan-2-ol and tetrahydrofuran). Experiments to obtain information of the influence of solvents on the activity of this α-D-galactosidase and the long-term enzyme stability were performed. The activity of the α-galactosidase decreased to 37 % in the presence of 25.5 % *tert*-butyl alcohol after 28 hours, while the activity decrease in the presence of 32.5 % acetone was much more significant (only 2 % of residual activity were detected after 28 hours). The decrease of glycosidase stability with increasing cosolvent concentration is a well known phenomenon (*Brena et al., 2003*). Therefore, the enzyme was added to the reaction mixture in several portions during the biotransformation. 2-Methylpropan-2-ol was the cosolvent of choice, as it was affecting both the activity and the stability to the smallest extent. Despite its alcoholic moiety, it is generally considered to be unable to act as an acceptor in enzymatic glycosylations due to a severe steric hindering (no reports on its enzymatic α-galactosylation are known).

Accordingly, a preparative scale enzymatic reaction was run using *p*-nitrophenyl α-D-galactopyranoside (**13**) as a sugar donor and **14** as a sugar acceptor in the presence of 25.5% v/v 2-methylpropan-2-ol (**Scheme 5**). No similar transformation could be performed with the β-galactosyl derivative **16** as an acceptor because its solubility was too low (2.0 - 2.7 mg/ml, the solubility of α-galactosyl derivate **14** in transglycosylation reaction was 36.4 mg/ml) even in the presence of a significant amount of 2-methylpropan-2-ol or of other cosolvents. Usual work-up of this reaction and purification by gel chromatography followed by flash chromatography allowed the isolation of the expected *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside (**41**). The presence of more polar compounds was also observed and by a HPLC comparison with standards identified them as

products of autocondensation of *p*-nitrophenyl α-D-galactopyranoside **13**: *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-α-D-galactopyranoside (**44**) and *p*-nitrophenyl α-D-galactopyranosyl-(1→6)-α-D-galactopyranoside (**43**), which cannot be avoided.

Another less polar product was isolated in a 7.3 % yield and, to our surprise, it was identified as 2-methylpropyl α-D-galactopyranoside (**42**). This result was quite unexpected due to the fact that 2-methylpropan-2-ol is an alcohol with sterically hindered OH group quite inert to enzymes (*van Rantwijk et al., 1999*) (for instance it can be used as a solvent in transesterification reactions catalyzed by hydrolases in organic media) and acting as a quite weak nucleophile in chemical reactions. This finding forced us to use acetone, the second best cosolvent, in order to avoid the formation of this by-product (**42)**. Accordingly, a transglycosylation reaction was performed in the presence of 32.5 % v/v acetone and the disaccharide *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside (**41**) was obtained in a 5 % yield (**Scheme 5**). Isolated yields of this mono-acylated disaccharide are low in comparison with the results previously obtained when our bi-enzymatic strategy was applied (regioselective acylation in organic solvents followed by regioselective transglycosylation) (*Hušáková et al., 2001; Weignerová et al., 1999b*). However, it has to be pointed out that the monoacylated (and, therefore, selectively protected) derivative **41** can not be synthesized in an alternative simple approach. For instance, the enzymatic acylation of the corresponding disaccharide **44** would produce derivatives mono-acylated at the non-reducing sugar moiety (*i.e.* *p*-nitrophenyl 6-*O*-acetyl-α-D-galactopyranosyl-(1→3)-α-D-galactopyranoside). Additionally, the difference of polarity between the single mono-acylated disaccharide **41** and the mixture of the disaccharides (**43** and **44**) obtained by autocondensation of **13** allows its easy isolation from the reaction mixture.



Scheme 5: Transglycosylation reaction using α-D-galactosidase from *T. flavus*

In conclusion, we have shown the possibility of using selectively acylated glycosyl acceptors for the synthesis of *p*-nitrophenyl galabioses. These compounds may have practical application in the preparation of glycoconjugates exploiting the nitrophenyl group for the attachment (*e.g.* by reduction to $–NH_2$ and transformation to a reactive –SCN group using $CSCl_2$). We have shown that some cosolvents, *e.g.* 2-methylpropan-2-ol and acetone, may be used in transglycosylation reactions to increase the solubility of modified substrates. However, the unexpected finding that 2-methylpropan-2-ol can be used as an acceptor in transglycosylation reactions catalyzed by the α-galactosidase from *T. flavus* can be of synthetic relevance and will be investigated in more detail.

5. 3. 3 α-L-Rhamnosidases (EC 3.2.1.40)

5. 3. 3. 1 Developing of α-L-Rhamnosidase Library

We prepared more than 30 different α-L-rhamnosidase samples and we characterised them by its substrate specificity and their stability in the presence of organic solvents, due to the low substrate solubility in water. To exploit this enzymatic library for synthetic applications, the presence of contaminating α-L-arabinosidases and β-D-glucosidases was investigated. The latter activities were found in several preparations, while α-L-arabinosidase content was generally quite low. According to the results a selective derhamnosylation of a saponin desglucoruscin was performed on a preparative scale.

5. 3. 3. 2 β-D-Glucosidase and α-L-Arabinosidase Activities in the α-L-Rhamnosidase Preparations

For biological studies selectively derhamnosylated substances (*e.g.* desrhamnodes-glucoruscin **46**) were required. Unfortunately most of the rhamnosidase preparations contained β-D-glucosidase and α-L-arabinosidase activity, which would cause total deglycolysation of the substrates. Therefore, preparations void of these activities were used.

The activity of β-D-glucosidase and α-L-arabinosidase in the preparations of the library was measured using the corresponding *p*-nitrophenyl glycosides (**Fig. 12**). It was shown that the α-L-arabinosidase(s) content was quite low in the entire library, while the presence of β-D-glucosidase(s) was detected in many preparations. The production of β-D-glucosidases was unrelated to the use of glucose-containing glycosides as inducers (rutin, hesperidin, naringin), since it was observed also in L-rhamnose-induced cultures. Nevertheless, cultures obtained using different inducers showed highly variable levels of contaminating β-D-glucosidases, in most cases the highest levels of production being observed with naringin.

Therefore, a rational choice of the inducer can lead to a production of enzyme preparations with low level of contaminating activities, thus avoiding extensive enzyme purifications and providing a "ready-to-use" library.

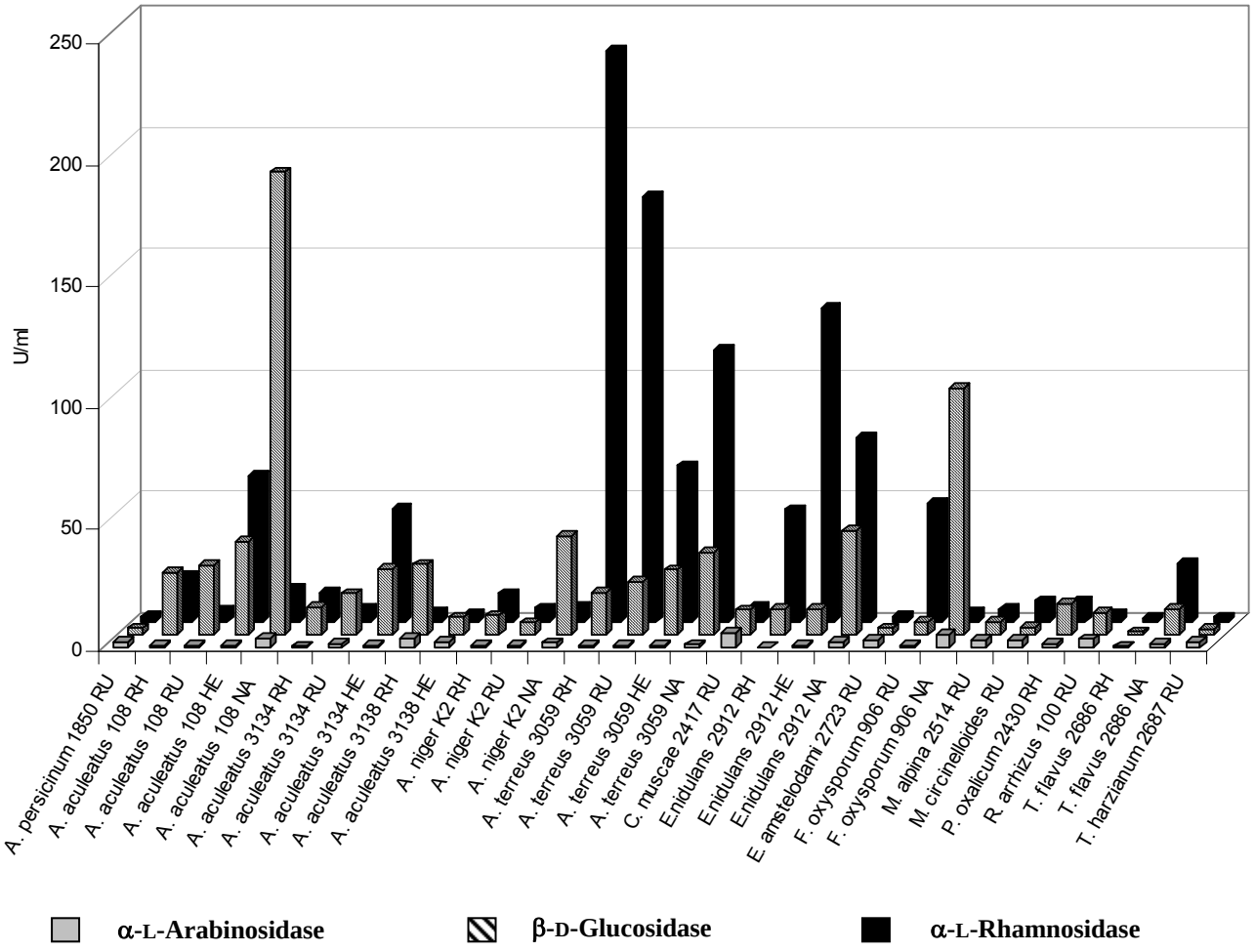


Fig. 12: Screening of contaminating β-D-glucosidases and α-L-arabinosidases in the α-L-rhamnosidase library.
Inducers: RU, rutin; RH, L-rhamnose; HE, hesperidin; NA, naringin.

5. 3. 3. 3 Substrate Specificity of the Library of α-L-Rhamnosidases

The substrate specificities of the different preparations of the library were investigated using six L-rhamnose-containing natural glycosides – rutin (**7**), quercitrin (**8**), naringin (**9**), hesperidin (**10**), ginsenoside Re (**45**), and asiaticoside (**6**) – as substrates.

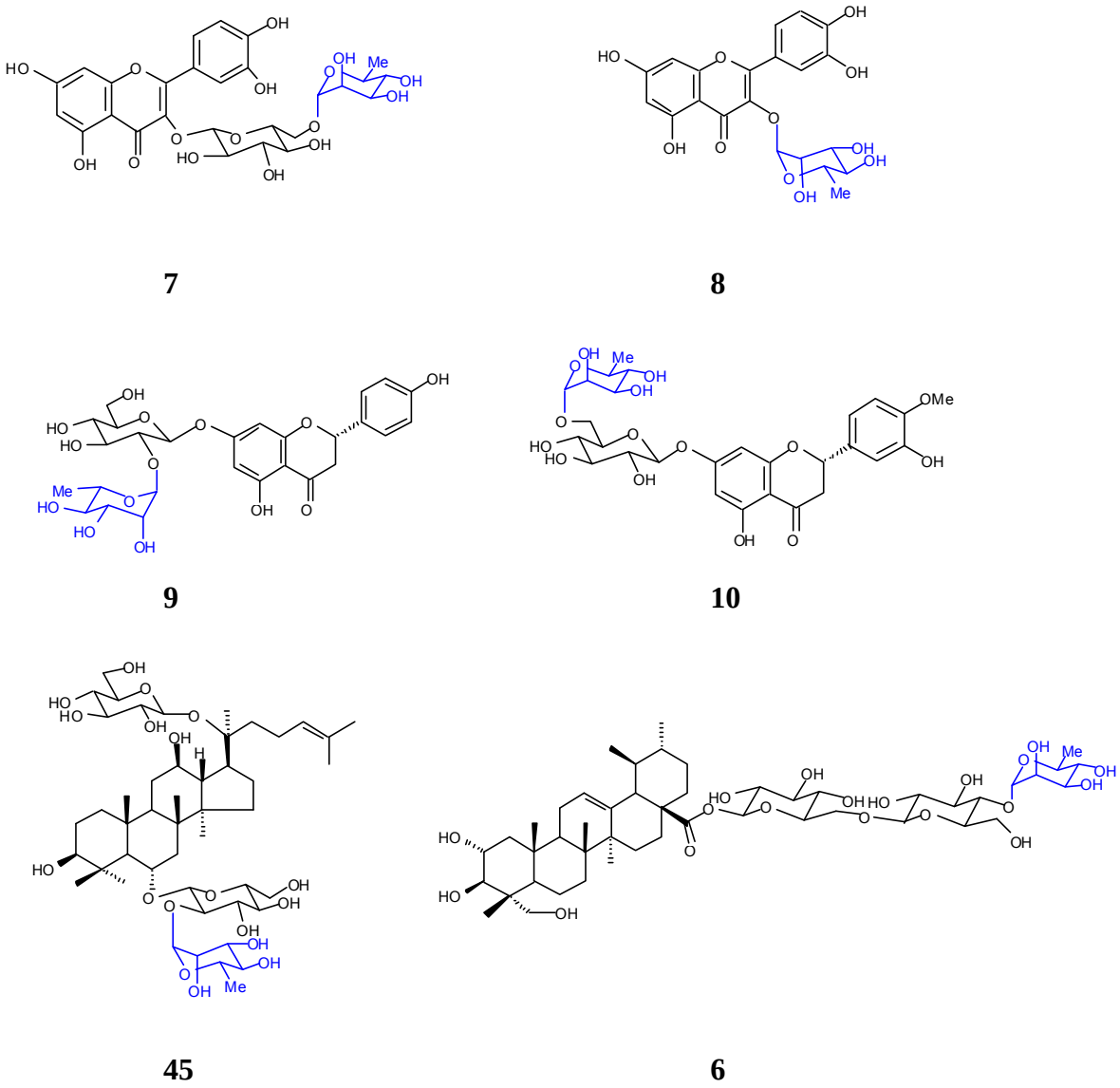


Fig. 13: Natural substrates of α-L-rhamnosidase

These compounds have quite different aglycon structures. Rutin, hesperidin, naringin, and quercitrin are flavonoid derivatives, while ginsenoside Re is a saponin and asiaticoside is a terpenoid glycoside. Furthermore, L-rhamnose is directly linked to the aglycon moiety in quercitrin, while all the other substrates are more complex glycosides, in which L-rhamnose is linked to a β-D-glucopyranosyl unit through different glycosidic bonds (α-1,6 in **7** and **10**, α-1,2 in **9** and **45**, α-1,4 in **6**).

A first set of reactions was performed with the suspensions of each substrate in potassium phosphate buffer, the TLC control of the reaction mixtures allowing a fast screening of the substrate preferences of the different enzyme preparations.

