

# Chemical biology studies on retaining exo- $\beta$ -glucosidases Su, Q.

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# **Chapter 1**

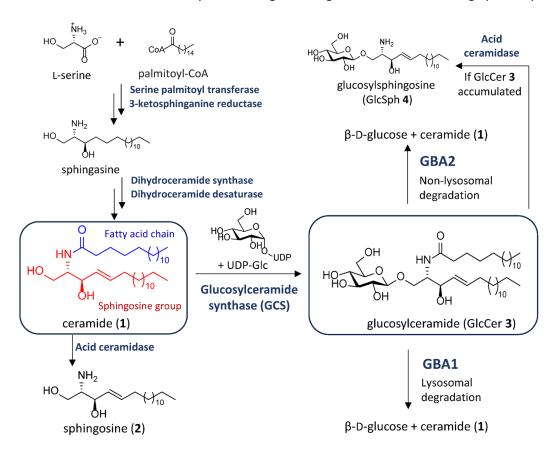
**General introduction** 

#### Glycosphingolipids

Glycosphingolipids (GSLs) are essential structural components of mammalian cell membranes and play a role in many (patho)physiological processes including cellular signaling, cell adhesion/recognition processes, and modulation of signal transduction processes. Glycosphingolipids are composed of carbohydrates linked to the 1-hydroxyl group of ceramide (1) which itself consists of a sphingoid base which is *N*-acylated with a fatty acid (see Scheme 1). The most abundant sphingoid base in mammals is sphingosine (2) containing 18 carbons and one double bond (d18:1), while sphingoid bases encompassing 12 to 26 carbons also exist. Of all mammalian GSLs, over 90% are derived from glucosylceramide (GlcCer, 3, also known as glucocerebroside), while the remainder is derived from galactosylceramide (GalCer). Solution of signal transduction processes.

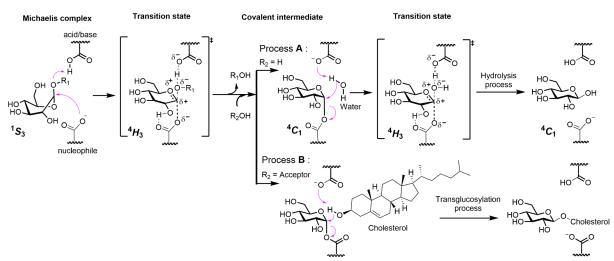
The first step in the biosynthesis of ceramide (1), the precursor from which GlcCer is formed, comprises<sup>1,3</sup> (Scheme 1) condensation of the amino acid L-serine and an acyl-CoA thioester (usually palmitoyl-CoA) into 3-ketosphinganine. The resultant 3-ketosphinganine is then reduced into sphinganine, acylation of which gives dihydroceramide, and desaturation then gives ceramide. After completing its synthesis at the cytosolic side of the ER membrane, ceramide 1 is transported to the cytosolic side of the cis-Golgi apparatus membrane where it is transformed into GlcCer 3 by the membrane bound glucosylceramide synthase<sup>4</sup> (GCS) using UDP-glucose as the donor glycoside. The synthesized GlcCer 3 is then transported to the luminal side of the Golgi membrane, where it is galactosylated to form lactosylceramide (LacCer). LacCer is an important branching point from which a series of complex glycosphingolipids such as the gangliosides, neolactosides, and lactosides are synthesized by stepwise elongation with various monosaccharides.

In humans, the catabolism of GlcCer (3) is mainly mediated by the lysosomal retaining exo-β-glucosidase, GBA1. The resultant ceramide 1 is further processed into sphingosine 2 and a free fatty acid by acid ceramidase (ASAH1) inside the lysosome. The generated sphingosine is exported from lysosomes and can be reused in the cytosol for regenerating ceramide via the salvage pathway.



