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Exploring the versatility of human β -glucosidases and related glycosylated metabolites with novel chemical tools

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6-*O*-Alkyl 4-methylumbelliferyl- β -D-glucosides as selective substrates for GBA1 in the discovery of glycosylated sterols

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2.1 Introduction

The lysosomal retaining β -D-glucosidase (EC. 3.2.1.45), glucocerebrosidase (*aka* GCase or GBA1), is a 497-amino acid glycoprotein responsible for removing the glucose moiety from glucosylceramide (GlcCer), an essential step in lysosomal breakdown of most glycosphingolipids (GSLs).^{1,2} Mutations in the *GBA1* gene at locus 1q21 can lead to cellular deficiency of glucocerebrosidase activity and cause Gaucher disease (GD). In GD patients, there is prominent lysosomal accumulation of GlcCer in tissue macrophages, which undergo transformation into enlarged viable “Gaucher cells”, a process accompanied with the manifestation of a wide range of symptoms.³⁻⁵ The most frequent phenotypic manifestation of GD in Caucasian populations is the non-neuronopathic variant of the disease, often indicated as type 1.⁵ More severe neuronopathic variants of GD, such as type 2 and 3, also occur. The heteroallelic presence of the N370S GBA1 substitution is associated with type 1 GD, while homoallelic presence of the L444P GBA1 substitution is associated with pathology of the central nervous system.^{2,6} Currently, there are two registered therapies for the treatment of type 1 GD: enzyme replacement therapy (ERT) and substrate reduction therapy (SRT).^{7,8} ERT involves the intravenous administration of recombinant rhGBA1 every two weeks, resulting in a decrease of visceral symptoms including organomegaly and haematological abnormalities. Due to the inability of the enzyme to pass the blood brain barrier, ERT does not correct neurological symptoms present in neuronopathic variants of GD. SRT, concerning the daily oral administration of glucosylceramide synthase (GCS) inhibitors (Miglustat or Eliglustat) focuses on reducing GSL synthesis. As a consequence of GCS inhibition, the residual GBA1 activity of GD patients is then sufficient and prevents accumulation of GlcCer.^{7,8} It has been recognized that mutations in the *GBA1* gene, even when only one allele has been mutated, pose a risk factor for developing Parkinson’s disease (PD).^{9,10} However, the link between GBA1 defects and PD is still enigmatic.

Early therapeutic intervention in GD is crucial to prevent severe complications like massive fibrous splenomegaly, avascular necrosis, and pathological fractures.⁴ Recently, an international network of expert centres has reviewed type 1 GD laboratory diagnosis and recommends the measurement of reduced GBA1 activity as the central confirmatory assessment of the disease.⁵ Genotyping and the analysis of circulating biomarkers are suggested as secondary tests. The most common method for laboratory diagnosis of GD involves measuring GBA1 activity in white

blood cells or fibroblasts using the artificial fluorogenic substrate, 4-methylumbelliferyl- β -D-glucopyranoside (4MU- β -Glc) and benchmarking it against a standard.⁵ However, presence of both the non-lysosomal cytosol-facing membrane bound GBA2 and broad substrate specific cytosolic GBA3 enzymes in materials contribute to the released 4MU, thereby complicating the analysis.^{11, 12} The use of specific substrates for GBA1 could provide clearer insight in the contribution of GBA1 to the released 4MU from 4MU- β -Glc. However, the restricted availability of highly specific GBA1 inhibitors, e.g. covalent inhibitor **ME656**,¹³ limits the use of the more sophisticated GBA1 activity measurement in biological materials. Therefore, there is a need for a fluorogenic 4MU-substrate specific for GBA1 that is accessible to the GD biomedical community. It has been previously established that 4-methylumbelliferyl- β -D-xylopyranoside (4MU- β -Xyl) is a specific substrate of GBA1; however, it exhibits a significantly lower $V_{\max}$ (~50 fold) when compared to 4MU- β -Glc.¹⁴ In terms of specificity, the CH₂OH group at C-6 of the sugar appears highly relevant. This observation is further supported by C-6-modified cyclophellitol based inhibitors (carbohydrate numbering) which selectively target GBA1 over GBA2 and GBA3 and exhibit a 100-fold higher potency compared to unmodified cyclophellitol-epoxide.^{15,16} The same approach of extending the C-6 position of the sugar has also been used by Vocablo and coworkers in the design and synthesis of his fluorescence-quenched substrates for live cell imaging of human glucocerebrosidase activity.¹⁷ Building upon this knowledge a series of 6-*O*-acylated and alkylated 4MU- β -Glc derivatives were designed and synthesized with the aim of developing candidate substrates that specifically interact with GBA1. The observed hydrolysis of 6-*O*-acyl-4MU- β -Glc substrates by GBA1 prompted the search for the naturally occurring glycosylated sterols and their acylated derivatives in human spleen by LC-MS/MS analysis. The outcome of the investigation is here reported and was instrumental in the discovery of natural 6-*O*-acyl-glycosyl-sterols and 6-*O*-acyl-glycosyl-phytosterols as natural substrates of GBA1. Of interest, the occurrence of glycosylated sterols in man was earlier described by Akiyama and colleagues¹⁸ and Marques and coworkers¹⁹ as well that of plant type glycosylated β -sitosterol.²⁰