Five of the 31 preparations of the library, *i.e. Acremonium persicinum* CCF 1850, inducer: rutin (**7**); *Circinella muscae* CCF 2417, inducer: L-rhamnose (**2**); *Eurotium amstelodami* CCF 2723, inducer: rutin (**7**); *Rhizopus arrhizus* CCF 100, inducer: rutin (**7**); *Trichoderma harzianum* CCF 2687, inducer: rutin (**7**) were inactive towards all the substrates tested, showing that, in some cases, flavonoid rhamnosides could act as inducers but not be cleaved by the enzymes produced. On the contrary, the other 26 enzymes were active towards at least two different rhamnosides. The results (**Table 7**) demonstrate rather different specificity for preparations obtained from different microbial strains. For example, the enzymes obtained from the *A. aculeatus* strains showed broad substrate specificity and were active towards five different compounds. Moreover, these preparations were effective in releasing L-rhamnose also from quercitrin (**8**), that was not hydrolysed by other α-L-rhamnosidase preparation from the same species (*Manzanares et al., 2001*), a fact which may be ascribed to the strain variability or to the alternative cultivation conditions (induction of specific activity). Despite the possible sterical hindrance of this compound, due to the direct α-L-rhamnosidic linkage to the aglycon, other enzymes, *i.e.* those obtained from the *Mortierella alpina, P. oxalicum* and *T. flavus* strains, were able to accept quercitrin as a substrate, while its hydrolysis had been described so far only using bacterial (*Hashimoto and Murata, 1998; Jang* and *Kim, 1996; Miake et al., 2000*) and animal (*Kurosawa et al., 1973*) α-L-rhamnosidases.

The complex glycoside ginsenoside Re (**45**) was efficiently derhamnosylated by the *A. aculeatus*, *Emericella nidulans* and *P. oxalicum* preparations. Interestingly, the only α-L-rhamnosidase able to hydrolyse ginsenosides described so far was obtained from an *Absidia* sp. 39 strain cultured in the presence of an aqueous extract of ginseng roots (Yu *et al.*, 2002), while our active preparations were obtained in the presence of nonstructurally related inducers.

Finally, the terpenoid glycoside asiaticoside (**6**) was accepted as a substrate by the *A. terreus*, *E. nidulans* and *F. oxysporum* preparations and by the preparation obtained by induction with naringin of the *T. flavus* strain. The *A. aculeatus* and *A. niger* preparations, which showed a good activity on the other substrates, were completely inactive towards

asiaticoside (α-1,4 glycosidic bond), therefore showing a marked preference for the hydrolysis of other (α-1,2 and α-1,6) glycosidic bonds.

In some cases, the preparations obtained from the same strain in the presence of different inducers showed also different substrate specificities. For instance, the preparation obtained by induction with naringin (**9**) of the *T. flavus* CCF 2686 strain showed a broader substrate specificity than that one obtained from the same strain by induction with L-rhamnose (**2**), which was active only on rutin (**7**) and quercitrin (**8**). The preparations obtained from the *E. nidulans* CCF 2912 strain under induction of L-rhamnose, hesperidin and naringin showed also a different substrate specificity, the one induced by hesperidin was able to accept a wider number of substrates.

The use of different microbial strains and different inducers affected not only the production levels of the desired enzymes, but also the "quality" of the enzymatic preparations in terms of substrate specificity. This might be likely due to the different induction susceptibility of various isoenzymes whose occurrence in fungi has been described (*Luonteri et al., 1998; Mileto et al., 1998*) and should be taken into account for the full exploitation of their metabolic versatility.

Table 7 Screening of the substrate specificity of the α-L-rhamnosidase library in the presence or in the absence of THF (20 % v/v)

		Substrate					
Strain	Inductor	Rutin	Hesperidin	Naringin	Quercitrin	Ginsenoside Re	Asiaticoside
Aspergillus aculeatus CCF 108	L-Rhamnose	●	●	●	○	○	-
	Rutin	●	●	●	○	○	-
	Hesperidin	●	●	●	○	○	-
	Naringin	●	●	●	○	○	-
Aspergillus aculeatus CCF 3134	L-Rhamnose	●	●	●	○	○	-
	Rutin	●	●	●	○	○	-
	Hesperidin	●	●	●	○	○	-
Aspergillus aculeatus CCF 3138	L-Rhamnose	●	●	●	○	○	-
	Hesperidin	●	●	●	○	○	-
Aspergillus niger CCIM K2	L-Rhamnose	●	●	●	-	-	-
	Rutin	●	●	●	-	-	-
	Naringin	●	●	●	-	-	-
Aspergillus terreus CCF 3059	L-Rhamnose	●	○	○	-	-	○
	Rutin	●	○	○	-	-	○
	Hesperidin	●	○	○	-	-	○
	Naringin	●	○	○	-	-	○
Emericella nidulans CCF 2912	L-Rhamnose	○	-	○	-	-	○
	Hesperidin	●	●	●	-	○	○
	Naringin	●	-	●	-	○	○
Fusarium oxysporum CCF 906	Rutin	●	○	○	-	-	○
	Naringin	○	○	○	-	-	○
Mortierella alpina CCF 2514	Rutin	●	-	-	○	-	-
Mucor circinelloides griseo-cyanus CCIM	Rutin	-	●	●	-	-	-
Penicillium oxalicum CCF 2430	L-Rhamnose	-	-	○	○	○	-
Talaromyces flavus CCF 2686	L-Rhamnose	●	-	-	○	-	-
	Naringin	○	-	○	○	-	○

- ● The substrate is hydrolysed by the respective enzyme preparation both in the presence and in the absence of 20% THF
- ○ The substrate is hydrolysed by the respective enzyme preparation only in the absence of 20% THF
- \- The substrate is not hydrolysed by the respective enzyme preparation neither in the presence nor in the absence of 20% THF

5. 3. 3. 4 Influence of Organic Solvents on the α-L-Rhamnosidase Stability

To improve the solubility of the natural glycosides used in the screening, different water-miscible organic cosolvents, *i.e.* CH_3CN, DMF, MeOH, THF and DMSO, can be used for the hydrolytic reactions. However, a negative effect on the enzymatic activity cannot be

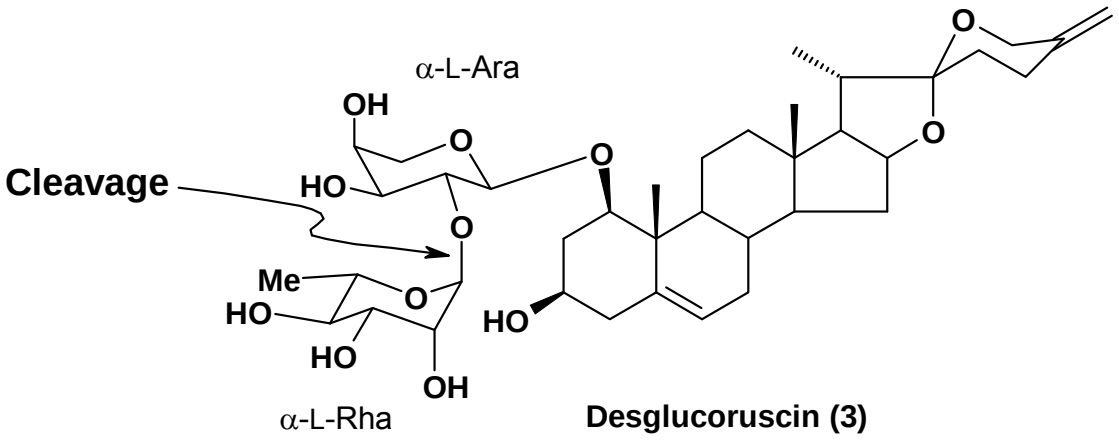
excluded a priori. For instance, as shown in **Table 7**, after the addition of 20% (v/v) THF some α-L-rhamnosidases, *e.g.* those obtained from the *P. oxalicum* strain, were completely inactivated. Most of the preparations maintained their catalytic power towards only some of the substrates. For example, the *A. aculeatus* preparations became inactive towards quercitrin (**8**) and ginsenoside Re (**45**), but were still able to hydrolyse rutin (**7**), hesperidin (**10**) and naringin (**9**). Analogously, *A. terreus* preparations kept their activity against rutin (**7**) but were inactive towards hesperidin (**10**) and naringin (**9**).

These observations suggest that the apparently broad substrate specificity of some preparations, for example, the ones from the *A. aculeatus* strains, might be likely due to the coexistence in the same sample of different enzymes or of different isoforms of the same enzyme with different substrate specificity and different stability towards organic cosolvents. Furthermore, in some cases the preparations obtained from the same strain using different inducers showed a different stability in the presence of this cosolvent (*e.g.* those from the *E. nidulans* and *T. flavus* strains).

5. 3. 3. 5 Application of the Library of α-L-Rhamnosidases

The final target of the study of α-L-rhamnosidases was to establish an enzyme library able to selectively derhamnosylate complex natural compounds without affecting different glycosidic bonds. Chemical methods (*e.g.* acidic hydrolysis) for restricted glycolysis of substrates, *e.g.* desglucoruscin (**3**) and asiaticoside (**6**) are impractical due to the concomitant hydrolysis of all glycosidic bonds. When using glycosidases, the possible presence of contaminating activities able to cleave glycosidic linkages different from the desired one (α-L-Rha) can lower the selectivity and, therefore, these activities have to be eliminated or suppressed. As an alternative, the screening of a broad enzyme library can provide preparations void of contaminants.

The saponin derivative desglucoruscin (**3**) bears an α-(1,2)-L-rhamnopyranosyl moiety linked to an α-L-arabinopyranoside. Our previous attempts of selective hydrolysis of **3** with commercial naringinase and hesperidinase were unsuccessful, and therefore this glycoside was a challenging example for testing our rhamnosidase library, taking into account the extremely low α-L-arabinosidase content in our enzyme preparations.



Scheme 6: Selective trimming of desglucoruscin

Due to the presence of hydrophobic and bulky aglycon, desglucoruscin has a very low water-solubility and, therefore, the use of organic solvents was indispensable. The screening was performed either in the presence of 20 % v/v water-miscible organic cosolvent (THF, CH_3CN, DMF, MeOH and DMSO) or in the presence of ethyl acetate (biphasic system). Concerning the homogeneous reactions, only THF (20% v/v) was able to solubilize desglucoruscin to a sufficient extent to perform the hydrolysis reaction. Moreover, using this cosolvent, detection of the derhamnosylation product desrhamnodesglucoruscin (**46**) by TLC was observed only with the *A. niger* CCIM K2 preparation (inducer: L-rhamnose). As this preparation also possessed the highest activity in the AcOEt/buffer biphasic system (compared to the other enzymes that were able to hydrolyse desglucoruscin under these reaction conditions), this rhamnosidase was selected for a preparative reaction.

Table 8. Derhamnosylation of desglucoruscin in AcOET/buffer or 20% v/v THF

Strain (the source of respective enzyme)	**Inducer**	**AcOEt/buffer**	**20% v/v THF**
Aspergillus aculeatus CCF 108	L-Rhamnose	+	-
	Rutin	+	-
	Hesperidin	+	-
	Naringin	+	-
Aspergillus aculeatus CCF 3134	L-Rhamnose	+	-
	Rutin	+	-
	Hesperidin	+	-
Aspergillus aculeatus CCF 3138	L-Rhamnose	+	-
	Hesperidin	+	-
Aspergillus niger CCIM K2	L-Rhamnose	++	+
	Rutin	+	-
	Naringin	+	-
Mortierella alpine CCF 2514	Rutin	+	-

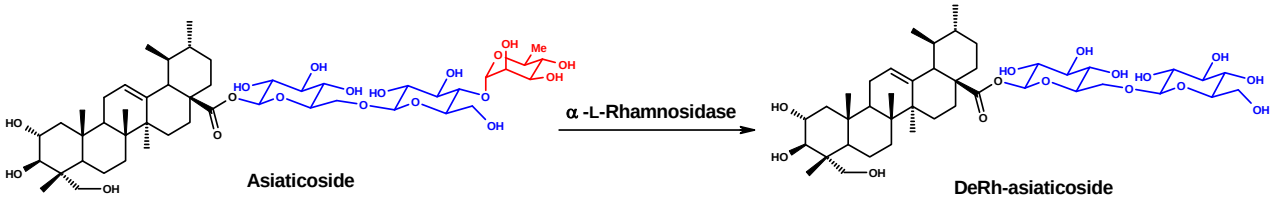
\+ Desglucoruscin is derhamnosylated by the respective enzymatic preparation

\- Desglucoruscin is not derhamnosylated by the respective enzymatic preparation

++ Desglucoruscin is derhamnosylated by the respective enzymatic preparation with a high conversion (about 70 %)

As expected, no significant α-L-arabinosidase contamination was observed and the formation of a single product was monitored. The selective derhamnosylation of desglucoruscin reached about 70 % conversion after 4 days and structural analysis of the isolated product confirmed its identity with the desired desrhamno derivative. This example shows the effectiveness of this practical approach in the search of highly selective biocatalysts.

Library of α-L-rhamnosidases was used for selective trimming of saponin derivative asiaticoside (**6**) (**Scheme 7**) (*Monti et al., 2005*).



α-L-rhamnosidases: from *Aspergillus terreus* CCF 3059
Emericella nidulans CCF 2912
Fusarium oxysporum CCF 906
Talaromyces flavus CCF 2686

Scheme 7: Selective trimming of asiaticoside

5. 3. 3. 6 α-L-Rhamnosidase Is an Inverting-Type Glycosidase

Glycosidases can be of inverting or retaining type according to the anomeric configuration of the monosaccharide produced upon hydrolysis (*Withers, 2001*). α-L-Rhamnosidase from *A. aculeatus* was determined to be an inverting glycosidase, *e.g.* L-rhamnose liberated is in its β-form (*Pitson et al., 1998*).

A series of fungal extracellular α-L-rhamnosidases from our enzyme library, which were used for the enzymatic reactions namely – *Aspergillus aculeatus* CCF 108, *Fusarium oxysporum* CCF 906, two strains *Aspergillus terreus* CCF 3059 –differing in inducer, was tested. By continuous NMR-^{1}H scanning of the α-L-rhamnosidase cleavage of *p*-nitrophenyl α-L-rhamnopyranoside first appearance of β-L-rhamnose was detected (**Fig. 14** and **15**, here assays with *Aspergillus aculeatus* CCF 108 are shown) that clearly indicates invertion of α-(substrate) into the β-(product). β-L-Rhamnose immediately undergoes mutarotation into α-anomer, however kinetic plot of both components indicates the inverting type of the enzyme. Our results are in correspondence with finding of Pitson *et al. (1998*).

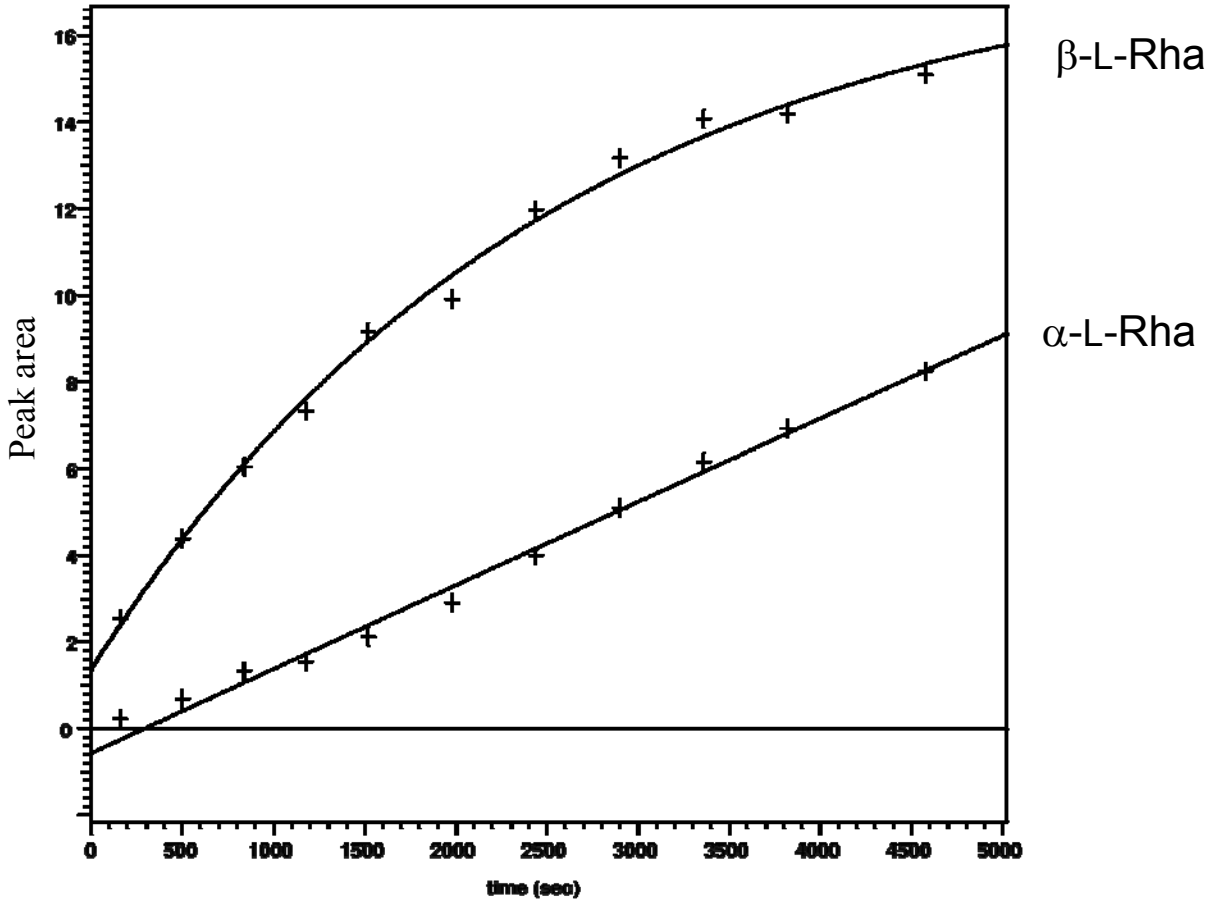


Fig. 14: Time profile of *p*-nitrophenyl α-L-rhamnopyranoside cleavage by α-L-rhamnosidase from *Aspergillus aculeatus* CCF 108 – appearance of β-L-rhamnopyranose in the first moments of reaction and the whole profile prove inverting character of the glycosidase.