**Scheme 1.** A brief schematic representation of GSLs biosynthesis and catabolism. Metabolic enzymes in blue.

Mammalian cells may contain several retaining exo-β-glucosidases. All cells express the lysosomal GBA1, many cell types express the cytosol-facing membrane-associated GBA2 and specific cells express the cytosolic GBA3. These retaining  $\beta$ -glucosidases all hydrolyze GlcCer 3 through a Koshland double displacement mechanism, which involves the formation of a covalent glucosyl enzyme intermediate.<sup>5</sup> The hydrolysis process (Figure 1) commences with attack of the nucleophilic carboxylic acid residue towards the substrate anomeric center, while simultaneously the catalytic acid/base protonates the aglycon, thereby making it a better leaving group. As the result, an intermediate of covalent glycosylenzyme adduct is formed with inversion of the anomeric configuration of the substrate glycoside. The aglycon then leaves the enzyme active site, generating space for a water molecule to enter. In a reversal of steps, the covalent enzyme-substrate intermediate is then hydrolyzed, during which the water molecule is deprotonated by the catalytic acid-base residue. The stereochemistry at the anomeric position of the glucose moiety is again inverted, resulting in a net retention of anomeric stereochemistry.<sup>6-8</sup> In this process, the substrate β-glucopyranoside adopts a <sup>1</sup>S<sub>3</sub> skew-boat conformation in the initial Michaelis complex with the enzyme active site, placing the aglycon in an axial position allowing for ensuing nucleophilic displacement. During this first S<sub>N</sub>2 displacement, the sugar is distorted to a <sup>4</sup>H<sub>3</sub> half-chair transition state (TS) conformation from which after formation of the covalent enzyme-substrate acylal adduct the pyranose conformation turns to 4C<sub>1</sub>.9,10 Notably, retaining β-glucosidases have the intrinsic capacity to transfer glucose from their substrate to water (giving hydrolysis process A of Figure 1), but also to other acceptor alcohols, leading to an overall transglycosylation event. For instance, GBA1 as well as GBA2 has been shown to be able to transfer glucose from GlcCer to cholesterol (see Figure 1 process B) to give, also with net retention of anomeric configuration, cholesterol β-glucoside as the transglycosylation product.<sup>11</sup>



**Figure 1.** Koshland double-replacement catalytic mechanism of retaining  $\beta$ -glucosidases. Process A: hydrolysis, process B: transglycosylation.

#### Gaucher disease, a common lysosomal storage disorder caused by genetic deficiency in GBA1

Lysosomal storage disorders (LSDs) are rare inherited metabolic disorders that are caused by dysfunction of specific lysosomal metabolic processes. Lysosome dysfunction may result from defects in the formation or stability of lysosomes, export of degradation products or defects in specific enzymatic activities. Often, dysfunction of lysosomes is caused by the mutation of a gene encoding a specific glycosidase that is responsible for the hydrolysis of substrates inside lysosomes, or that of related lysosomal activator proteins, lysosomal transporters, or integral membrane proteins.

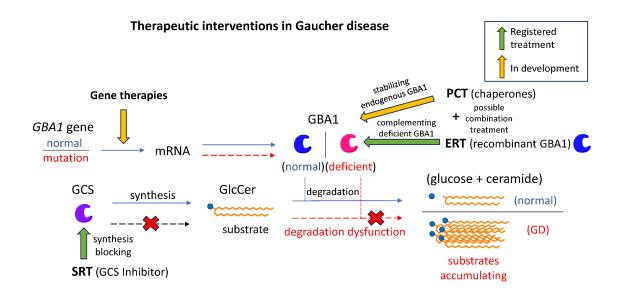
Gaucher disease (GD) is the most common LSD, $^{16}$  and was first described by Phillipe C. E. Gaucher, who in his doctoral thesis described a patient with an unexplained hepatosplenomegaly in 1882. GD is caused by genetic deficiency in lysosomal acid  $\beta$ -glucosidase GBA1 (Enzyme Commission (EC) number 3.2.1.45), resulting in accumulation of GlcCer (3) in lysosomes of tissue macrophages that then transform into lipid-laden Gaucher cells. As a consequence of accumulating GlcCer, also

glucosylsphingosine (GlcSph **4**) levels increase through the action of acid ceramidase (Scheme 1),<sup>16,17</sup> and GlcSph, which is present in very low levels in healthy individuals, is now viewed as a valuable GD biomarker.<sup>18</sup> GD storage macrophages are thought to underlie characteristic symptoms of GD patients such as hepatosplenomegaly and pancytopenia (shortage of red blood cells and platelets). GD has an average frequency of 1 in 50,000–200,000 births and occurs at a much higher frequency among the Ashkenazi Jewish population (1:1,000).<sup>12</sup>