2.2 Results

2.2.1 Synthesis and biochemical characterization of substrates 2-10

Several different 6-*O*-acyl-4MU- β -Glc derivatives (**2**: caproyl; **3**: palmitoyl; **4**: stearoyl; and **5**: oleoyl, Figure 1A) were first synthesized. For this purpose, commercial 4MU- β -Glc was acylated using the corresponding carboxylic by means of an enzymatic reaction catalysed by lipase Novozyme 435.

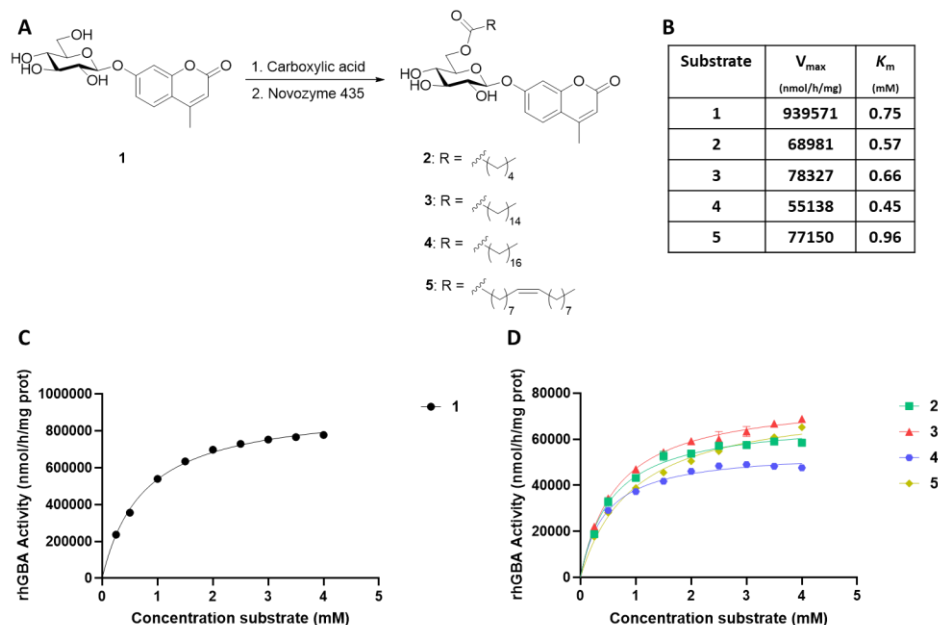


Figure 2.1. **A**) Enzymatic synthesis of 6-*O*-acyl-4MU- β -Glc derivatives with variable lipids (**2**: caproyl; **3**: palmitoyl; **4**: stearoyl and **5**: oleoyl). **B**) Maximum rate of hydrolysis ($V_{\max}$) and the Michaelis constant (K_m) for fluorogenic substrates **1-5**. **C**) rhGBA activity using 4MU- β -Glc (**1**). **D**) rhGBA activity using 6-*O*-acyl-4MU- β -Glc substrates **2-5**.