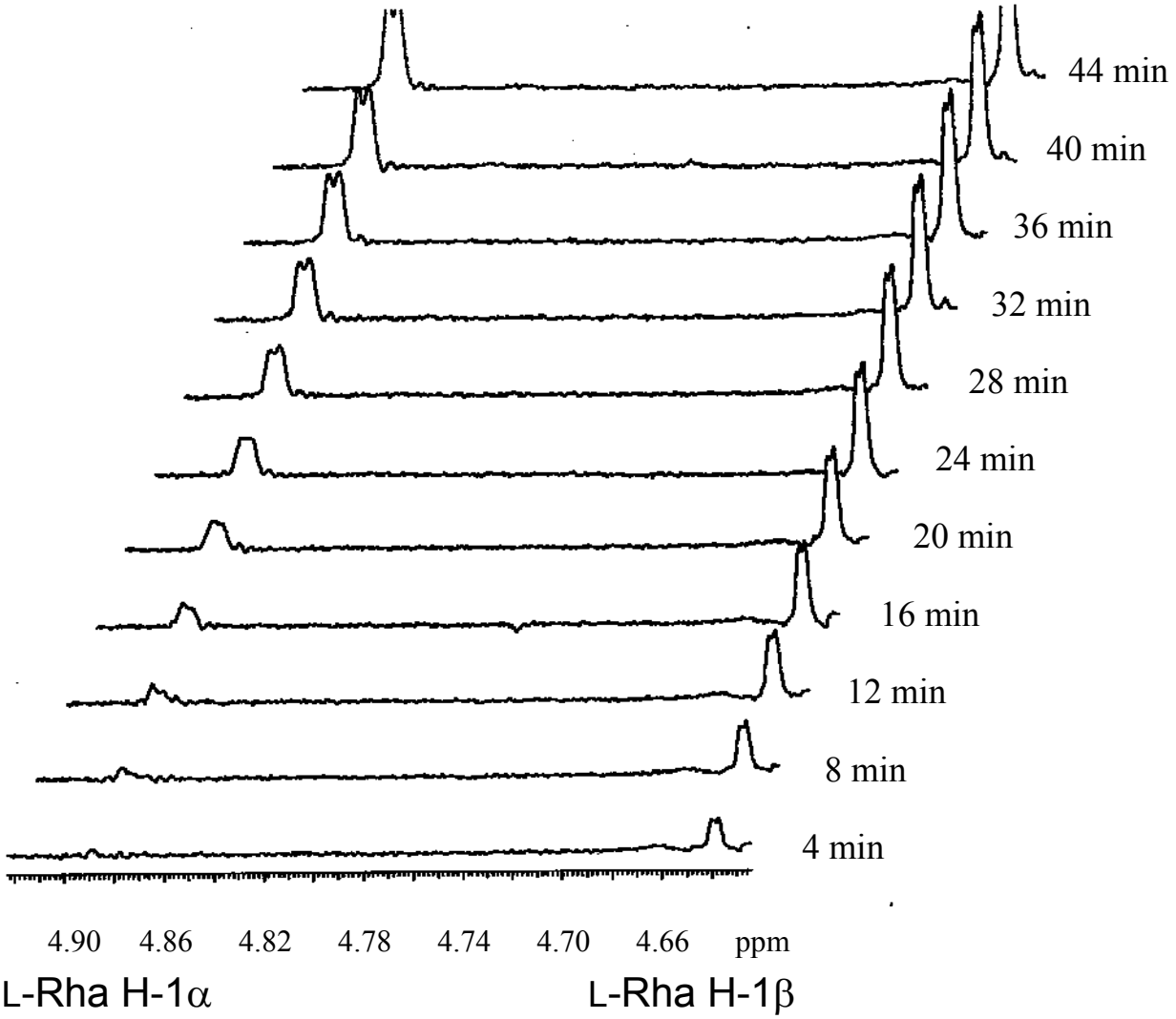


Fig. 15: Continuous NMR-^{1}H scanning of the α-L-rhamnosidase from *Aspergillus aculeatus* CCF 108 cleavage of *p*-nitrophenyl α-L-rhamnopyranoside; dublet resonating at 4.66 ppm belongs to anomeric proton of L-rha H-1β; dublet at 4.90 ppm belongs to that of L-rha H-1α

6. CONCLUSION

- Major target of this work was to create a large versatile library of well characterised glycosidases applicable for various tasks. Large collections of fungal strains and bacteria were created and kept and they were screened for enzyme production. Production of the most useful enzymes was scaled up.
- Novel methods for enzymatic synthesis of bioactive glycosides were developed using complementary approaches, *e.g.* biphasic systems, cosolvents, high salt concentrations. New reaction conditions were tested in enzymatic glycosylation to improve their effectiveness. Regioselectivity of enzymatic glycosylations was increased using partly protected sugars by chemoenzymatic acylation. Enzymatic modification of sugars and glycosidic derivatives was performed using not only glycosidases, but also lipases and proteases.

Reproducible quality of enzymes was achieved using defined culture media and cultivation conditions. Enzymes were isolated from the culture supernatant by precipitation by ammonium sulfate (typically by 20 - 80% saturation) at the maximum activity of respective glycosidase, typically after 4-14 days of growth. Precipitated enzymes were centrifuged and stored under saturated solution of $(NH_4)_2SO_4$ at 4 °C. The precipitates were very stable (under described conditions even for a few years) and they were of satisfactory purity suitable for most synthetic applications or for further purification. The enzymes were available in satisfactory and stable quality and quantity. Moreover, the production of the desired glycosidase activity could be stimulated by respective cultivation conditions, as *e.g*. by different inducers. Using easy production method a large number of the enzymes was produced and screened by the simple method, *e.g.* colorimetric micro-well procedure (using corresponding *p*-nitrophenyl substrate). We developed cheap screening method using micro-well plates enabling to test a large panel of the enzymes in the short time.

6. 1. β-*N*-Acetylhexosaminidases

Chitooligomers, which were prepared by controlled acid-catalysed chitin hydrolysis, were found to be the best inducers of β-*N*-acetylhexosaminidase production, even though GlcNAc induced β-*N*-acetylhexosaminidase production as well, although to a lower extent than the crude chitin hydrolysates, but with a higher specific activity. By use of the chitooligomers a large panel of β-*N*-acetylhexosaminidases (more than one hundred) from various fungal strains (*e.g.* genera *Aspergillus, Penicillium, Mucor, Fusarium, Talaromyces, Trichoderma, Mortierella, and Acremonium*) was developed. β-*N*-Acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 prepared using GlcNAc as the inducer was studied for its properties and for its gene sequence (*Plíhal et al., 2007*).

Fifty-five fungal strains having high β-*N*-acetylhexosaminidase activity were screened for their β-GalNAc-ase/β-GlcNAc-ase ratio. Generally, the β-GalNAc-ase/β-GlcNAc-ase ratio in most of β-*N*-acetylhexosaminidases was under 0.5. Higher activity of β-GalNAc-ase was found only in very few enzymes tested, the best one being from *Penicillium oxalicum* CCF 2430. A substantial increase of β-GalNAc-ase activity was observed between the 12^{th} and 13^{th} day of cultivation, when the β-GalNAc-ase/β-GlcNAc-ase ratio reached 2.3 - 2.8. After precipitation of the enzyme by ammonium sulfate (80 % saturation) from the cultivation medium the ratio decreased to 1.2 - 1.5. These differences may be caused by possible changes in refolding or in the hydration envelope of the protein. Different pH optima for β-GalNAc-ase (pH optimum 4.5) and β-GlcNAc-ase (pH optimum 5.0) were found. The highest β-GalNAc-ase/β-GlcNAc-ase ratio was at the pH value 4.5. These data were important for the design of the reaction conditions when using β-GalNAc-ase for the synthetic purposes. The influence of selected inorganic salts, *e.g.* $(NH_4)_2SO_4$, $MgSO_4$ and LiCl [in higher concentrations influencing water activity and/or enzyme hydration (*Rajnochová et al., 1997*)] on β-GlcNAc-ase and β-GalNAc-ase (and their ratio) activity of β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 was studied. $(NH_4)_2SO_4$, $MgSO_4$ and LiCl suppressed only β–GlcNAc-ase activity. The maximum ratio of 3.35 was observed in the presence of 20 % (w/v) of $MgSO_4$. We also studied the influence of aminosugars (GlcN·HCl, GlcNAc and GalNAc) on the β *N*-acetylhexosaminidase from *P. oxalicum* CCF 2430. All tested aminosugars decreased the β-GalNAc-ase/β-GlcNAc-ase ratio. These compounds are known feedback inhibitors of this enzyme. This was an important finding

because during enzymatic reactions (synthesis, enzyme determination) these sugars (GlcNAc or GalNAc) were liberated into the reaction medium, thus changing these parameters.

We used GlcNAc (**4**) as an acceptor for β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430. The reaction was performed in 20% (w) $MgSO_4$ to increase the β-GalNAc-ase/ β-GlcNAc-ase ratio. The reaction gave 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**28**, 26.5%) and 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (**29**, 19%). Even though these regioisomers had the same molecular weight, the compounds could be separated simply by gel chromatography on Toyopearl HW40F. This fact could be explained by different exclusion volumes (shape, hydration envelope) of both disaccharides. The results showed that this enzyme represented a β-*N*-acetylhexosaminidase with a very high β-GalNAc-ase/β-GlcNAc-ase ratio. To the best of our knowledge this enzyme has not been used yet for transglycosylation reactions. We used GalNAc (**5**) as an acceptor under the same reaction conditions. In this case only a single isomer 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-galactopyranose (**30**) was formed in an excellent yield of 87%.

The transglycosylation activity of forty-three extracellular fungal β-*N*-acetylhexosaminidases were screened for their ability to transfer the β-GlcNAc moiety onto *galacto*-type acceptors, *e.g.* D-galactose (**1**), 2-acetamido-2-deoxy-D-galactopyranose (**5**) and lactose (**40**). The β-*N*-acetylhexosaminidases from *Aspergillus flavofurcatus* CCF 3061, *A. tamarii* CCF 1665 and *A. oryzae* CCF 1066 gave the best yields and thus they were selected for semi-preparative procedures.

Under the catalysis of the β-*N*-acetylhexosaminidase from the *A. flavofurcatus* CCF 3061 with D-galactose and lactose as acceptors and *p*NP-β-D-GlcpNAc as a donor we synthesised a unique, non-reducing disaccharide β-D-Glc*p*NAc-(1→1)-β-D-Gal*p* (**34**) and trisaccharides β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→1)-β-D-Gal*p* (**35**), β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc (**38**) and β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→1)-β-D-Glc*p*NAc (**39**). Using D-galactose as the acceptor we obtained two main products disaccharide β-D-Glc*p*NAc-(1→1)-β-D-Gal*p* (**34**) and trisaccharide β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→1)-β-D-Gal*p* (**35**). Using lactose as the acceptor non-reducing lacto-oligosaccharides β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc (**38**) and β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→1)-β-D-Glc*p*NAc (**39**). Method of selective anomeric deacetylation was used to separate these

trisaccharides. This method leads to removal of acetyls only on anomeric hydroxyls and thus to the change of polarity of reducing saccharides and the polarity of non-reducing trisaccharides remains unchanged. Thus modified saccharides were separated by the silica gel chromatography, which enabled to separate them from the non-reducing ones. Thanks to this method separation of non-reducing trisaccharides from reducing one was achieved. The use of GalNAc (**5**) as an acceptor with the β-*N*-acetylhexosaminidases from *A. flavofurcatus* CCF 3061, *A. oryzae* CCF 1066 and *A. tamarii* CCF 1665 afforded only β-D-Glc*p*NAc-(1→6)-D-Gal*p*NAc (**47**).

Twenty extracellular β-*N*-acetylhexosaminidases of fungal origin were screened for the regioselectivity by the reverse hydrolysis with GlcNAc (**4**) as a substrate. Almost all enzymes exhibited a rather high selectivity for β(1→6) bond formation at a GlcNAc concentration of 0.3 - 1.4 M. The β-*N*-acetylhexosaminidase from *A. flavofurcatus* CCF 3061 displayed a selective preference for the β(1→4) product formation (**32**) from all β-*N*-acetylhexosaminidases tested. The three β-*N*-acetylhexosaminidases from *A. tamarii* CCF 1665, *P. funiculosum* CCF 2325 and *P. funiculosum* CCF 1994 produced relatively a high proportion of a rare chitobiose isomer β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**31**). We examined the β-*N*-acetylhexosaminidase from *P. funiculosum* CCF 1994 as the enzyme with the most pronounced formation of **31** regarding the following criteria: the influence of reaction time and GlcNAc concentration on the respective isomer (**31**, **32**, and **33**) production. In the course of the first three days of the reaction (from the total reaction time 8 days, when we obtained maximum yield of isomer **31**), exclusively β-D-Glc*p*NAc-(1→6)-D-Glc*p*NAc (**33**) was formed. During next five days another isomers β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc (**32**) (from the fourth day) and β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc (**8**) (from the fifth day) were produced. Doubling GlcNAc concentration (0.6 to 1.0 M), the formation of **31** was tripled (1.3 to 4.0 %). A further increase of the substrate concentration caused the decrease of the product formation, probably due to the enzyme inhibition by substrate. The influence of inorganic salts [$(NH_4)_2SO_4$ and $MgSO_4$] on the product formation was studied. They stimulated strongly the β(1→6) isomer production. The isomer **31** was formed only at low $(NH_4)_2SO_4$ concentration (0.1 - 0.2 M). The formation of product **32** was totally suppressed by the inorganic salt addition. The starting material GlcNAc (**4**) can easily be recovered from the reaction mixture by gel filtration, the separation of respective isomers was not trivial. The course of the GlcNAc condensation could not be monitored by HPLC due to the similar retention time values

of isomers (19.26 min for **31** and 19.76 min for **33**). However, the formation of β(1-3), β(1-4), β(1-6) linkages (depending on the enzyme source) could be monitored by TLC giving a clear resolution (R_f 0.58 for **31**, R_f 0.54 for **32** and R_f 0.67). The compounds could be separated by silica gel chromatography in their peracetylated forms.