The clinical phenotype of GD patients is heterogenous, even in some cases of homozygotic twins. <sup>19</sup> The common visceral symptoms include hepatosplenomegaly, thrombocytopenia, abnormalities in coagulation, anemia, and skeletal manifestations (for instance, bone pain and bone fractures). Three different clinical types of this disorder are generally discerned. Type I GD is the most common form. This chronic non-neurological phenotype is characterized by organomegaly, bone disease (for instance, avascular necrosis, bone pain) and cytopenia. Type II GD patients show acute neurological manifestations already in the first year of life, while type III GD patients develop sub-acute, progressive neurological symptoms at a later age. The distinction of GD in these three phenotypes is not very strict and nowadays the existence of a continuum of phenotypes is proposed. <sup>20</sup>

#### Therapeutic strategies to treat GD

There has been considerable progress in the development of therapeutic strategies to treat GD in the past decades, making GD perhaps the best-studied LSD from a clinical perspective. Yet, no curative treatment for this disease exist and as well neuropathological (type II, III) manifestations of the disease are difficult to treat by any of the developed therapies. 12,14,21,22 Two therapeutic strategies are clinical practice and comprise enzyme replacement therapy (ERT) and substrate reduction therapy (SRT). Besides these, several alternative strategies are in (pre)clinical experimental phases, including pharmacological chaperone therapy (PCT). The concept of ERT is to complement endogenous, defective enzyme with a functional, recombinant one.<sup>16</sup> Macrophages, including GD cells, express mannose receptors on the surface, which can be utilized to bind high-mannose type N-glycans in recombinant GBA1 which are then delivered to lysosomes. ERT has been approved for GD by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA). The available therapeutic enzymes include  $Imiglucerase^{23}$ , Velaglucerase  $alfa^{24}$ , and Taliglucerase  $alfa^{25}$  ERT is however effective only for the non-neuronopathic phenotype (type I) and does not prevent neurological symptoms in patients with types II and III, this because recombinant enzyme is unable to penetrate into the brain. The concept of SRT is to use small molecules to inhibit the build-up of storage molecules in lysosomes by (partially) blocking their biosynthesis. <sup>16</sup> In the context of GD, the registered small-molecule drugs imiglustat and eliglustat, both glucosylceramide synthase (GCS) inhibitors, have been approved for the treatment of type I Gaucher disease.<sup>26</sup> SRT drugs have the advantage over recombinant enzymes (which are administered intravenously) that they are oral medications. However, small compound SRT drugs have a slower onset of efficacy than ERT. The concept of PCT is to use small molecules to improve the folding of misfolded protein in patients.<sup>27</sup> These small molecules (named pharmacological chaperones) can favorably interact with mutant proteins by assisting folding in the ER. In an alternative version merging PCT with ERT, small molecule pharmacological chaperones are used to stabilize recombinant enzyme in circulation, thereby elevating protein levels that end up in disease tissue. <sup>16</sup> Migalastat as a competitive inhibitor of  $\alpha$ -galactosidase A is the first approved PCT drug by the FDA,<sup>28</sup> but there is no approved PCT drug for GD at present. Other therapies in development include gene therapy, both in vivo transduction (termed AAV therapy) and ex vivo transduction (termed lentiviral gene therapy). 12,21,22



**Figure 2.** Therapeutic strategies to treat GD.

# Properties of retaining exo-β-glucosidases in human cells

Human cells contain four reported retaining exo-β-glucosidases. The lysosomal acid β-glucosidase GBA1 (glucosylceramidase; glucocerebrosidase; GCase, EC. 3.2.1.45, GH30), is encoded by the GBA1 gene located on locus 1q21 of the human chromosome 1. GBA1 is a 497 amino acid glycoprotein with four N-linked glycans and has a characteristic  $(\alpha/\beta)_8$  TIM barrel catalytic domain. <sup>30</sup> In this domain, Glu 340 and Glu 235 are employed as catalytic nucleophile and acid/base residues. 31 Nascent GBA1 that emerges from ER-associated ribosomal protein synthesis undergoes N-glycosylation to yield, within the ER lumen, newly formed glycoprotein featuring three to four high mannose-type N-glycans. After proceeding through the ER quality control system (the calnexin-calreticulin cycle) and the Golgi apparatus, at which stage three of the high-mannose-type N-glycans are trimmed and reglycosylated to yield complex N-glycans, the GBA1 protein is shuttled to lysosomes by means of the mannose 6phosphate independent receptor. During this process, GBA1 associates with the lysosomal integral membrane protein 2 (LIMP2) chaperone, from which it detaches once in the acidic environment of lysosomes. 32 GBA1 has an optimal catalytic activity at around pH 5.2 ex vivo 33 and requires the sphingolipid activator protein, saposin C, to hydrolyze GlcCer located in the lysosomal membrane.<sup>34</sup> As described above, deficiency of GBA1 as caused by inherited mutations of the GBA1 gene are at the basis of GD. Of note, clinical and genetic evidence has shown that GBA1 mutations are also a risk factor for the development of Parkinson Disease. 35,36