Compounds **2-5** were tested as substrates for GBA1 using pure recombinant GBA1 (rhGBA, Cerezyme[®]), revealing that rhGBA1 retains hydrolytic activity towards substrates with extensions at C-6 (Figure 2.1B and 2.1D). Specifically, rhGBA1 processes 6-*O*-acyl glucosides **2-5** with about equal efficiency as it does 4MU- β -Glc, irrespective of the chain length of the 6-*O*-acyl group. When comparing the maximum hydrolysis rate ($V_{\max}$) of GBA1 with 4MU- β -Glc and with the 6-*O*-acyl glucosides **2-5**, there was approximately a 12-fold decrease.

The ester linkage in compounds **2-5** is intrinsically vulnerable to esterase-mediated processing in cells and cell extracts, which may be a caveat for use of the substrates

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in such complex biological samples. With this in mind a second generation of 6-*O*-acyl glucosides was synthesized based on 6-*O*-palmitoyl-4MU- β -Glc (**3**) (Figure 2.2). Substrate **3** was used as parent compound since it showed the most favourable kinetics independent of the chain length. Five different chemical linkages were selected for diversification: **6** with an amide linkage, **7** with a thioester linkage, **8** with a thioether linkage, **9** with an ether linkage and **10** with an amine linkage.

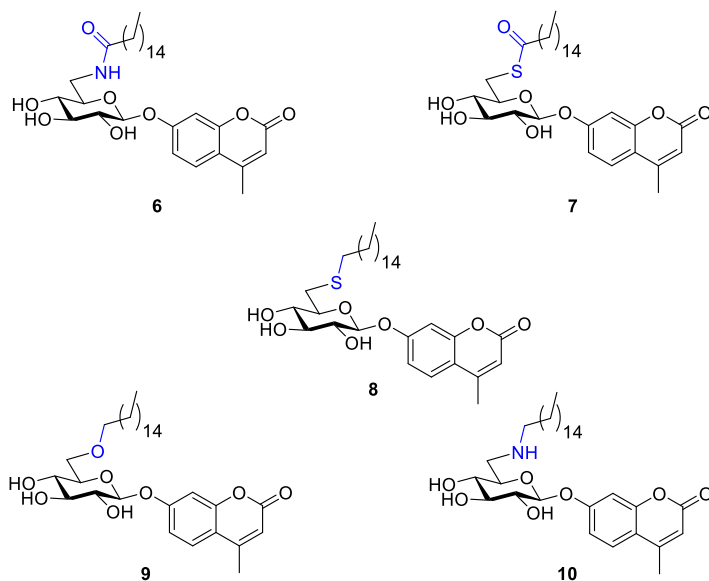
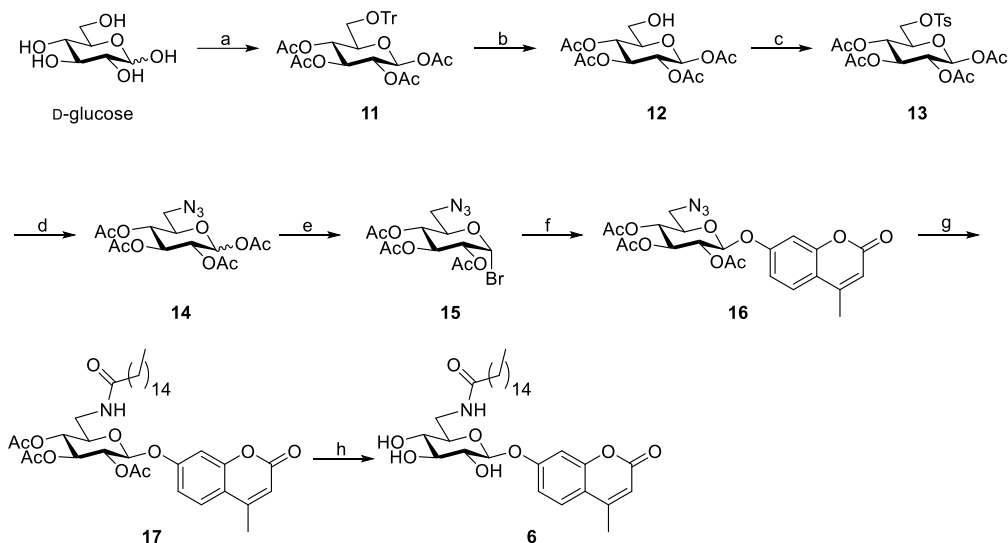