6. 2. α-D-Galactosidases

A library of fungal α-D-galactosidases (*e.g.* from genera *Aspergillus, Penicillium, Talaromyces, Trichoderma, Micromucor, and Circinella*) was prepared and tested for its properties and application in synthetic reactions. α-D-Galactosidase from *Talaromyces flavus* CCF 2686 strain was, however, unique, not only because of an unusual inducer, but also for its properties. It was induced solely by 6-deoxyglucose. Raffinose (as well as melibiose) was absolutely ineffective in this case. This was a big drawback, because 6-deoxyglucose was very expensive and its synthesis was rather laborious.

Enzymatic glycosylations catalyzed by glycosidases are absolutely stereoselective but they often suffer from poor regioselectivity (*Weignerová et al., 1999a; Křen and Thiem, 1997*). This is especially problematic when using saccharide acceptors, which carry more reactive OH's groups. Generally, primary OH's groups are better acceptors than the secondary ones As a consequence; the selective protection of the primary hydroxyls (*e.g.* 6-OH in hexopyranoses) in the acceptor saccharide was performed by enzymatic regioselective esterification catalyzed by hydrolases (lipases or proteases) in organic solvents. Glycosidases can tolerate these modified substrates. Nevertheless, it has to be considered that any further substitution (acylation) on less soluble sugars, *i.e*. *galacto*-derivatives diminishes their solubility, thus making necessary the use of cosolvents. Enzymatic transglycosylation of partially acylated saccharide acceptors was performed.

One of the main problems related to the enzymatic acylation of free mono- and disaccharides is the low solubility of these compounds in the organic solvents that are more suitable to preserve the catalytic activity of lipases and proteases. The use of polar solvents, like pyridine or DMF, substantially decreases the number of enzymes that can be employed. The selective acylation of the *N*-acetylhexosamines, such as 2-acetamido-2-deoxy-D-glucopyranose (**4**), 2-acetamido-2-deoxy-D-galactopyranose (**5**) and 2-acetamido-2-deoxy-D-mannopyranose (**11**) was achieved. In the reaction of acylation of GlcNAc, the conversion

was satisfactory and the product, isolated in 62 % yield, was found to be 2-acetamido-6-*O*-acetyl-2-deoxy-D-glucopyranose (**17**) according to ^{1}H and ^{13}C-NMR (*see Appendix*). In the case of GalNAc, two monoesters, 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose (**18**), the expected main product and 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactofuranose (**19**), by-product, were isolated in 51 and 5 % yields, respectively, and characterised by NMR. The acetylation of ManNAc also gave two products, **20** and **21**, in 59 and 13 % yields, respectively. The main product **20** was the expected 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose, as unambiguously determined by NMR. The by-product **21** was 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-D-mannopyranose, also determined by NMR. The acylation of 2-acetamido-2-deoxy-D-mannopyranose **(11)** was repeated using trifluoroethyl butanoate as an activated ester and the corresponding butanoyl derivatives 2-acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranose **(22**) and 2-acetamido-3,6-di-*O*-butyryl-2-deoxy-D-mannopyranose **(23**) were recovered in good yields. The solubility of the mono-acetylated aminosugars 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose (**18**) and 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose **(20**) was higher in organic solvents that are more suitable to preserve lipases activity. In order to evaluate the regioselectivity of these enzymes towards the secondary free OH's at C-3 and C-4 of **18** and **20**, these compounds were dissolved in the mixture of acetone : pyridine (8 : 1 v/v) containing trifluoroethyl acetate. The samples of lipases were then added to the reaction mixtures. The formation of a defined new product was observed only with 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose (**20**) using *Candida antarctica* lipase B (Novozym 435). Compound containing three acetyls 2-acetamido-1,6-di-*O*-acetyl-2-deoxy-α-D-mannopyranose (**26**) was isolated in 18 % yield. Similarly, Novozym 435-catalysed the butanoylation of 2-acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranose (**22**)**,** which gave 2-acetamido-1,6-di-*O*-butanoyl-2-deoxy-α-D-mannopyranose (**27**) in 19 % yield. Finally, for further synthetic applications, we reacted *p*-nitrophenyl 2-acetamido-2-deoxy-*β*-D-galactopyranoside (**24**) with Novozym 435 in a mixture of pyridine-acetone-vinyl acetate, in this case an almost quantitative conversion to the 6-*O*-acetate **25** (166 mg, 0.4 mmol, 53.3 % isolated yield) was observed. *p*-Nitrophenyl 6-*O*-acetyl-α-D-galactopyranoside (**14**) and *p*-nitrophenyl 6-*O*-acetyl-β-D-galactopyranoside (**16**), were obtained in good yields (95.5 % for **14**, 73.8 % for **16**) in a regioselective enzymatic transesterification catalyzed by lipase PS in acetone-pyridine with vinyl acetate as an acyl donor. All obtained products were separated by flash column chromatography, except *p*-nitrophenyl 6-*O*-acetyl-β-D-galactopyranoside (**16**) and *p*-nitrophenyl 2-acetamido-6-*O*-

acetyl-2-deoxy-β-D-galactopyranoside (**25**), which were removed by the crystallisation from pyridine.

We have shown that a careful choice of the reaction conditions (solvent, enzyme, acylating agent) can allow the efficient regioselective acylation of poorly soluble carbohydrates like *N*-acetylhexosamines and their derivatives.

We investigated the performance of the α-galactosidase from *Talaromyces flavus* CCF 2686 when *p*-nitrophenyl 6-*O*-acetyl-α-D-galactopyranoside (**14**) and *p*-nitrophenyl 6-*O*-acetyl-β-D-galactopyranoside (**16**) were used in transglycosylation reactions, as we have found that this enzyme accepts a large variety of acceptors and it was found to be particularly suitable for synthetic applications. Solubilities of **14** and **16** were quite low in the aqueous medium and, as a result, we had to use a significant amount of water-miscible organic solvents to reach a sufficient concentration (acetone, acetonitrile, *N*,*N*-dimethylformamide, dimethylsulfoxide, dioxane, 2-methoxyethanol, pyridine, 2-methylpropan-2-ol and tetrahydrofuran). 2-methylpropan-2-ol was the cosolvent of choice, as it was affecting both the activity and the stability to the smallest extent. Despite its alcoholic moiety, it is generally considered to be unable to act as an acceptor in enzymatic glycosylations due to a severe steric hindering (no reports on its enzymatic α-galactosylation are known).

A preparative scale enzymatic reaction was run using *p*-nitrophenyl α-D-galactopyranoside (**13**) as a sugar donor and **14** as a sugar acceptor in the presence of 25.5% v/v 2-methylpropan-2-ol. No similar transformation could be performed with the β-galactosyl derivative **16** as an acceptor because its solubility was too low (2.0-2.7 mg/ml, the solubility of α-galactosyl derivate **14** in transglycosylation reaction was 36.4 mg/ml) even in the presence of a significant amount of 2-methylpropan-2-ol or of other cosolvents. Usual work-up of this reaction and purification by gel chromatography followed by flash chromatography allowed the isolation of the expected *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside (**41**). The presence of more polar compounds was also observed and by a HPLC comparison with standards identified them as products of autocondensation of *p*-nitrophenyl α-D-galactopyranoside **13**: *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-α-D-galactopyranoside (**44**) and *p*-nitrophenyl α-D-galactopyranosyl-(1→6)-α-D-galactopyranoside (**43**), which cannot be avoided. Another less polar product was isolated in a 7.3 % yield and, to our surprise, it was identified as 2-methylpropyl α-D-galactopyranoside (**42**). This result was quite unexpected due to the fact that 2-methylpropan-2-ol is an alcohol with sterically hindered OH group quite inert to

enzymes (*van Rantwijk et al., 1999*) (for instance it can be used as a solvent in transesterification reactions catalyzed by hydrolases in organic media) and acting as a quite weak nucleophile in chemical reactions. This finding forced us to use acetone, the second best cosolvent, in order to avoid the formation of this by-product (**42**). Accordingly, a transglycosylation reaction was performed in the presence of 32.5 % v/v acetone and the disaccharide *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside (**41**) was obtained in a 5 % yield. The difference of polarity between the single mono-acylated disaccharide **41** and the mixture of the disaccharides (**43** and **44**) obtained by autocondensation of **13** allows its easy isolation from the reaction mixture. In conclusion, we have shown the possibility of using selectively acylated glycosyl acceptors for the synthesis of *p*-nitrophenyl galabioses.

6. 3. α-L-Rhamnosidases

Sixteen different fungal strains were screened for their ability to produce α-L-rhamnosidases under different cultivation conditions in the presence of potential enzyme inducers. Several inducers: L-rhamnose (**2**) and L-rhamnose containing flavonoid glycosides rutin (**7**), naringin (**9**), and hesperidin (**10**) were tested. Despite the fact that fungi usually show low constitutive levels of glycosidase production (*Huňková et al., 1999*), none of the strains tested was able to produce α-L-rhamnosidases in the absence of inducers, while all the fungi used in this study produced this enzymatic activity in the presence of the respective inducers, showing that new fungal species and different induction conditions could be successfully combined for the rapid obtainment of wide glycosidase libraries. Moreover, some of the α-L-rhamnosidase positive strains (*Acremonium persicinum*, *Circinella muscae*, *Emericella nidulans*, *Eurotium amstelodami*, *Mortierella alpina*, *Rhizopus arrhizus*, *Talaromyces flavus*, and *Trichoderma harzianum*) belong to species or genus where the occurrence of α-L-rhamnosidases has never been detected. α-L-Rhamnosidases from the majority of species tested were induced by more inducers, even though one of them was always superior. Enzymes with good activity (more than 30) were harvested by ammonium sulfate precipitation and tested for their properties, *e.g*. substrate specificity and stability towards organic cosolvents, due to the low substrate solubility in water. To exploit this enzymatic library for synthetic applications, the presence of contaminating

α-L-arabinosidases and β-D-glucosidases was investigated. The latter activities were found in several preparations, while α-L-arabinosidase content was generally quite low.

The use of different microbial strains and different inducers affected not only the production levels of the desired enzymes, but also the "quality" of the enzymatic preparations in terms of substrate specificity and stability in organic cosolvents. Furthermore, in some cases the preparations obtained from the same strain using different inducers showed a different stability in the presence of cosolvents (*e.g.* those from the *E. nidulans* and *T. flavus* strains). In the presence of organic cosolvents most of the preparations maintained their catalytic power towards only some of the substrates. These observations suggest that the apparently broad substrate specificity of some preparations, for example, the ones from the *A. aculeatus* strains, might be likely due to the coexistence in the same sample of different enzymes or of different isoforms [isoenzymes whose occurrence in fungi has been described (*Luonteri et al., 1998; Mileto et al., 1998*)] of the same enzyme [isoenzymes whose occurrence in fungi has been described (*Luonteri et al., 1998; Mileto et al., 1998*)] with different substrate specificity and different stability towards organic cosolvents.

The saponin derivative desglucoruscin (**3**) was selectively derhamnosylated using α-L-rhamnosidase from *A. niger* CCIM K2 (inducer: L-rhamnose). Due to the presence of hydrophobic and bulky aglycon, desglucoruscin has a very low water-solubility and, therefore, the use of organic solvents was indispensable. From a few organic cosolvents only THF (20% v/v) was able to solubilize desglucoruscin to a sufficient extent to perform the hydrolysis reaction. Moreover, using this cosolvent, detection of the derhamnosylation product desrhamnodesglucoruscin (**46**) by TLC was observed only with the *A. niger* CCIM K2 preparation (inducer: L-rhamnose). As this preparation also possessed the highest activity in the AcOEt/buffer biphasic system (compared to the other enzymes that were able to hydrolyse desglucoruscin under these reaction conditions), this rhamnosidase was selected for a preparative reaction. As expected, no significant α-L-arabinosidase contamination was observed and the formation of a single product was monitored. The selective derhamnosylation of desglucoruscin reached about 70 % conversion after 4 days and structural analysis of the isolated product confirmed its identity with the desired desrhamno derivative.

A series of fungal extracellular α-L-rhamnosidases from our enzyme library, which were used for the enzymatic reactions namely – *Aspergillus aculeatus* CCF 108, *Fusarium oxysporum* CCF 906, two strains *Aspergillus terreus* CCF 3059 –differing in inducer, was

tested for the type of the enzyme (inverting/retaining). By continuous NMR-^{1}H scanning of the α-L-rhamnosidase cleavage of *p*-nitrophenyl α-L-rhamnopyranoside first appearance of β-L-rhamnose was detected, that clearly indicates invertion of α(substrate) into the β-(product). β-L-Rhamnose immediately undergoes mutarotation into α-anomer, however kinetic plot of both components indicates the inverting type of the enzyme. Our results are in correspondence with finding of Pitson *et al. (1998*).

6. 4. Screening for α/β-*N*-Acetylmannosaminidase Activity

Due to the occurrence of saccharidic chains with β-ManNAc moieties in biological structures it was supposed that there should exist an enzyme able to hydrolyse these linkages. However**,** β-*N*-acetylmannosaminidase has not been described so far. For screening of such enzyme, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-mannopyranoside as a substrate was employed. This substrate is not available commercially, therefore it was synthesized in our laboratory (*Krist et al., 2003*) by a multi-step synthesis in a sufficient amount for the screening of about 30 species of bacteria (*e.g. Acinetobacter sp.*, *Pseudomonas sp., Rhodococcus sp., Bacillus sp., and Corynebacterium sp*.) and for the screening for the enzyme in snail gut juice, which is known to be rich in various hydrolytic enzymes. *p*-Nitrophenyl 2-acetamido-2-deoxy-α-D-mannopyranoside was synthesized as well (*Krist et al., 2003*), therefore, we performed screening for both α- and β-*N*-acetylmannosaminidase activities. For extensive screening bacteria were cultivated for various time periods (24, 48, 72 and 96 h). Induction of tentative β-*N*-acetylmannosaminidase by ManNAc – analogously as in the case of β-*N*-acetylhexosaminidase – was attempted. However, no induction effect or enhancement of its production was observed. Activity of this enzyme was screened both in the medium (extracellular enzyme) and in disintegrated cells (intracellular enzyme) using protease inhibitors (protease inhibitor cocktail, Sigma). In this case we used method using multi-well plates. In the cultivation media neither α-*N*-acetylmannosaminidase nor β-*N*-acetylmannosaminidase activity was found. However, very low intracellular activity of both, α-*N*-acetylmannosaminidase and β-*N*-acetylmannosaminidase was found in preparations from a few bacteria species. The highest activity was found in the cells of *Rhodococcus equi* A5. The highest

α-*N*-acetylmannosaminidase activity (0.006 U/ml) and β-*N*-acetylmannosaminidase activity (0.008 U/ml) was found in the 72h-old culture, even though both activities already appeared after 48 h of growth and they remained detectable till fourth day of growth. Detection of these activities was repeated, however, the production of this enzyme was not increased by any means (*e.g.* ManNAc induction).