The non-lysosomal β-glucosidase GBA2 (EC 3.2.1.45, GH116) is thought to locate closely to the cell plasma membrane<sup>37</sup> and on the cytosolic surface of the ER and Golgi apparatus.<sup>38</sup> It is a protein encompassing 927 amino acids and is encoded by the GBA2 gene located on human chromosome 9 at position p13.3.<sup>39</sup> The enzyme employs E527 as catalytic nucleophile and D677 as acid/base and a retaining mechanism in catalyzing β-glucoside hydrolysis.<sup>31</sup> Ex vivo, GBA2 shows an optimal hydrolysis ability at around pH 5.8.<sup>40</sup> In humans, GBA2 is mainly expressed in brain, heart, skeletal muscle, kidney and placenta and at lower levels also in liver, spleen, small intestine and lung. GBA2 has been considered to play a compensatory role in GD through hydrolysis of accumulating GlcCer. GBA1 deficient mice and cells from GD patients show elevated GBA2 levels as well as an increase in in vitro GBA2 activity.<sup>41,42</sup> The action of GBA2 in GBA1 deficient GD patients may actually be harmful and inhibition of GBA2 may be beneficial. GBA2 deletion in type I GD mice has been reported to rescue the disease phenotype, despite an increase in GlcCer and GlcSph levels.<sup>43</sup> The mechanism for the observed beneficial action of GBA2 deletion in GD mice is not fully understood yet. Mutations in GBA2 have furthermore been associated with spastic paraplegia and cerebellar ataxia.<sup>44-46</sup>

The cytosolic  $\beta$ -glucosidase GBA3 (EC 3.2.1.21, GH1), also referred as non-specific  $\beta$ -glucosidase, broad-specificity  $\beta$ -glycosidase, and Klotho related protein (KLrP), is a retaining  $\beta$ -glucosidase composed of 469 amino acid and having a broad substrate specificity. GBA3 is encoded by the *GBA3* gene located in locus 4p15.2, and is expressed as cytosolic protein in human liver, kidney, intestine, and spleen. GBA3 employs E373 as catalytic nucleophile and E165 as catalytic acid/base<sup>31,48</sup> and has an optimum pH at around 6 to 7. It has been reported that GBA3 can efficiently hydrolyze the artificial C6-NBD-GlcCer but natural GlcCer only at a very low rate. Besides  $\beta$ -glucosides, GBA3 is also able to hydrolyze  $\beta$ -D-galactosides,  $\beta$ -D-fucosides,  $\alpha$ -L-arabinosides and  $\beta$ -D-xylosides. However, the endogenous substrates of GBA3 in human are not known. Beyond GBA1, GBA2 and GBA3, intestinal lactase-phlorizin hydrolase (LPH, EC 3.2.1.23/62, GH1) exclusively found on the brush border of the mammalian small intestine, features retaining  $\beta$ -glucosidase activity. This enzyme was not subject of the research described in this Thesis.