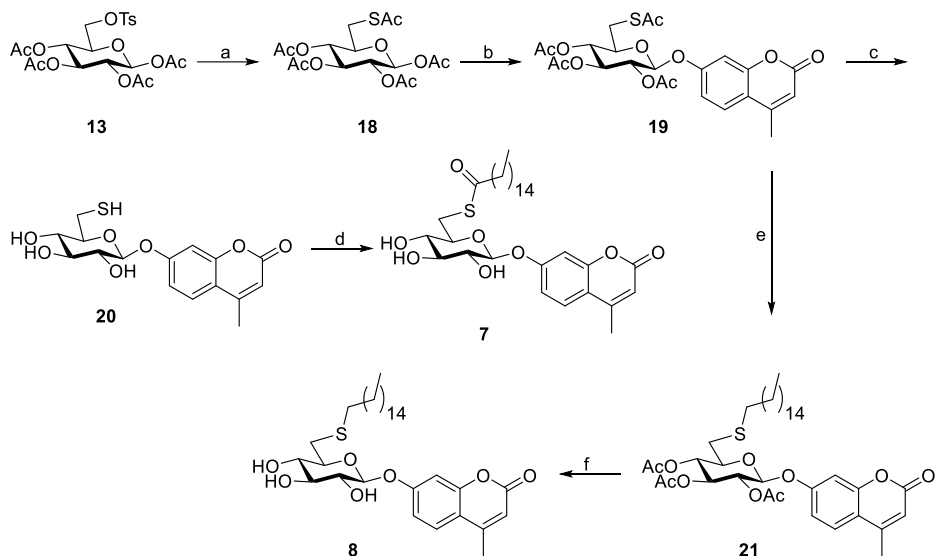
Figure 2.2. Chemical structures of second generation GBA1 substrates **6-10**.



Scheme 2.1. Synthesis of 6-*O*-amide-4MU- β -Glc **6**. Reagent and conditions: a) i. TrCl, dry pyridine, 80 °C, 18 h; ii. Ac₂O, 0 °C to r.t., 18 h, 38% (2 steps). b) HBr (33% in AcOH), AcOH, 0 °C, 5 min, 58%. c) TsCl, dry pyridine, 0 °C to r.t., 18 h, 70%. d) NaN₃, 15-crown-5, DMF, 50 °C, 18 h, 93% (α : β 1:0.4). e) TMSBr, BiBr₃, CH₂Cl₂, 0 °C to r.t., 18 h, 91%. f) 4-MU, NaOH, Acetone/H₂O (1:1), r.t., dark, 18 h, 62%. g) i. palmitic acid, HOBT, EDC, MeCN/CH₂Cl₂, 0 °C, 5 min; ii. **16**, PBu₃, dark, 5.5 h, 79%. h) NaOMe, MeOH/CH₂Cl₂, r.t., 4 h, 77%.

The synthesis towards 6-*O*-amide-4MU- β -Glc substrate **6** started with a one-pot C-6 hydroxyl tritylation and subsequent per-acetylation of D-glucose. The trityl group was then deprotected with 33% HBr in acetic acid to generate intermediate **12**. Tosyl chloride in dry pyridine was used to tosylate the primary hydroxyl, which was reacted with sodium azide and 15-Crown-5 ether to generate azido intermediate **14**. Of note, the basic conditions lead to an epimerization of the anomeric bond. The anomeric position was then brominated using a mixture of TMSBr and bismuth tribromide after which the fluorogenic moiety was coupled using sodium hydroxide in a mixture of acetone and water. Intermediate **16** was treated with palmitic acid in the presence of HOBT, EDC and tributylphosphine to yield intermediate **17** in 79% yield. This reaction was performed in one-pot to prevent the acetyl migration from C4 to C6, which was observed when using Staudinger ligation conditions. Final deprotection of the acetyl protecting groups then yielded **6** in 0.05% over 9 steps.