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8. ABBREVIATIONS

Ac	acetyl
AcOEt	ethylacetate
Ac_2O	acetanhydride
α-L-Rha	α-L-rhamnosidase
CAZy	The Carbohydrate-Active Enzymes database
CCF	Culture Collection of Fungi
CCIM	Culture Collection of the Institute of Microbiology
CCM	Czech Collection of Microorganisms
COSY	Correlation Spectroscopy
N,*N'*-diacetylchitobiose	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose
N,*N'*,*N''*-triacetylchitotriose	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleid acid
DTT	dithiothreitol
EDTA	ethylendiamintetraacetic acid
ESI-MS	ElectroSpray-Ionization Mass Spectrometry
Et	ethyl
α-L-Fuc	α-L-fucosidase
α-D-Gal	α-D-galactosidase
Gal	galactose
α-D-Gal*p*(1→3)-6-*O*-Ac-α-D-Gal*p*-*O*-*p*-NP	*p*-nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside
α-Gal-(1→3)-β-Gal	α-galactopyranosyl-(1→3)-β-galactopyranose
α-D-Gal*p*-(1→1)-α-D-Glc*p*	α-D-galactopyranosyl-(1→1)-α-D-glucopyranoside
β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→1)-β-D-Glc*p*NAc	β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→1)-2-acetamido-2-deoxy-β-D-glucopyranoside
β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc	β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-2-acetamido-2-deoxy-β-D-glucopyranoside

GalNAc	2-acetamido-2-deoxy-D-galactopyranose
β-GalNAc-ase activity β-GalNAc-ase/β-GlcNAc-ase ratio	β-*N*-acetylgalactosaminidase activity ratio of β-*N*-acetylgalactosaminidase and β-*N*-acetylglucosaminidase activities
Glc	glucose
Glc-β-(1→4)-GlcNAc	β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose
GlcNAc	2-acetamido-2-deoxy-D-glucopyranose
$(GlcNAc)_{n=2-6}$	$[(2\text{-acetamido-2-deoxy-β-D-glucopyranosyl})]_{n=2-6}$
β-GlcNAc-ase activity	β-*N*-acetylglucosaminidase activity
β-D-Glc*p*NAc-(1→1)-β-D-Gal*p*	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→1)-β-D-galactopyranoside
β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucopyranose
β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose
β-D-Glc*p*NAc-(1→6)-D-Glc*p*NAc	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose
β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→1)-β-D-Gal*p*	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→1)-β-D-galactopyranoside
β-D-Glc*p*NAc-(1→1)-β-D-Man*p*	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→1)-D-mannopyranoside
GlcNAc-β-(1→4)-ManNAc	2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-mannopyranose
GC	Gass Chromatography
β-HexNAc	β-*N*-acetylhexosaminidase
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HPLC	High Performance Liquid Chromatography
IUB-MB	International Union of Biochemistry and Molecular Biology
K_i	inhibition constant
Le^x antigen	$Lewis^x$ antigen
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry

Man	mannose
α-Man	α-mannosidase
ManNAc	2-acetamido-2-deoxy-D-mannopyranose
ManNAc-β-(1→4)-GlcNAc	2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→4)-D-glucopyranosa
ManNAc-β-(1→4)-Gly	2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→4)-D-glycopyranosa
ManNAc-β-(1→4)-L-Rha	2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→4)-L-rhamnopyranosa
ManNAc-β-(1→2)-L-Rha	2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→2)-L-rhamnopyranosa
Me	methyl
MPA	Meat-Pepton Agar
NAD^+	nicotinamide adenine dinucleotide
NANA or Neu5Ac	*N*-acetylneuraminic acid
NK	Natural Killer cell
NKR-P1	Natural Killer Receptor Protein-1
NMR	Nuclear Magnetic Resonance
*p*NP α-Gal	*p*-nitrophenyl α-D-galactopyranoside
*p*NP β-Gal	*p*-nitrophenyl β-D-galactopyranoside
*p*NP β-D-GalNAc	*p*-nitrophenyl.2-acetamido-2-deoxy-β-D-galactopyranoside
$OD_{610\ nm}$	Optical Density (value of absorbance of cell suspension measured spectrophotometricaly at 610 nm)
Py	pyridine
Rha	L-rhamnose
α-L-Rham	α-L-rhamnosidase
R_f	retardation factor
TBDMS	*tert*-butyldimethylsilyl
TCEB	trichloroethyl butyrate
TFEA	trifluoroethyl acetate
THF	tetrahydrofuran
TLC	Thin-Layer Chromatography
TOSCY	TOtal correlation SpectroscopY
TRIS	tris(hydroxymethyl)aminomethan
U	unit of enzyme activity (μmol min^{-1})

APPENDIX

p-Nitrophenyl 6-O-*acetyl-α-D-galactopyranoside* (**14**): m.p. 164-165°C. ^{1}H NMR (D_2O, 30 °C): 1.632 (3 H, s, Ac), 3.832 (1 H, dd, J_1 = 3.6 Hz, J_2 = 9.8 Hz, *H-2*), 3.872 (1 H, dd, J_1 = 0.9 Hz, J_2 = 3.3 Hz, H-4), 3.896 (1 H, dd, J_1 = 3.3 Hz, J_2 = 9.8 Hz, H-3), 3.938 (1 H, dt, J_1 = 0.9 Hz, J_2 = 6.3 Hz, H-5), 4.027 (2 H, m, H-6), 5.661 (1 H, d, J = 3.6 Hz, H-1), 7.073 and 8.070 (4 H, AA'BB', arom.). ^{13}C NMR (D_2O, 30 °C): 20.19 (q, Ac), 63.95 (t, C-6), 67.85 (d, C-2), 69.04 (d, C-3 or C-4), 69.38 (d, C-3 or C-4), 69.80 (d, C-5), 96.61 (d, C-1), 173.86 (s, CO), 117.15 (d), 126.15 (d), 142.58 (s), 161.30 (s) (arom.); $[\alpha]_D^{25}$ = 206.4 (c = 0.2, H_2O).

p-Nitrophenyl 6-O-*acetyl-β-D-galactopyranoside* (**16**): m.p. 216-217 °C. ^{1}H NMR (CD_3OD, 30 °C): 2.087 (3 H, s, Ac), 3.642 (1 H, dd, J_1 = 3.4 Hz, J_2 = 9.7 Hz, *H-3*), 3.861 (1 H, dd, J_1 = 7.7 Hz, J_2 = 9.7 Hz, H-2), 3.922 (1 H, dd, J_1 = 1.2 Hz, J_2 = 3.4 Hz, H-4), 4.014 (1 H, ddd, J_1 = 1.2 Hz, J_2 = 4.6 Hz, J_3 = 7.8 Hz, H-5), 4.266 (1 H, dd, J_1 = 4.6 Hz, J_2 = 11.5 Hz, H-6a), 4.351 (1 H, dd, J_1 = 7.8 Hz, J_2 = 11.5 Hz, H-6b), 5.054 (1 H, d, J = 7.7 Hz, H-1), 7.251 and 8.242 (4 H, AA'BB', arom.). ^{13}C NMR (CD_3OD, 30 °C): 20.9 (q, Ac), 64.9 (t, C-6), 70.3 (d, C-4), 72.1 (d, C-2), 74.7 (d, C-5), 74.8 (d, C-3), 102.2 (d, C-1), 117.9 (d), 126.8 (d), 144.3 (s), 164.1 (s) (arom.); $[\alpha]_D^{25}$ = 35.2 (c = 1.0, MeOH).

2-Acetamido-6-O-*acetyl-2-deoxy-D-glucopyranose* (**17**): m.p. 179-181°C. ^{1}H NMR (DMSO+D_2O, 30 °C): 1.85 (3 H, s, NAc), 2.03 (3H, s, Ac), 3.12 (1 H, t, J = 8 Hz), 3.55 (2 H, m), 3.80 (1 H, dd, J_1 = 7 Hz, J_2 = 2 Hz, H-5), 4.05 (1 H, dd, J_1 = 11 Hz, J_2 = 7 Hz, H-6b), 4.25 (1 H, dd, J_1 = 11 Hz, J_2 = 2 Hz, H-6a), 4.90 (1 H, d, J = 3 Hz, H-1), 7.68 (1 H, d, J = 10 Hz, NH). ^{13}C NMR (DMSO, 30 °C): 20.7 (Ac), 22.5 (NAc), 54.1 (C-2), 63.9 (C-6), 69.2 (C-5), 70.1 (C-3), 70.9 (C-4), 90.5 (C-1), 169.9 (NCO), 170.6 (CO).

*2-Acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose* (**18**): m.p. 101-102 °C. ^{1}H NMR (D_2O, 30 °C) - α-pyranose : β-pyranose : α-furanose : β-furanose = 50:41:2:7. *α-pyranose:* 1.833 (3 H, s, NAc), 1.904 (3 H, s, OAc), 3.713 (1 H, dd, $J_{1,2}$=3.2 Hz, $J_{2,3}$=11.1 Hz, H-3), 3.817 (1 H, dd, $J_{2,3}$=3.2 Hz, $J_{3,4}$=1.0 Hz, H-4), 3.925 (1 H, dd, $J_{1,2}$=3.7 Hz, $J_{2,3}$=11.1 Hz, H-2), 4.017 (1 H, dd, $J_{5,6a}$=9.1 Hz, $J_{6a,6b}$=12.9 Hz, H-6a), 4.068 (1 H, m, H-5), 4.096 (1 H, dd, $J_{5,6b}$=4.8 Hz, $J_{6a,6b}$=12.2 Hz, H-6b), 5.017 (1 H, d, $J_{1,2}$=3.7 Hz, H-1); *β-pyranose:* 1.831 (3 H, s, NAc), 1.908 (3 H, s, OAc), 3.514 (1 H, dd, $J_{2,3}$=10.8 Hz, $J_{2,3}$=3.3 Hz, H-3), 3.666 (1 H, dd, $J_{1,2}$=8.4 Hz, $J_{2,3}$=10.8 Hz, H-2), 3.757 (1 H, dd, $J_{3,4}$=3.3 Hz, $J_{4,5}$=1.3 Hz, H-4), 3.684 (1 H, m, H-5), 4.435 (1 H, d, $J_{1,2}$=8.4 Hz, H-1); *α-furanose*: 5.946 (1 H, d, $J_{1,2}$=5.6 Hz, H-1); *β-furanose*: 5.875 d (1 H, $J_{1,2}$=4.2 Hz, H-1). ^{13}C NMR (D2O, 30 °C): *α-pyranose:* 20.4 (OAc), 22.1 (NAc), 50.3 (C-2), 64.2 (C-6), 67.4 (C-3), 68.2 (C-5), 68.7 (C-4), 91.3 (C-1), 174.1 (6-C=O), 174.9 (2-C=O); *β-pyranose:* 20.4 (OAc), 22.1 (NAc), 53.8 (C-2), 64.2 (C-6), 67.9 (C-4), 71.0 (C-3), 72.4 (C-5), 96.7 (C-1), 174.1 (6-CO), 174.9 (2-CO).

*2-Acetamido-6-*O*-acetyl-2-deoxy-D-galactofuranose* (**19**): m.p. 95-96 °C. ^{1}H NMR (D_2O, 30 °C) - α-furanose : β-furanose : α-pyranose : β-pyranose = 71:6:13:10; *α-furanose*: 1.912 (3 H, s, Ac), 3.768 (1 H, m, H-5), 3.786 (1 H, dd, $J_{3,4}$=7.4 Hz, $J_{4,5}$=4.7 Hz, H-4), 3.946 (1 H, dd, $J_{5,6a}$=6.3 Hz, $J_{6a,6b}$=11.7 Hz, H-6a), 4.036 (1 H, dd, $J_{5,6b}$=3.8 Hz, $J_{6a,6b}$=11.7 Hz, H-6b), 4.108 (1 H, dd, $J_{2,3}$=9.6 Hz, $J_{3,4}$=7.4 Hz, H-3), 4.327 (1 H, dd, $J_{1,2}$=4.7 Hz, $J_{2,3}$=9.6 Hz, H-2), 5.953 (1 H, d, $J_{1,2}$=4.7 Hz, H-1); *β-furanose*: 5.886 (1 H, d, $J_{1,2}$=4.6 Hz, H-1);. *α-pyranose:* 5.017 (1 H, d, $J_{1,2}$=3.7 Hz, H-1); *β-pyranose:* 4.434 (1 H, d, $J_{1,2}$=8.4 Hz, H-1). ^{13}C NMR (D_2O, 30 °C): 20.3 (OAc), 21.7 (NAc), 57.4 (C-2), 65.3 (C-6), 69.0 (C-5), 71.6 (C-3), 82.5 (C-4), 94.3 (C-1).

*2-Acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose* (**20**); colourless oil: ^{1}H NMR (CD_3OD, 30 °C) - α-furanose : β-furanose : α-pyranose : β-pyranose = 3.1 : 2.9 : 55.3 : 11.1; *α-pyranose:* 2.029 (3 H, s, 2-Ac), 2.078 (3 H, s, 6-Ac), 3.542 (1 H, dd, $J_{3,4}$ = 9.6 Hz, $J_{4,5}$=9.9 Hz, H-4), 3.998 (1 H, ddd, $J_{4,5}$ = 9.9 Hz, $J_{5,6a}$ = 7.0 Hz, $J_{5,6b}$ = 2.2 Hz, H-5), 4.022 (1 H, dd, $J_{2,3}$ = 4.8 Hz, $J_{3,4}$ = 9.6 Hz, H-3), 4.263 (1 H, dd, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 4.8 Hz, H-2), 4.275 (1 H, dd, $J_{5,6a}$ = 7.0 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.388 (1 H, dd, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 5.026 (1 H, d, $J_{1,2}$ = 1.7 Hz, H-1); *β-pyranose:* 2.072 (3 H, s, 2-Ac), 2.078 (3 H, s, 6-Ac), 3.411 (1 H, dd, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 9.6 Hz, H-4), 3.480 (1 H, ddd, $J_{4,5}$ = 9.6 Hz, $J_{5,6a}$ = 2.2 Hz, $J_{5,6b}$ = 7.0 Hz, H-5), 3.674 (1 H, dd, $J_{2,3}$ = 4.4 Hz, $J_{3,4}$ = 9.2 Hz, H-3), 4.417 (1 H, dd, $J_{5,6a}$ = 2.2 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.279 (1 H, dd, $J_{5,6b}$ = 7.0 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 4.429 (1 H, dd, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 4.4 Hz, H-2), 4.873 (1 H, d, $J_{1,2}$ = 1.7 Hz, H-1); α-furanose: 5.264 (1 H, d, J = 5.7 Hz, H-1); *β-furanose*: 5.243 (1 H, d, J = 5.0 Hz, H-1). ^{13}C NMR (CD_3OD, 30 °C) - *α-pyranose*: 21.04 (6-Ac), 22.87 (2-Ac), 55.65 (C-2), 65.79 (C-6), 69.58 (C-4), 70.56 (C-3), 71.70 (C-5), 94.99 (C-1), 173.26 (6-CO), 174.44 (2-CO); *β-pyranose*: 21.01 (6-Ac), 23.07 (2-Ac), 56.05 (C-2), 65.70 (C-6), 69.38 (C-4), 74.55 (C-3), 76.40 (C-5), 95.37 (C-1), 173.26 (6-CO), 175.98 (2-CO).

*2-Acetamido-3,6-di-*O*-acetyl-2-deoxy-D-mannopyranose* (**21**); colourless oil: ^{1}H-NMR (DMSO, 30 °C): 1.90, 1.95 and 2.05 (3 H each, s, 3 x Ac), 3.62 (1 H, t, J = 9.8 Hz, H-4), 3.92 (1 H, br t, J = 8.5 Hz, H-5), 4.15 (1 H, dd, J_1 = 12.6 Hz, J_2 = 7.8 Hz, H-6a), 4.23 (1 H, br d, J = 4.64 Hz, H-2), 4.35 (1 H, br d, J = 12.6 Hz, H- 6b), 4.84 (1 H, br s, H-1α), 5.02 (1 H, dd, J_1 = 4.6 Hz, J_2 = 9.8 Hz, H-3). ^{13}C-NMR (DMSO, 30 °C) δ : 20.6, 20.7 and 22.3 (3 x Ac), 50.4 (C-2), 63.9 (C-6), 64.2 (C-4), 69.9 (C-5), 71.6 (C-3), 92.4 (C-1), 170.6 and 171.2 (2 x CO).