**Table 1.** General overview of the human retaining exo-β-glucosidases, GBA1, GBA2 and GBA3.

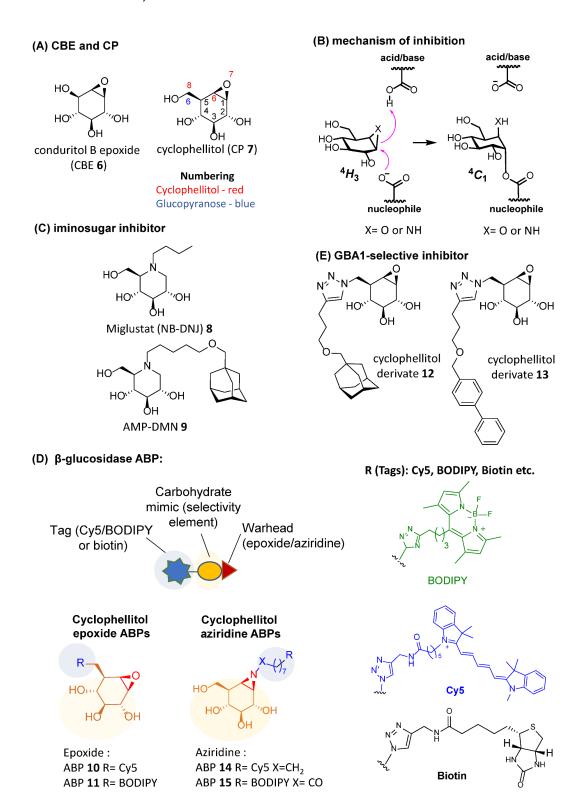
Enzyme	GBA1	GBA2	GBA3
GH family	30	116	1
Sub-cellular localization	lysosome	plasma membrane <sup>37</sup> / cytosolic surface of ER, Golgi <sup>38</sup>	cytosol
Tissue distribution	ubiquitous	brain, heart, skeletal muscle, kidney and placenta	liver, kidney, intestine, and spleen
Optimum pH	5.2	5.8	6.0 - 7.0
Additives for activity	additives needed <sup>33</sup>	no additives <sup>40</sup>	no additives
Gene mutant consequence	Gaucher Disease	Associated with spastic paraplegia and cerebellar ataxia	unknown

#### Chemical tools for studying retaining exo-β-glucosidases

Over the past decades, a series of chemical tools have been developed for retaining exo- $\beta$ -glucosidase (activity) detection, inhibition, and visualization, including fluorogenic substrates, inhibitors, and activity-based probes (ABPs). The artificial substrate, 4-methylumbelliferyl- $\beta$ -D-glucopyranoside **5** (4-MU- $\beta$ -D-Glc) is composed of a glucose and a 4-methylumbelliferone (4-MU) for group (which is non-fluorescent when attached to the sugar). It can be employed to reflect the  $\beta$ -glycosidic bond hydrolysis activity of retaining exo- $\beta$ -glucosidases through detecting the released, fluorescent 4-MU.

Figure 3. 4-Methylumbelliferyl- $\beta$ -D-glucopyranoside 5 fluorogenic substrate.