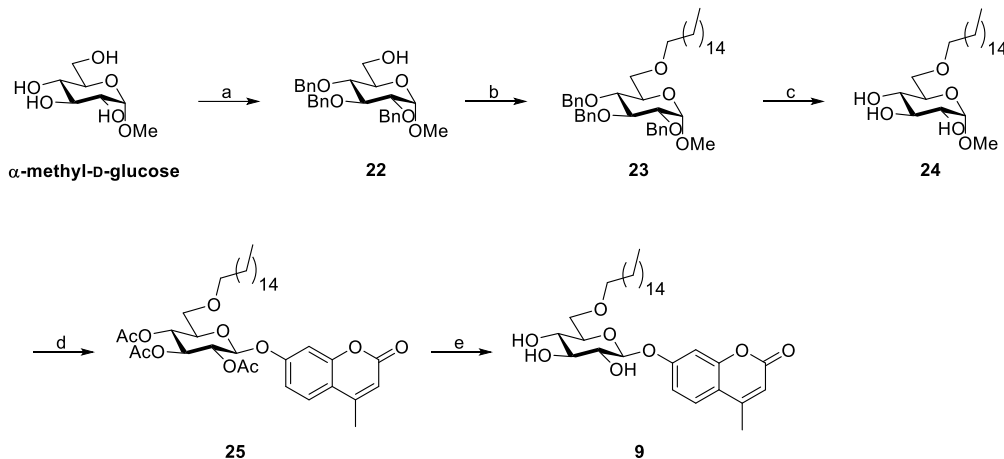
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Scheme 2.2. Synthesis of 6-*O*-thioester-4MU-β-Glc and 6-*O*-thioether-4MU-β-Glc substrates **7** and **8**, respectively. Reagent and conditions: a) KSac, DMF, r.t., 18 h, 73%. B) i. HBr (33% in AcOH), CH₂Cl₂, 0 °C to r.t., 5 h; ii. 4MU, NaOH, Acetone/H₂O (1:1), r.t., dark, 18 h, 32% (2 steps). C) NaOMe, MeOH/CH₂Cl₂, r.t., 4 h, 78%. D) palmitoyl chloride, Et₃N, DMF/CH₂Cl₂ (2:1), 0 °C to r.t., 6 h, 95% (brsm). E) i. NH₂NH₂·H₂O, AcOH, DMF, r.t., 1 h; ii. 1-bromohexadecane, Et₃N, CH₂Cl₂, 0 °C to r.t., 18 h, 87% (2 steps brsm). F) NaOMe, MeOH/CH₂Cl₂, r.t., 4 h, 77%.

Thio-derived 4MU-β-Glc substrates **7** and **8** were synthesized from intermediate **13**. A thioacetate was first installed at C6 by treatment of intermediate **13** with potassium ethanethiolate in DMF. The anomeric position was subsequently brominated with 33% of HBr in acetic acid since bismuth tribromide and TMSBr lead to decomposition of the brominated intermediate. The crude bromide was immediately coupled after a simple work up to 4MU using sodium hydroxide in a mixture of acetone and water. Peracetylated intermediate **19** was then used to generate both products **7** and **8**. Substrate **7** was synthesized by first full deprotection of the acetyl protecting groups using sodium methoxide in methanol followed by selective acylation of the thiol with palmitoyl chloride. Precautions were taken to mitigate excessive acylation of the secondary hydroxyl groups and unreacted starting material could easily be recovered in this final step leading to a yield of 95% based on recovered starting material. For the synthesis of alkylated product **8**, the thioacetate in **19** was first deprotected by treatment with hydrazine and subsequently alkylated without purification using 1-bromohexadecane and

triethylamine to yield intermediate **21** in 87% over 2 steps. Final deprotection using sodium methoxide yielded the desired final compound **8**.



Scheme 2.3. Synthesis of 6-O-alkyl-4MU- β -Glc linked substrate **9**. Reagent and conditions: a) i. TBSCl, pyridine, 0 °C to r.t., 2 h; ii. BnBr, NaH, DMF, 70 °C, 3 h; iii. HCOOH/H₂O (4:1), THF, 0 °C to r.t., 3 h, 62% (3 steps). b) 1-bromohexadecane, NaH, DMF, 0 °C to r.t., 16 h, 89%. c) Pd/C, H₂, HCl, EtOAc/EtOH (1:1), r.t., 4 h, 96%. d) i. H₂SO₄, Ac₂O/AcOH, 0 °C to r.t., 18 h; ii. TMSBr, BiBr₃, CH₂Cl₂, 0 °C to r.t., 18 h; iii. 4MU, NaOH, Acetone/H₂O (1:1), r.t., dark, 18 h, 36% (3 steps). e) NaOMe, MeOH/CH₂Cl₂, r.t., 4 h, 74%.