*2-Acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranose* (**22**); colourless oil. ^{1}H NMR (CD_3OD, 30 °C) - α-furanose : β-furanose : α-pyranose : β-pyranose =5 : 4 : 77 : 14; *α-pyranose:*: 0.977 (3H, t, *J*=7.4 Hz, 6-$COCH_2CH_2$ CH_3), 1.672 (2H, m, 6-$COCH_2CH_2CH_3$), 2.028 (3H, s, 2-Ac), 2.351 (2H, t, *J*=7.4 Hz, 6-$COCH_2CH_3CH_3$), 3.540 (1H, dd, $J_{3,4}$=9.6 Hz, $J_{4,5}$=9.9 Hz, H-4), 3.996 (1H, ddd, $J_{5,6b}$= 2.2 Hz, $J_{5,6a}$=7.0 Hz, $J_{4,5}$=9.9 Hz, H-5), 4.025 (1H, dd, $J_{2,3}$=4.7 Hz, $J_{3,4}$=9.6 Hz, H-3), 4.271 (1H, dd, $J_{5,6a}$=7.0 Hz, $J_{6a,6b}$=11.7 Hz, H-6a), 4.413 (1H, dd, $J_{5,6b}$=2.2 Hz, $J_{6a,6b}$=11.7 Hz, H-6b), 4.255 (1H, dd, $J_{1,2}$=1.7 Hz, $J_{2,3}$=4.7 Hz, H-2), 5.031 (1H, d, $J_{1,2}$=1.7 Hz, H-1); *β-pyranose:* 0.981 (3H, t, *J*=7.4 Hz, 6-$COCH_2CH_2CH_3$), 1.677 (2H, m, 6-$COCH_2CH_2CH_3$), 2.072 (3H, s, 2-Ac), 2.351 (2H, t, *J*=7.4 Hz, 6-$COCH_2CH_2CH_3$), 3.483 (1H, ddd, $J_{4,5}$=9.9 Hz, $J_{5,6a}$=7.2 Hz, $J_{5,6b}$=2.2 Hz, H-5), 3.409 (1H, dd, $J_{3,4}$=9.2 Hz, $J_{4,5}$=9.9 Hz, H-4), 3.677 (1H, dd, $J_{2,3}$=4.4 Hz, $J_{3,4}$=9.2 Hz, H-3), 4.281 (1H, dd, $J_{5,6a}$=7.2 Hz, $J_{6a,6b}$=11.8 Hz, H-6a), 4.425 (1H, dd, $J_{1,2}$=1.7 Hz, $J_{2,3}$=4.4 Hz, H-2), 4.433 (1H, dd, $J_{5,6b}$=2.2 Hz, $J_{6a,6b}$=11.8 Hz, H-6b), 4.874 (1H, d, $J_{1,2}$=1.7 Hz, H-1); *α-furanose*: 5.266 (1 H, d, $J_{1,2}$=5.7 Hz, H-1); *β-furanose:* 5.244 (1 H, d, $J_{1,2}$=5.0 Hz, H-1). ^{13}C NMR (CD_3OD, 30 °C): *α-pyranose*:14.22 (6-$COCH_2CH_2$ CH_3), 19.70 (6-$COCH_2CH_2CH_3$), 22.90 (2-Ac), 37.18 (6-$COCH_2$ CH_2CH_3), 55.67 (C-2), 65.58 (C-6), 69.60 (C-4), 70.54 (C-3), 71.75 (C-5), 94.90 (C-1), 174.40 (2-CO), 175.74 (6-CO); *β-pyranose:* 14.22 (6-$COCH_2CH_2CH_3$), 19.70 (6-$COCH_2CH_2CH_3$), 23.10 (2-Ac), 37.15 (6-$COCH_2CH_2CH_3$), 56.37 (C-2), 65.53 (C-6), 69.41 (C-4), 74.55 (C-3), 76.44 (C-5), 95.31 (C-1), 175.58 (2-CO), 175.64 (6-CO); *α-furanose*: 102.10 (C-1); *β-furanose*: 97.29 (C-1).

2-Acetamido-3,6-di-O-*butyryl-2-deoxy-D-mannopyranose* (**23**); colourless oil: ^{1}H NMR (CD_3OD, 30 °C) - α-pyranose : β-pyranose = 83 : 17; *α-pyranose* 0.964 (3H, t, *J*=7.4 Hz, 3-$COCH_2CH_2CH_3$), 0.983 (3H, t, *J*=7.4 Hz, 6-$COCH_2CH_2CH_3$), 1.602 – 1.725 (4H, m, 3-$COCH_2CH_2CH_3$, 6-$COCH_2CH_2CH_3$), 2.003 (3H, s, 2-Ac), 2.305 (2H, t, *J*=7.3 Hz, 3-$COCH_2CH_2CH_3$), 2.362 (2H, t, *J*=7.3 Hz, 6-$COCH_2CH_2CH_3$), 3.718 (1H, dd, $J_{3,4}$=10.0 Hz, $J_{4,5}$=10.0 Hz, H-4), 4.121 (1H, ddd, $J_{4,5}$=10.0 Hz, $J_{5,6a}$=7.0 Hz, $J_{5,6b}$=2.3 Hz, H-5), 4.319 (1H, dd, $J_{5,6a}$=7.0 Hz, $J_{6a,6b}$=11.7 Hz, H-6a), 4.417 (1H, dd, $J_{5,6b}$=2.3 Hz, $J_{6a,6b}$=11.7 Hz, H-6b), 4.480 (1H, dd, $J_{1,2}$=1.7 Hz, $J_{2,3}$=4.6 Hz, H-2), 4.994 (1H, d, $J_{1,2}$=1.7 Hz, H-1), 5.224 (1H, dd, $J_{2,3}$=4.6 Hz, $J_{3,4}$=10.0 Hz, H-3); *β-pyranose*: 0.969 (3H, t, *J*=7.4 Hz, 3-$COCH_2CH_2CH_3$), 0.983 (3H, t, *J*=7.4 Hz, 6-$COCH_2CH_2CH_3$), 1.602 – 1.725 (4H, m, 3-$COCH_2CH_2CH_3$, 6-$COCH_2CH_2CH_3$), 2.038 (3H, s, 2-Ac), 2.298 (2H, t, *J*=7.3 Hz, 3-$COCH_2CH_2CH_3$), 2.362 (2H, t, *J*=7.3 Hz, 6-$COCH_2CH_2CH_3$), 3.589 (1H, ddd, $J_{4,5}$=9.4 Hz, $J_{5,6a}$=7.0 Hz, $J_{5,6b}$=2.1 Hz, H-5), 3.638 (1H, dd, $J_{3,4}$=9.5 Hz, $J_{4,5}$=9.4 Hz, H-4), 4.319 (1H, dd, $J_{5,6a}$= 7.0 Hz, $J_{6a,6b}$=11.7 Hz, H-6a), 4.438 (1H, dd, $J_{5,6b}$=2.1 Hz, $J_{6a,6b}$=11.7 Hz, H-6b), 4.581 (1H, dd, $J_{1,2}$=1.7 Hz, $J_{2,3}$=4.3 Hz, H-2), 4.857 (1H, dd, $J_{2,3}$= 4.3 Hz, $J_{3,4}$=9.5 Hz, H-3), 4.973 (1H, d, $J_{1,2}$=1.7 Hz, H-1). ^{13}C NMR (CD_3OD, 30 °C) - *α-pyranose*: 14.21 (3-$COCH_2CH_2$ CH_3, 6-$COCH_2CH_2$ CH_3), 19.52 (3-$COCH_2CH_2CH_3$), 19.70 (6- $COCH_2CH_2CH_3$), 22.73 (2-Ac), 37.17 (6-$COCH_2CH_3CH_3$), 37.34 (3-$COCH_2CH_3CH_3$), 52.92 (C-2), 65.45 (C-6), 67.03 (C-4), 71.82 (C-5), 73.47 (C-3), 95.01 (C-1), 173.83 (2-CO), 175.01 (3-CO), 175.75 (6-CO); *β-pyranose*: 14.24 (3-$COCH_2CH_2CH_3$, 6-$COCH_2CH_2CH_3$), 19.40 (3-$COCH_2CH_2CH_3$), 19.70 (6-$COCH_2CH_2CH_3$), 22.91 (2-Ac), 37.14 (6-$COCH_2CH_2CH_3$), 37.26 (3-$COCH_2CH_2CH_3$), 53.31 (C-2), 65.33 (C-6), 66.73 (C-4), 76.14 (C-3), 76.38 (C-5), 94.71 (C-1), 174.6 (2-CO), 175.33 (3-CO), 175.64 (6-CO).

*p-Nitrophenyl 2-acetamido-6-*O*-acetyl-2-deoxy-β-D-galactopyranoside* (**25**); amorphous white solid: ^{1}H-NMR (CD_3OD, 30 °C): 1.996 (3 H, s, 2-NAc), 2.091 (3 H, s, 6-OAc), 3.811 (1 H, dd, $J_{2,3}$=10.7 Hz, $J_{3,4}$=3.3 Hz, H-3), 3.934 (1 H, dd, $J_{3,4}$=3.3 Hz, $J_{4,5}$=1.2 Hz, H-4), 3.998 (1 H, ddd, $J_{4,5}$=1.2 Hz, $J_{5,6a}$=4.6 Hz, $J_{5,6b}$=7.7 Hz, H-5), 4.237 (1 H, dd, $J_{1,2}$=8.4 Hz, $J_{2,3}$=10.7 Hz, H-2), 4.290 (1 H, dd, $J_{5,6a}$=4.6 Hz, $J_{6a,6b}$=11.5 Hz, H-6a), 4.368 (1 H, dd, $J_{5,6b}$=7.7 Hz, $J_{6a,6b}$=11.5 Hz, H-6b), 5.234 (1 H, d, $J_{1,2}$=8.4 Hz, H-1), 7.190 & 8.229 (4 H, AA'BB', ΣJ=9.3 Hz, pNP). ^{13}C NMR (CD_3OD): 20.8 (OAc), 23.0 (NAc), 54.0 (C-2), 64.7 (C-6), 69.6 (C-4), 72.5 (C-3), 74.6 (C-5), 102.2 (C-1), 117.8 (2 C, 2 x C-*ortho*), 126.7 (2 C, 2 x C-*meta*), 144.2 (C-*para*), 163.8 (C-*ipso*), 172.7 (6-CO), 174.4 (2-CO).

*2-Acetamido-1,6-di-*O*-acetyl-2-deoxy-α-D-mannopyranose* (**26**); colourless oil. ^{1}H NMR (DMSO, 30 °C): 1.886 (3 H, s, 2-NAc), 2.008 (3 H, s, 6-OAc), 2.077 (3 H, s, 1-OAc), 3.539 (1 H, dd, $J_{3,4}$=9.2 Hz, $J_{4,5}$=9.7 Hz, H-4), 3.653 (1 H, ddd, $J_{4,5}$=9.7 Hz, $J_{5,6a}$=7.0 Hz, $J_{5,6b}$=2.1 Hz, H-5), 3.763 (1 H, dd, $J_{2,3}$=4.9 Hz, $J_{3,4}$=9.2 Hz, H-3), 4.041 (1 H, ddd, $J_{1,2}$=2.1 Hz, $J_{2,3}$=4.9 Hz, $J_{2,NH}$=7.9 Hz, H-2), 4.097 (1 H, dd, $J_{5,6a}$=7.0 Hz, $J_{6a,6b}$=11.9 Hz, H-6a), 4.256 (1 H, dd, $J_{5,6b}$=2.1 Hz, $J_{6a,6b}$=11.9 Hz, H-6b), 5.746 (1 H, d, $J_{1,2}$=2.1 Hz, H-1), 7.774 (1 H, d, $J_{2,NH}$=7.9 Hz, NH). ^{13}C NMR (DMSO, 30 °C): 20.7 (1-Ac, 6-Ac), 22.4 (2-Ac), 51.4 (C-2), 63.6 (C-6), 66.5 (C-4), 67.9 (C-3), 72.7 (C-5), 91.9 (C-1), 168.6 (1-CO), 169.8 (2-CO), 170.3 (6-CO).

*2-Acetamido-1,6-di-*O*-butyryl-2-deoxy-D-mannopyranose* (**27**); brownish oil. ^{1}H NMR (CD_3OD, 30 °C): 0.962 (3 H, t, J = 7.4 Hz, 6-$COCH_2CH_2CH_3$), 1.005 (3 H, t, J = 7.4 Hz, 1-$COCH_2CH_2CH_3$), 1.649 (2 H, m, 6-$COCH_2CH_2CH_3$), 1.700 (2 H, m, 1-$COCH_2CH_2CH_3$), 2.046 (3 H, s, 2-Ac), 2.326 (2 H, t, J = 7.3 Hz, 6-$COCH_2CH_2CH_3$), 2.400 (2 H, t, J = 7.2 Hz, 1-$COCH_2CH_2CH_3$), 3.622 (1 H, dd, $J_{3,4}$ = 9.5 Hz, $J_{4,5}$ = 9.9 Hz, H-4), 3.816 (1 H, ddd, $J_{4,5}$ = 9.9 Hz, $J_{5,6a}$ = 7.3 Hz, $J_{5,6b}$ = 2.2 Hz, H-5), 3.975 (1 H, dd, $J_{2,3}$ = 4.9 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 4.265 (1 H, dd, $J_{5,6a}$ = 7.3 Hz, $J_{6a,6b}$ = 11.9 Hz, H-6a), 4.279 (1 H, dd, $J_{1,2}$ = 1.8 Hz, $J_{2,3}$ = 4.9 Hz, H-2), 4.404 (1 H, dd, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 11.9 Hz, H-6b), 5.962 (1 H, d, $J_{1,2}$ = 1.8 Hz, H-1). ^{13}C NMR (CD_3OD, 30 °C): 14.15 (1-$COCH_2CH_2CH_3$), 14.20 (6-$COCH_2CH_2CH_3$), 19.67 (1-$COCH_2CH_2CH_3$), 19.72 (6-$COCH_2CH_2CH_3$), 22.78 (2-Ac), 37.16 (1-$COCH_2CH_2CH_3$, 6-$COCH_2CH_2CH_3$), 53.82 (C-2), 65.15 (C-6), 68.95 (C-4), 70.56 (C-3), 74.39 (C-5), 93.69 (C-1), 173.15 (1-CO), 174.49 (2-CO), 175.56 (6-CO).