Small molecule β-glucosidase inhibitors have received considerable attention in studying the mechanism and active site residues of retaining  $\beta$ -glucosidases, as well as their potential value for therapeutic application. Conduritol B epoxide (CBE 6) and cyclophellitol (CP 7) have been reported as retaining β-glucosidase-selective inhibitors decades ago (see for their chemical structures Figure 4). CBE 6 and CP 7 both are cyclitol epoxides that covalently and irreversibly reacts with the catalytic nucleophile of retaining  $\beta$ -glucosidases. <sup>57,58</sup> CBE **6** and cyclophellitol **7** are close structural homologues and their mode of action in inhibiting retaining β-glucosidases is very similar. The inactivation involves attack of the catalytic nucleophile at the pseudo-anomeric position, resulting in opening of the epoxide and the formation of a stable and irreversible enzyme-inhibitor ester adduct. This process is facilitated by protonation of the epoxide by the general acid/base residue (Figure 4B). Compared to CBE 6, CP 7 has an extra methylene, breaking the symmetry inherent to CBE 6. As a result, and in contrast to CBE 6, CP 7 has considerable selectivity for retaining beta-glucosidases (note that rotation of CBE over the axis bisecting C3-C4 and C1-C6 yields a close alpha-glucoside analogue, explaining the inhibitory effect of CBE also on retaining  $\alpha$ -glucosidases). An important class of competitive GBA1-3 inhibitors are the N-alkylated deoxynojirimycins such as N-butyl-1-deoxynojirimycin (NB-DNJ, Miglustat, 8) and Nadamantanemethyloxypentyl-deoxynojirimycin (AMP-DNM, 9), both originally developed as GCS inhibitors that were subsequently shown to be effective GBA2 inhibitors as well.<sup>61,62</sup> Activity-based probes (ABPs) finally are powerful chemical tools<sup>63-66</sup> to investigate, retaining  $\beta$ -glucosidases in vitro (also in cellular extracts), in cells and in living organisms (see Figure 4).<sup>64-69</sup> They are composed of a reactive chemical warhead (to react within the enzyme active site to form a covalent and irreversible enzyme-inhibitor adduct), a recognition element (here a glucopyranose-like structural element) and a reporter tag (fluorescent tag or affinity tag for visualization or purification). The design of  $\beta$ -glucosidase ABPs exploits the scaffold of cyclophellitol 7<sup>70,71</sup> onto which a reporter (fluorescent or affinity tag) at C8 of cyclophellitol (for GBA1 selectivity) or at he nitrogen atom of cyclophellitol aziridine (for broadspectrum reactivity towards retaining β-glucosidases) is grafted. C8-modified cyclophellitol ABPs 10 and 11 potently and selectively label GBA1 over other the other two human retaining β-glucosidases, GBA2 and GBA3.<sup>60,72</sup> Selective labelling of GBA1 by these ABPs was observed in all tested lysates, except in those of the small intestine, in which LPH, and fragments thereof, where also found to be modified.<sup>72</sup> Vocadlo and co-workers designed fluorogenic substrates equipped with a fluorophore at C6 of a βglucoside, the aglycon of which carried a fluorescence quencher, compounds that proved to be efficient GBA1-selective substrates able to image GBA1 activity in situ.<sup>59</sup> Artola and co-workers generated cyclophellitol-C8-modified inhibitors (12, 13) that proved to be potent and selective GBA1 inhibitors both in vitro and in vivo. 60 Considering that the other two retaining β-glucosidases did only accept C8modified cyclophellitols, cyclophellitol aziridine scaffolds were developed in which the nitrogen atom of the aziridine was utilized to introduce a fluorescent reporter group (Cy5, BODIPY).73 Cyclophellitol aziridine ABPs 14 and 15 proved to be broad-spectrum retaining  $\beta$ -glucosidase ABPs that label, besides GBA1, also GBA2, GBA3, and LPH. 73,74 These ABPs report on retaining β-glucosidases irrespective of their origin, this due to the result of conservation of the catalytic pockets. This has allowed the study of retaining β-glucosidases in plants, 75,76 zebrafish (Danio rerio), 60 and mice. 72,73 At the onset of the studies presented in this Thesis, however, no mechanism-based, covalent and irreversible inhibitors, and ABPs derived thereof, selective for either GBA2 or GBA3 were known.



**Figure 4.** Retaining β-glucosidase inhibitors and ABPs. (A) Structure of CBE **6** and CP **7.** (B) Mechanism by means of which cyclophellitol and cyclophellitol aziridine inhibit retaining β-glucosidases. (C) The competitive GBA1-3 inhibitors Miglustat **8** and AMP-DNM **9**. (D) Structure of β-glucosidase ABPs. (E) Cyclophellitol-C8-modified GBA1-selective inhibitors **12** and **13**.

#### Thesis outline

The overall aim of the research described in this Thesis was to employ activity-based protein profiling (ABPP) to study retaining  $\beta$ -glucosidases. Part of the research comprises the discovery and application of inhibitors and activity-based probes (ABPs) for each of the three human retaining βglucosidases, GBA1, GBA2 and GBA3. These studies are complemented by research in which the inhibitors and probes are used to study homologous retaining β-glucosidase activities from other species. Chapter 2 comprises a study in which three structurally and configurationally closely related cyclitol epoxides and aziridines, namely those derived from conduritol B epoxide, β-D-xylocyclophellitol and cyclophellitol, are compared for their activity and selectivity as GBA1 inhibitors and activity-based probes. Chapter 3 studies β-D-arabinofuranose-configured cyclophellitol aziridine ABPs as selective reporters of mammalian GBA2 activities in vitro and in situ. Chapter 4 concerns the exploration of potential GBA3-selective fluorogenic substrates and fluorescent ABPs. Chapter 5 reports on the use of the established cyclophellitol-based ABPs to probe Caenorhabditis elegans (C. elegans) samples for β-glucosidase activities and to establish similarities and differences between these and the mammalian enzymes (GBA1, GBA2). Chapter 6 utilizes the same set of ABPs to study the activity of a putative β-glucosidase (termed B56) from the tobacco plant Nicotiana tabacum. Chapter 7 discusses the results of the experiments described in Chapters 2-6 and presents some prospects for future research. This thesis is concluded with Appendices that include a list of publications and a Curriculum vitae of the author.

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