The synthesis of compound **9** started from α -methyl-D-glucose and partially protected intermediate **22** was obtained following described procedures.²¹ Subsequent alkylation of **22** with bromohexadecane using sodium hydride in DMF lead to formation of compound **23** in 89%. The benzyl protecting groups were removed using standard palladium on carbon hydrogenation conditions in 96% yield. Intermediate **24** was per-acetylated using sulfuric acid in an acetic acid and acetic anhydride mixture, and the crude intermediate was subsequently brominated using TMSBr and a catalytic amount of bismuth tribromide. After work up, the unstable brominated intermediate was immediately coupled to 4MU using NaOH to generate intermediate **25** in 37% over 3 steps. Methanolysis of the acetates using sodium methoxide gave compound **9** in 11% yield over 9 steps.

on carbon and hydrogen gas. Final column chromatography lead to the isolation of the desired product **10** in a 2% yield over the last five steps.

The newly synthesized 4MU- β -Glc derivatives **6-10** were subsequently assessed as substrates for rhGBA1 (Figure 2.3). To ensure proper solubility of the substrates, all samples were supplemented with 2.5% DMSO (v/v) and 0.5% Triton X-100 (v/v). Surprisingly, only the ether-linked derivative **9** was processed by rhGBA1 while all other derivatives proved to be poor substrates for GBA1. The maximum rate of the enzymatic cleavage ($V_{\max}$) for 6-*O*-alkyl-4MU- β -Glc **9** was around 3-fold lower than that for 4MU- β -Glc but approximately 5-fold higher than its parent 6-*O*-palmitoyl-4MU- β -Glc substrate **3**. Furthermore, the affinity of **9** for the enzyme, as indicated by the lower K_m value, is higher for **9** when compared to both **3** and 4MU- β -Glc. This clearly indicates that the removal of the carbonyl moiety in the ester-linked substrate **3** enhances binding to the active site.

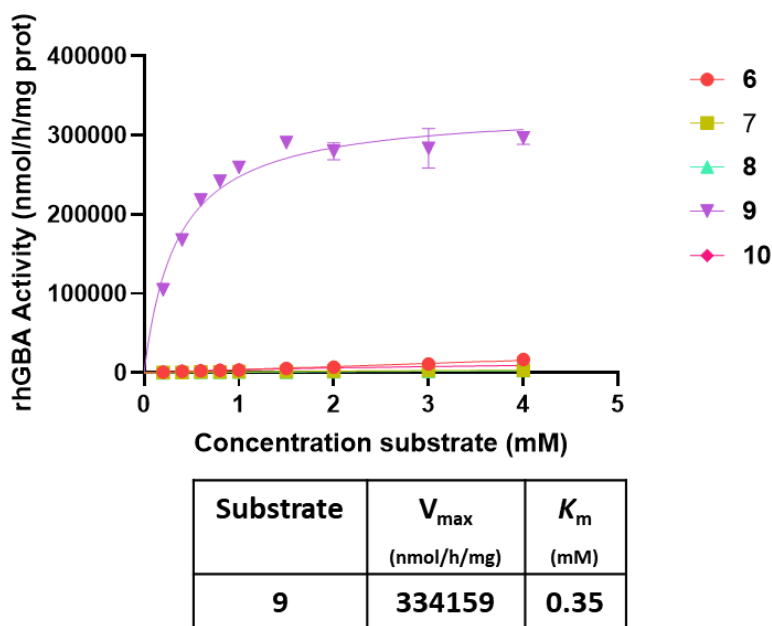


Figure 2.3. Maximum rate of hydrolysis ($V_{\max}$) and the Michaelis constant (K_m) for fluorogenic substrates **6-10**.

The pH-optima of fluorogenic substrates **1**, **3** and **9** were then determined (Figure 2.4A-C). While 4MU- β -Glc showed a pH-optimum of 5.4 (Figure 2.4A), a broader pH-optimum between 4.8 and 5.6 was observed for substrates **3** and **9** (Figures 2.4B

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and 2.4C), and the pH-optima coincide with the acidic pH of lysosomes where GBA1 resides.