2-Acetamido-2-β-D-glucopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucopyranose (**31**) characterised as peracetate **31a**: m.p. 25 °C. ^{1}H NMR ($CDCl_3$, 30 °C) - α-anomer : β-anomer = 64:36; *α-anomer:* 1.937, 2.008, 2.018, 2.050, 2.065, 2.071, 2.079, 2.200 (24 H, s, Ac), 3.165 (1 H, ddd, *J* = 7.2, 7.9, 10.4 Hz, H-2‘), 3.774 (1 H, ddd, *J* = 2.5, 4.1, 10.0 Hz, H-5‘), 3.953 (1 H, dd, *J* = 9.1, 10.5 Hz, H-3), 3.996 (1 H, ddd, *J* = 2.4, 4.3, 10.2 Hz, H-5), 4.053 (1 H, dd, *J* = 2.5, 12.4 Hz, H-6‘a), 4.077 (1 H, dd, *J* = 2.4, 12.4 Hz, H-6a), 4.247 (1 H, dd, *J* = 4.3, 12.4 Hz, H-6b), 4.445 (1 H, dd, *J* =4.1, 12.4 Hz, H-6‘b), 4.480 (1 H, ddd, *J* = 3.7, 9.2, 10.5 Hz, H-2), 5.003 (1 H, dd, *J* = 9.2, 10.0 Hz, H-4‘), 5.026 (1 H, dd, *J* = 9.1, 10.2 Hz, H-4), 5.201 (1 H, d, *J* = 7.9 Hz, H-1‘), 5.586 (1 H, d, *J* = 9.2 Hz, 2-NH), 5.649 (1 H, dd, *J* = 9.2, 10.4 Hz, H-3‘), 5.792 (1 H, d, *J* = 7.2 Hz, 2‘-NH), 6.108 (1H, d, *J* = 3.7 Hz, H-1); *β-anomer* 1.946, 2.012, 2.018, 2.029, 2.061, 2.086, 2.116 (24 H, s, Ac), 3.394 (1 H, ddd, *J* = 7.6, 8.1, 10.5 Hz, H-2‘), 3.710 (1 H, ddd, *J* = 2.6, 4.5, 9.9 Hz, H-5‘), 3.839 (1 H, ddd, *J* = 2.3, 4.9, 9.8 Hz, H-5), 3.840 (1 H, ddd, *J* = 8.3, 8.4, 9.7 Hz, H-2), 4.118 (1 H, dd, *J* = 2.6, 12.5 Hz, H-6‘a), 4.127 (1 H, dd, *J* = 2.3, 12.4 Hz, H-6a), 4.237 (1 H, dd, *J* = 8.8, 9.7 Hz, H-3), 4.284 (1 H, dd, *J* = 4.9, 12.4 Hz, H-6b), 4.334 (1 H, dd, *J* = 4.5, 12.5 Hz, H-6‘b), 5.001 (1 H, dd, *J* = 8.8, 9.8 Hz, H-4), 5.008 (1 H, dd, *J* = 9.2, 9.9 Hz, H-4‘), 5.091 (1 H, d, *J* = 8.1 Hz, H-1‘), 5.470 (1 H, dd, *J* = 9.2, 10.5 Hz, H-3‘), 5.792 (1 H, d, *J* = 7.6 Hz, 2‘-NH), 5.835 (1 H, d, *J* = 8.4 Hz, 2-NH), 5.958 (1 H, d, *J* = 8.3 Hz, H-1). ^{13}C NMR ($CDCl_3$, 30 °C) *α-anomer:* 51.4 (C-2), 56.7 (C-2‘), 61.6 (C-6), 61.8 (C-6'), 67.8, 68.7 (C-4, C-4‘), 69.6 (C-5), 70.7 (C-3‘), 71.4 (C-5‘), 75.5 (C-3), 91.0 (C-1), 98.7 (C-1‘); *β-anomer*: 55.3 (C-2), 55.9 (C-2‘), 61,7 (C-6, C-6‘), 67,8, 68,7 (C-4, C-4‘), 71,5 (C-3‘), 71.7 (C-5‘), 72.4 (C-5), 76.8 (C-3), 91.8 (C-1), 98.7 (C-1‘)

2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→1)-β-D-galactopyranoside (**34**):
m.p. 25 °C. ^{1}H NMR (D_2O, 30 °C): 1.829 (3 H, s, 2-Ac), 3.26 (2 H, m, H-4, H-5), 3.300 (1 H, dd, *J* = 8.0, 10.0 Hz, H-2‘), 3.353 (1 H, dd, *J* = 8.9, 10.3 Hz, H-3), 3.430 (1 H, dd, *J* = 3.5, 10.0 Hz, H-3‘), 3.464 (1 H, ddd, *J* = 1.0, 4.8, 7.2 Hz, H-5‘), 3.53 (1 H, m, H-6a), 3.541 (1 H, dd, *J* = 8.6, 10.3 Hz, H-2), 3.56 (2 H, m, H-6‘), 3.697 (1 H, dd, *J* = 1.0, 3.5 Hz, H-4‘), 3.709 (1 H, dd, *J* = 1.7, 12.3 Hz, H-6b), 4.468 (1 H, d, *J* = 8.0 Hz, H-1‘), 4.673 (1 H, d, *J* = 8.6 Hz, H-1). ^{13}C NMR (D_2O, 30 °C): 22.52 (2-Ac), 55.53 (C-2), 60.90 (C-6), 61.32 (C-6‘), 68.85 (C-4‘), 70.07 (C-4), 70.47 (C-2‘), 72.85 (C-3‘), 74.09 (C-3), 75.52 (C-5‘), 76.24 (C-5), 98.20 (C-1), 100.01 (C-1‘), 175.24 (2-CO); MALDI-TOF MS: C_{14} $H_{25}NO_{11}$ (383.14): *m/z* 383.20 $[M + H]^+$; [α]D20 = -6.98 (c = 0.63, H2O). Vicinal coupling constants showed that both sugars moieties responsible for two contiguous spin systems found by COSY have β-*galacto* and β-*gluco* configurations. A crosspeak between C-1 and H-1‘ observed in HMBC indicated a (1→1) glycosidic bond.

*2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→1)-β-D-galactopyranoside (***35***):*
m.p. 25 °C. ^{1}H NMR (D_2O, 30 °C): 1.811 (s, 3 H, 2-Ac), 1.840 (s, 3 H, 2‘‘-Ac), 3.236 (1 H, dd, *J* = 8.4, 9.7 Hz, H-4‘‘), 3.26 (1 H, m, H-5‘‘), 3.284 (1 H, dd, *J* = 7.9, 10.1 Hz, H-2‘), 3.301 (1 H, ddd, *J* = 2.2, 4.6, 9.6 Hz, H-5), 3.343 (1 H, dd, *J* = 8.4, 10.3 Hz, H-3‘‘), 3.408 (1 H, dd, *J* = 8.1, 9.6 Hz, H-4), 3.415 (1 H, dd, *J* = 3.5, 10.1 Hz, H-3‘), 3.44 (1 H, m, H-6b), 3.450 (1 H, dt, *J* = 0.9, 7.1 Hz, H-5‘), 3.492 (1 H, dd, *J* = 8.1, 10.3 Hz, H-3), 3.517 (1 H, dd, *J* = 8.4, 10.3 Hz, H-2‘‘), 3.52 (1 H, m, H-6‘‘a), 3.54 (2 H, m, H-6‘), 3.563 (1 H, dd, *J* = 8.2, 10.3 Hz, H-2), 3.622 (1 H, dd, *J* = 2.2, 12.2 Hz, H-6a), 3.652 (1 H, dd, *J* = 0.9, 3.5 Hz, H-4|), 3.70 (1 H, m, H-6‘‘b), 4.361 (1 H, d, *J* = 8.4 Hz, H-1‘‘), 4.440 (1 H, d, *J* = 7.9 Hz, H-1‘), 4.658 (1 H, d, *J* = 8.2 Hz, H-1). ^{13}C NMR (D_2O, 30 °C) HMQC and HMBC readouts: 24.7 (2‘‘-Ac), 24.8 (2-Ac), 54.9 (C-2), 55.6 (C-2‘‘), 60.3 (C-6), 60.8 (C-6‘‘), 61.2 (C-6‘), 68.8 (C-4‘), 70.1 (C-4‘‘), 70.6 (C-2‘), 72.8 (C-3), 72.9 (C-3‘), 73.8 (C-3‘‘), 74.8 (C-5), 75.5 (C-5‘), 76.2 (C-5‘‘), 79.6 (C-4), 98.1 (C-1), 100.0 (C-1‘), 101.7 (C-1‘‘), 174.8 (2‘‘-CO), 175.2 (2-CO); MALDI-TOF MS: $C_{22}H_{38}N_2O_{16}$ (586.22): *m/z* 586.28 [*M* + H]+; $[\alpha]_D^{20}$ = -20.75 (*c* = 0.4, H2O). ^{1}H NMR spectrum consists of three spin systems (COSY). As judged from the extracted vicinal couplings, one of them has β-*galacto* and two β-*gluco* configuration. Heteronuclear couplings (HMBC) of H-1‘ to C-1 and H-1‘‘ to C-4 determine the glycosidic bonds thus confirming the structure **35**.

β-D-Galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-2-acetamido-2-deoxy-β-D-glucopyranoside (β-D-Galp-(1→4)-β-D-Glcp-(1→1)-β-D-GlcpNAc) (**38**) characterised as peracetate (**38a**): ^{1}H NMR ($CDCl_3$, 30 °C): 1.925, 1.972, 2.019, 2.021, 2.036, 2.049, 2.052, 2.067, 2.091, 2.147, 2.155 (all s, each 3 H, Ac), 3.627 (1 H, ddd, *J* = 2.2, 5.1, 9.7 Hz, H-5), 3.700 (1 H, ddd, *J* = 8.1, 8.6, 10.4 Hz, H-2‘‘), 3.717 (1 H, ddd, *J* = 2.2, 4.9, 9.9, H-5‘‘), 3.794 (1 H, dd, *J* = 9.0, 9.7 Hz, H-4), 3.877 (1 H, ddd, *J* = 1.1, 6.5, 7.1 Hz, H-5‘), 4.081 (1 H, dd, *J* = 7.1, 11.2 Hz, H-6‘a), 4.115 (1 H, dd, *J* = 2.2, 12.2 Hz, H-6‘‘a), 4.134 (1 H, dd, *J* = 6.5, 11.2 Hz, H-6‘b), 4.142 (1 H, dd, *J* = 5.1, 12.1 Hz, H-6a), 4.509 (1 H, dd, *J* = 2.2, 12.1 Hz, H-6b), 4.258 (1 H, dd, *J* = 4.9, 12.2 Hz, H-6‘‘b), 4.501 (1 H, d, *J* = 7.8 Hz, H-1‘), 4.758 (1 H, d, *J* = 7.8 Hz, H-1), 4.873 (1 H, dd, *J* = 7.8, 9.3 Hz, H-2), 4.970 (1 H, dd, *J* = 3.4, 10.4 Hz, H-3‘), 4.975 (1 H, d, *J* = 8.1 Hz, H-1‘‘), 5.039 (1 H, dd, *J* = 9.2, 9.9 Hz, H-4‘‘), 5.115 (1 H, dd, *J* = 7.8, 10.4 Hz, H-2‘), 5.214 (1 H, dd, *J* = 9.0, 9.3 Hz, H-3), 5.351 (1 H, dd, *J* = 1.1, 3.4 Hz, H-4‘), 5.392 (1 H, dd, *J* = 9.2, 10.4 Hz, H-3‘‘), 5.534 (1 H, d, *J* = 8.6 Hz, NH-2‘‘).

^{13}C NMR ($CDCl_3$, 30 °C) HMQC readouts: 54.6 (C-2‘‘), 60.7 (C-6‘), 61.5 (C-6), 61.9 (C-6‘‘), 66.6 (C-4‘), 68.4 (C-4‘‘), 69.1 (C-2‘), 70.7 (C-5‘), 70.9 (C-3‘), 71.0 (C-2), 71.9 (C-3‘‘, C-5‘‘), 72.4 (C-3), 73.0 (C-5), 75.7 (C-4), 97.6 (C-1‘‘), 97.9 (C-1), 100.9 (C-1‘); MALDI-TOF MS: $C_{40}H_{55}NO_{26}$ (965.30): *m/z* 965.60 [*M*+H]+; $[\alpha]_D^{20}$ = -0.75 (*c* = 0.4, $CHCl_3$). Three isolated spin systems were picked up by COSY and 1D-TOCSY experiments. According to the respective vicinal coupling constants they corresponded to β-*gluc*o, β-*galacto* and 2-acetamido-2-deoxy-β-*gluco* units. Up-field resonating H-4 indicated a β-Gal-(1 → 4)-β-Glc glycosidic bond.

β-D-Galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→1)-2-acetamido-2-deoxy-β-D-glucopyranoside (β-D-Galp-(1→4)-α-D-Glcp-(1→1)-β-D-GlcpNAc) (**39**) characterised as decaacetate (**39b**): ^{1}H NMR ($CDCl_3$, 30 °C): 1.958, 1.970, 2.024, 2.032, 2.049, 2.071, 2.090, 2.102, 2.126, 2.153 (all s, 3 H each, Ac), 3.575 (1 H, ddd, *J* = 8.0, 8.0, 10.7 Hz, H-2‘‘), 3.679 (1 H, dd, *J* = 8.4, 9.2 Hz, H-4), 3.758 (1 H, dd, *J* = 3.8, 8.7 Hz, H-2), 3.729 (1 H, m, H-5‘‘), 3.899 (1 H, ddd, *J* = 1.1, 6.3, 6.8 Hz, H-5‘), 4.113 (1 H, dd, *J* = 6.8, 11.3 Hz, H-6‘a), 4.115 (1 H, dd, *J* = 7.1, 14.1 Hz, H-6a), 4.200 (2 H, m, H-6‘‘), 4.207 (1 H, dd, *J* = 6.3, 11.3 Hz, H-6‘b), 4.511 (1 H, d, *J* = 7.9 Hz, H-1‘), 4.418 (1 H, dd, *J* = 4.2, 14.1 Hz, H-6b), 4.965 (1 H, dd, *J* = 3.4, 10.5 Hz, H-3‘), 5.106 (1 H, dd, *J* = 7.9, 10.5 Hz, H-2‘), 5.053 (1 H, dd, *J* = 9.3, 10.0 Hz, H-4‘‘), 5.093 (1 H, d, *J* = 8.0 Hz, H-1‘‘), 5.314 (1 H, d, *J* = 3.8 Hz, H-1), 5.378 (1 H, dd, *J* = 1.1, 3.4 Hz, H-4‘), 5.406 (1 H, dd, *J* = 8.4, 8.7 Hz, H-3), 5.481 (1 H, dd, *J* = 9.3, 10.7 Hz, H-3‘‘), 5.780 (1 H, d, *J* = 7.4 Hz, NH-2‘‘). ^{13}C NMR (CDC_{l3}, 30 °C) HMQC readouts: 55.8 (C-2‘‘), 60.9 (C-6‘), 61.6 (C-6‘‘), 62.2 (C-6), 66.7 (C-4‘), 68.1 (C-5), 68.5 (C-4‘‘), 69.2 (C-2‘), 70.5 (C-3), 70.9 (C-3‘, C-5‘), 71.2 (C-3‘‘), 71.9 (C-5‘‘), 76.4 (C-4), 76.8 (C-2), 91.4 (C-1), 99.8 (C-1‘‘), 100.9 (C-1‘); MALDI-TOF MS: $C_{38}H_{53}NO_{25}$ (923.29): *m/z* 923.60 [M + H]+; $[\alpha]_D^{20}$ = -1.5 (*c* = 0.2, $CHCl_3$). The analysis of vicinal couplings observed in three spin systems present in ^{1}H NMR spectrum (COSY, 1D-TOCSY) determined the presence of α-*gluco*, β-*galacto* and 2-acetamido-2-deoxy-β-*gluco* moieties in the molecule. The molecular weight and 10 singlets observed in the acetyl region of ^{1}H NMR mean that the compound is decaacetate. Chemical shift of H-2 (3.758 ppm) indicates that C-2 position is not acetylated in this compound (compared to the respective shift in **38a** being 4.873 ppm).

*p-Nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside* **(41)**: ^{1}H NMR (D_2O, 30 °C): 1.632 (3 H, s, CH_3), 3.569 (1 H, d, *J* = 6.4 Hz, H-6‘), 3.686 (1 H, dd, *J* = 3.9, 10.3 Hz, H-2‘), 3.769 (1 H, dd, *J* = 3.3, 10.3 Hz, H-3‘), 3.829 (1 H, dd, *J* = 1.3, 3.3 Hz, H-4‘), 3.947 (1 H, m, H-5), 3.990 (1 H, dd, J = 3.4, 10.2 Hz, H-2), 4.020 (1 H, m, H-5‘), 4.033 (1 H, dd, J = 2.8, 10.2 Hz, H-3), 4.036 (2 H, m, H-6), 4.126 (1 H, d, J = 0.8, 2.8 Hz, H-4), 4.997 (1 H, d, J = 3.9 Hz, H-1‘), 5.718 (1 H, d, J = 3.4 Hz, H-1), 7.076 and 8.083 (4 H,AA’BB‘, arom.); ^{13}C NMR (D_2O, 30 °C) HMQC readouts: 20.2 (q, CH_3), 61.5 (t, C-6‘), 64.0 (t, C-6), 65.5 (d, C-4), 66.4 (d, C-2), 68.5 (d, C-2‘), 69.5 (d, C-4‘), 69.6 (d, C-3‘), 69.7 (d, C-5), 71.3 (d, C-5‘), 74.2 (d, C-3), 95.5 (d, C-1‘), 96,7 (d, C-1), 173.8 (s, CO), 117.4 (d), 126.2 (d), 142.7 (s), 161.2 (s) (arom.); MALDI-TOF MS: $C_{20}H_{28}NO_{14}$ (506.15): *m/z* 506.12 $[M + H]^+$. COSY and TOCSY spectra identified two contiguous spin systems of the OCH-(O)CH-(O)CH-(O)CH-(O)CH-CH_2O type and one AA’BB’. Extracted vicinal coupling constants are consistent with *galacto*-configuration of both sugar moieties. Proton signals assignment was transferred to carbons by means of HMQC experiment. HMBC correlation H-1 to carbon at 161.2 ppm confirmed the attachment of *p*-nitrophenyl to one sugar moiety. Acetylation at C-6 was confirmed by HMBC correlations H-6 and acetyl methyl to same C=O. Correlation H-1’ to C-3 confirmed glycosidic bond between the second and first sugar ring.