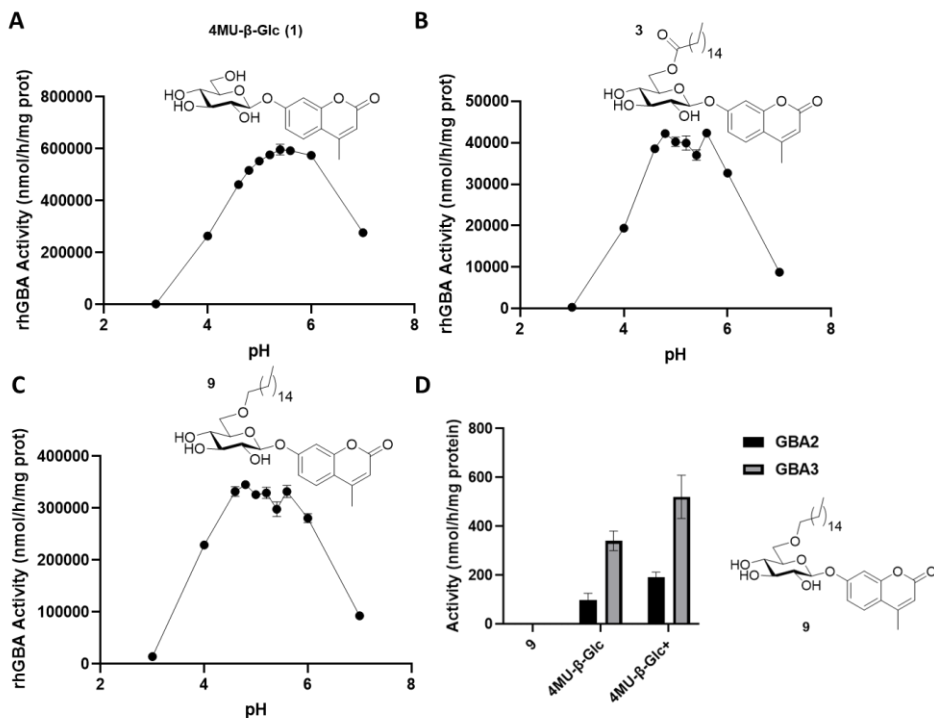


Figure 2.4. Profile of the pH-dependent processing by rhGBA1 of 4MU- β -Glc **1** (A), **3** (B) and **9** (C) at 1 mM of substrate. D) Hydrolysis of 1 mM of **9** (2.5% DMSO supplemented with 0.5% Triton X-100), 1 mM of 4MU- β -Glc, and 1 mM of 4MU- β -Glc + (2.5% DMSO supplemented with 0.5% Triton X-100) by GBA2 and GBA3 incubated for 30 minutes at 37 °C. For GBA2 activities, HEK293T cells overexpressing GBA2 with a concentration of 4.9 mg/mL and a pH of 5.8 were used, whereas for GBA3 HEK293T cells overexpressing GBA3 with a concentration of 5.4 mg/mL and an optimal pH of 7.0 were used.

After identifying a fluorogenic substrate that performs optimally at a pH level similar to that of lysosomes under physiological conditions, the specificity for lysosomal GBA1 was examined using various HEK293T cell lines overexpressing GBA2 or GBA3 (Figure 2.4D). Substrate concentrations of 1 mM were used exceeding in all cases the K_m values by two-fold. The ether-linked substrate **9** was not hydrolysed by GBA2 and GBA3, while 4MU- β -Glc was hydrolysed by GBA2 and GBA3 both with and

without the required additives to solubilize **9**, indicating that the synthesized substrate is a specific substrate for GBA1.