2-Methylpropyl α-D-galactopyranoside (**42**)**:** ^{1}H NMR (D_2O, 30 °C): 1.055 (9 H, s, $(CH_3)_3C$), 3.472 (1 H, dd, *J* = 7.2, 11.7 Hz, H-6a), 3.508 (1 H, dd, *J* = 5.4, 11.7 Hz, H-6b), 3.523 (1 H, dd, *J* = 4.1, 10.4 Hz, H-2), 3.631 (1 H, dd, *J* = 3.4, 10.4 Hz, H-3), 3.743 (1 H, dd, *J* = 1.3, 3.4 Hz, H-4), 3.890 (1 H, ddd, *J* = 1.3, 5.4, 7.2 Hz, H-5), 5.001 (1 H, d, *J* = 4.1 Hz, H-1). ^{13}C NMR (D_2O, 30 °C): 28.09 (q, $(CH_3)_3CO$), 61.43 (t, C-6), 68.71 (d, C-2), 69.65 (d, C-4), 69.85 (d, C-3), 70.51 (d, C-5), 76.20 (s, $(CH_3)_3CO$), 93.29 (d, C-1); MALDI-TOF MS: $C_{10}H_{20}O_6$ (236.13): *m/z* 263.15 $[M + H]^+$

p-Nitrophenyl α-D-galactopyranosyl-(1→3)-α-D-galactopyranoside (**44**) ^{1}H NMR (D_2O, 30 °C): 3.479 (1 H, dd, *J* = 5.0, 11.9 Hz, H-6a), 3.520 (1 H, dd, *J* = 7.3, 11.9 Hz, H-6b), 3.571 (1 H, d, *J* = 6.5 Hz, H-6‘), 3.683 (1 H, dd, *J* = 3.9, 10.3 Hz, H-2‘), 3.754 (1 H, m, H-5), 3.768 (1 H, dd, *J* = 3.3, 10.3 Hz, H-3‘), 3.827 (1 H, dd, J = 1.2, 3.3 Hz, H-4‘), 3.980 (1 H, dd, J = 3.5, 10.2 Hz, H-2), 4.025 (1 H, m, H-5‘), 4.032 (1 H, dd, J = 2.9, 10.2 Hz H-3), 4.095 (1 H, dd, J = 1.0, 2.9 Hz, H-4), 5.001 (1 H, d, J = 3.9 Hz, H-1‘), 5.689 (1 H, d, J = 3.5 Hz, H-1), 7.087 and 8.052 (4 H,AA’BB‘, arom.);

^{13}C NMR (D_2O, 30 °C) HMQC readouts: 61.2 (t, C-6), 61.5 (t, C-6‘), 65.7 (d, C-4), 66.6 (d, C-2), 68.4 (d, C-2‘), 69.4 (d, C-4‘), 69.5 (d, C-3‘), 71.3 (d, C-5‘), 72.2 (d, C-5), 74.3 (d, C-3), 95.5 (d, C-1‘), 97.1 (d, C-1), 117.2 (d), 126.4 (d), 142.6 (s), 161.7 (s) (arom.);

MALDI-TOF MS: $C_{18}H_{26}NO_{13}$ (464.14): *m/z* 464.12 $[M + H]^+$

Desrhamnodesglucoruscin (**46**):

^{1}H-NMR (CD_3OD, 30 °C) δ : 5.57 (1 H, br d, *J* = 5.6 Hz, H-6); 4.78 and 4.75 (1 H each, br s each, CH_2-27); 4.45 (1 H, m, H-16); 4.28 (1 H, d, *J* = 12.1 Hz, H_{eq}-26); 4.23 (1 H, d, *J* = 7.1 Hz, H-1’); 3.88 (1 H, dd, J_1 = 12.6 Hz, J_2 = 2.3 Hz, H_{eq} -5’); 3.83 (1 H, d, *J* = 12.1 Hz, H_{ax} -26); 3.80 (1 H, br s, H-4’); 1.12 (3 H, s, CH_3 –19); 0.96 (3 H, d, *J* = 7.0 Hz, CH_3 -21); 0.84 (3 H, s, CH_3 –18). ^{13}C-NMR (CD_3OD, 30 °C): ppm: 145.2; 139.8; 125.9; 110.7; 108.9; 102.0; 83.7; 82.5; 74.6; 72.7; 70.1; 69.1; 67.5; 65.8; 64.1; 57.9; 51.5; 43.5; 43.3; 42.9; 41.3; 41.2; 36.9; 34.1; 34.0; 32.9; 32.8; 29.5; 24.6; 17.0; 14.9. Analytical and spectroscopic data were identical to those of an authentic standard of desrhamnodesglucoruscin **(46)**. ESI-MS: desglucoruscin **(3)**: 729 $[M + Na]^+$, 319 [Rha-Ara + Na]$^+$; desrhamnodesglucoruscin **(46)**: 583 $[M + Na]^+$; 173 [Ara + Na]$^+$.

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10. A. Pišvejcová, J. Rauvolfová, V. Přikrylová, L. Hušáková, M. Kuzma, and V. Křen: Use of the library of fungal β-*N*-acetylhexosaminidases for synthesis of glycosaminoglycosides, *10th Bratislava Symposium on Saccharides,* Smolenice, SK, September 1 - 6, 2002

11. P. Simerská, L. Hušáková, A. Pišvejcová, L. Weignerová, M. Kuzma, S. Riva, and V. Křen: α-Galactosidase and their use for the synthesis of sterically hindered α-D-galactopyranosides, *10th Bratislava Symposium on Saccharides*, Smolenice, SK, September 1 - 6, 2002

12. P. Fialová, A. Pišvejcová, L. Weignerová, and V. Křen: Modified substrates for β-*N*-acetylhexosaminidases, *10th Bratislava Symposium on Saccharides*, Smolenice, SK, September 1 – 6, 2002

13. A. Pišvejcová, J. Rauvolfová, V. Přikrylová, L. Hušáková, M. Kuzma, and V. Křen: Use of the library of fungal β-*N*-acetylhexosaminidases for synthesis of glycosaminoglycosides, *Houby jako modelové organismy ve výzkumu a biotechnologii - II,* Olomouc, CZ, September 5 - 6, 2002

14. P. Simerská, L. Hušáková, A. Pišvejcová, L. Weignerová, M. Kuzma, S. Riva, and V. Křen: α-Galactosidase and their use for the synthesis of sterically hindered α-D-galactopyranosides, *2nd Czech-Swiss Symposium on Advanced Biotechnology*, Prague, CZ, November 7 - 9, 2002

15. K. Bezouška, P. Man, P. Pompach, P. Novák, V. Havlíček, R. Hilgenfeld, O. Plíhal, J. Sklenář, A. Pišvejcová, and V. Křen: Protein chemistry and mass spectrometry in structural analysis of large proteins, *5th Igler MS Tage*, Igls bei Innsbruck, AT, February 20 - 21, 2003

16. A. Pišvejcová, D. Monti, P. Sedmera, C. Tarabiono, M. Lama, S. Riva, and V. Křen: Library of α-L-rhamnosidases and its application, *The Carbohydrate Workshop 2003,* Guestrow, DE, March 27 - 30, 2003

17. A. Pišvejcová, D. Monti, P. Sedmera, C. Tarabiono, M. Lama, S. Riva, and V. Křen: Library of α-L-rhamnosidases and its application, *Cukrblik 2003 - Czech National Carbohydrate Symposium*, Prague, CZ, April 16, 2003

18. P. Simerská, A. Pišvejcová, L. Weignerová, M. Kuzma, M. Macková, S. Riva, and V. Křen: Enzymic synthesis of alkyl α-D-galactopyranosides, *Cukrblik 2003 - Czech National Carbohydrate Symposium*, Prague, CZ, April 16, 2003

19. O. Plíhal, J. Sklenář, P. Matoušek, P. Novák, P. Man, V. Havlíček, L. Weignerová, A. Pišvejcová, V. Křen, and K. Bezouška: Induction of β-*N*-acetylhexosaminidase and secretion pathway investigation in *Aspergillus oryzae*, *Cukrblik 2003 - Czech National Carbohydrate Symposium*, Prague, CZ, April 16, 2003

20. A. Pišvejcová, D. Monti, P. Sedmera, C. Tarabiono, M. Lama, S. Riva, and V. Křen: α-L-Rhamnosidases and their use in selective trimming of natural compounds, *BioTrans 2003 - 6th International Symposium on Biocatalysis and Biotransformations*, Olomouc, CZ, June 28 - July 3, 2003

21. L. Hušáková, J. Rauvolfová , A. Pišvejcová, R. Ettrich, K. Bezouška, V. Přikrylová, and V. Křen: *N*-Acetylmannosamine containing saccharides: Production using β-*N*-acetylhexosaminidases and their separation by ion-exchange/exclusion chromatography, *BioTrans 2003 - 6th International Symposium on Biocatalysis and Biotransformations*, Olomouc, CZ, June 28 - July 3, 2003

22. P. Simerská, A. Pišvejcová, S. Nicotra, M. Lama, M. Kuzma, M. Macková, S. Riva, and V. Křen: Selective enzymatic acylation of *N*-acetylhexosamines and their derivatives, *EuroCarb 12 - 12th European Carbohydrate Symposium*, Grenoble, FR, July 6 - 11, 2003

23. P. Fialová, A. Pišvejcová, L. Weignerová, and V. Křen: New transglycosylation reactions with modified substrates catalysed by β-*N*-acetylhexosaminidases, *EuroCarb 12 - 12th European Carbohydrate Symposium*, Grenoble, FR, July 6 - 11, 2003

24. A. Pišvejcová, D. Monti, P. Sedmera, C. Tarabiono, M. Lama, S. Riva, and V. Křen: α-L-Rhamnosidases and their use in selective trimming of natural compounds, *EuroCarb 12 - 12th European Carbohydrate Symposium*, Grenoble, FR, July 6 - 11, 2003

25. T. Macek, M. Kamlar, R. Ježek, A. Pišvejcová, C. Koncz, and L. Kohout: Studies on brassinosteroide hormone binding proteins from plants, *20th Conference on Isoprenoids 2003*, Liberec, CZ, September 13 - 18, 2003

26. S. Riva, M. Lama, D. Monti, A. Pišvejcová, and V. Křen: Produzione di una libreria di α-L-ramnosidasi e suo utilizzo per la modifica di glicosidi naturali, *VI Convegno Nazionale "GIORNATE DI CHIMICA DELLE SOSTANZE NATURALI",* Vietri sul Mare (SA), IT, September 29 - October 1, 2003

27. J. Rauvolfová, A. Pišvejcová, M. Kuzma, V. Přikrylová, and V. Křen: Enzymatic synthesis of *N*-acetylglucosaminobioses by reverse hydrolysis: Characterisation and application of the library of fungal β-*N*-acetylhexosaminidases, *The Carbohydrate Workshop 2004,* Borstel, DE, March 17 - 20, 2004

28. P. Simerská, A. Pišvejcová, M. Kuzma, M. Macková, and V. Křen: α-D-Galactosidase from *Talaromyces flavus* and its synthetic applications, (poster P71), *22nd International Carbohydrate Symposium*, Glasgow, UK, July 23 - 27, 2004

29. V. Křen, J. Rauvolfová, V. Přikrylová, M. Kuzma, and A. Pišvejcová: Glycosidase-catalysed synthesis of non-reducing saccharides, (poster P274), *22nd International Carbohydrate Symposium*, Glasgow, UK, July 23 - 27, 2004

30. O. A. Aiyelaagbe, W.-D. Fessner, A. Pišvejcová, and V. Křen: The "Natural strategy" for glycosidase-assisted glycoside synthesis. Part 2: Deoxysugar glycosides, (plenární přednáška), *Applied Biocatalysis – "Future of Biocatalysis in the European Union, COST D25 Meeting*, Athens, GR, September 30 - October 2, 2004

31. A. Pišvejcová, J. Rauvolfová, L. Weignerová, and V. Křen: Library of fungal glycosidases as a powerful synthetic tool, *Cukrblik 2005 - Czech National Carbohydrate symposium*, Prague, CZ, March 31, 2005, přednáška

32. D. Monti, C. Rossi, A. Pišvejcová, L. Hušáková, V. Křen, and S. Riva: Modulation of the β-1,4-galactosyltransferase donor and acceptor specificity by α-lactalbumin, *BioTrans 2005 - 7th International Symposium on Biocatalysis and Biotransformations*, Delpth, NL, July 3 - 8, 2005

Long-term visits abroad:

- September 17th - October 21st, 2001

  Regioselective enzymatic acylation of *N*-acetylhexosamines and their derivatives

  Istituto di Chimica del Riconoscimento Molecolare, CNR

  Milano, Italy, Laboratory of Dr. Sergio Riva

  CNR – AV ČR bilateral project

- September 9th - October 8th, 2002

  Production of fungal α-L-rhamnosidases, screening of α-L-rhamnosidase library and its application in selective trimming of saponin derivatives

  Istituto di Chimica del Riconoscimento Molecolare, CNR

  Milano, Italy, Laboratory of Dr. Sergio Riva

  EU COST D25/0001/02 project

- September 1st - October 31st, 2004

  Screening of acceptor and donor specificity of β-1,4-galactosyltransferase (β-1,4-GalT), the influence of α-lactalbumin on these specificities and the influence of solvents on the activity of the enzyme

  Istituto di Chimica del Riconoscimento Molecolare, CNR

  Milano, Italy, Laboratory of Dr. Sergio Riva

  NATO Science Programme

Courses:

- Course ”Mikroskopické saprofytické houby významné z hlediska člověka“, January 29th – February 2nd, 2001 and September 10th - 14th, 2001, Department of Botany, Charles University, Prague

- “Odborná a vědecká komunikace písmem v medicíně” (“Science Writing Workshop”), April 2nd, 9th, 16th a 23rd, 2002, 1st Medical Faculty, Charles University, Prague

- “Prezentace vědeckých výsledků”, May 28th - 30th, 2002, GAČR, Academy of Sciences of the Czech Republic, Prague

- “Nové metody a cíle organické syntézy”, April 4th - May 28th, 2003, Institute of organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague

- Symposium of Czech-Slovak Microscopic Society “Mikroskopie 2004“, March 11th – 12th, 2004, Nové Město na Moravě, Czech-Slovak Microscopic Society, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague

- “Moderní problémy v biotechnologii”, June 19th, 2003, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Prague

- “Molekulární biologie a genetika”, 2001, Department of Genetics and Microbiology, Charles University, Prague

Scholarships awarded:

- In 2001 a mobility grant from "Foundation Czech Literary Fond" for 5th International Symposium on Biocatalysis and Biotransformation (BioTrans 2001), Darmstadt, DE, September 2 - 7, 2001

- In 2003 a mobility grant from "Foundation Czech Literary Fond" for 12th European Carbohydrate Symposium (EuroCarb 12), Grenoble, FR, July 6 - 11, 2003