2.2.2 Diagnosis of Gaucher disease with 4MU substrates: GBA1 selectivity of substrate **9**

Human spleen is known to be rich in the β -glucosidases GBA1, GBA2 and GBA3.²⁴ To see how substrate **9** behaves in a complex system, the enzymatic activity in lysates of spleens from non-GD control individuals and GD patients was measured using 4MU- β -Glc **1**, the 6-*O*-alkyl-4MU- β -Glc substrate **9** and 4MU- β -Xyl as substrates. Assays were performed following pre-treatment with and without the GBA1-specific suicide inhibitor **ME656**.¹³ The activity measured with 4MU- β -Xyl, even without **ME656** treatment, was extremely low (<2 nmol/h/mg protein for control spleen lysates, Figure S2.1). The activity measured with the new substrate **9** was considerably higher (5 - 19 nmol/h/mg protein, Table 1). The table displays the total β -glucosidase activity in the spleen lysates towards 4MU- β -Glc **1** and **9**. Importantly, when using **9** as substrate, a clearly reduced activity was observed in the GD spleen lysates compared to the control ones. Pretreatment of the spleen lysates with **ME656** eliminated virtually all activity towards substrate **9**. This result further suggests that substrate **9** indeed allows specific assessment of GBA1 activity. Of note, the activity towards 4MU- β -Glc **1** detected in spleen lysates that were pretreated with **ME656** reflects the enzymes GBA2 and GBA3 activities, and this combined GBA2 and GBA3 activities in Gaucher spleen lysates is quite comparable to that in control spleen lysates.

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Table 2.1. β-Glucosidase activity in non-GD spleen lysates (samples C1-C5) and GD spleen lysates (samples GD1-GD8) using 4MU-β-Glc **1** and 6-O-alkyl-4MU-β-Glc substrate **9**, with or without pretreatment with selective and covalent GBA1 inhibitor ME656.

Spleen sample		Protein (mg/ml)	Activity substrate 1 – ME656 (nmol/h/mg protein)	Activity substrate 1 + ME656 (nmol/h/mg protein)	Activity substrate 9 – ME656 (nmol/h/mg protein)	Activity substrate 9 + ME656 (nmol/h/mg protein)
Control	C1	65.2	22.0 ± 2.4	5.4 ± 0.4	12.2 ± 1.1	0.0 ± 0.0
	C2	53.2	12.0 ± 1.6	3.8 ± 0.7	5.1 ± 0.6	-0.1 ± 0.0
	C3	37.0	13.2 ± 1.8	2.4 ± 0.4	6.2 ± 1.6	0.0 ± 0.0
	C4	50.0	26.5 ± 1.8	3.8 ± 0.2	19.0 ± 0.7	0.0 ± 0.0
	C5	39.6	22.7 ± 0.7	12.8 ± 1.8	8.9 ± 0.2	0.2 ± 0.0
Gaucher disease	GD1	52.6	5.0 ± 0.7	3.8 ± 0.6	0.9 ± 0.0	0.0 ± 0.0
	GD2	66.5	5.0 ± 0.7	2.9 ± 0.6	1.4 ± 0.1	0.0 ± 0.0
	GD3	51.4	3.2 ± 0.3	2.3 ± 0.3	0.8 ± 0.1	0.0 ± 0.0
	GD4	54.7	4.7 ± 0.7	4.4 ± 0.6	0.1 ± 0.1	-0.1 ± 0.0
	GD5	53.9	5.2 ± 0.5	3.9 ± 0.6	0.3 ± 0.0	-0.1 ± 0.0
	GD6	52.9	2.8 ± 0.6	1.9 ± 0.6	0.4 ± 0.6	-0.1 ± 0.6
	GD7	51.4	3.4 ± 0.3	2.0 ± 0.1	0.1 ± 0.0	-0.1 ± 0.0
	GD8	54.6	3.2 ± 0.4	2.3 ± 0.3	0.1 ± 0.0	-0.1 ± 0.0

2.2.2 Natural occurrence of 6-O-acyl-glucosyl-sterols and increased levels in GD spleens.

Plants contain specific sterols which can be conjugated with glucose and 6-O-acyl glucose, collectively named 6-O-acyl-sterolins.^{25,26} Except for the occurrence and metabolism of glucosylcholesterol (GlcChol) and galactosylcholesterol (GalChol), grouped as glycosylcholesterol (HexChol), the existence of glycosylated sterols in humans is still poorly studied.²⁷⁻²⁹ Knowing that GBA1 is able to process compounds **3** and **9**, the natural occurrence of 6-O-acyl-glucosyl-lipids and their activity as GBA1 substrates was evaluated. Plants contain high levels of glycosylated phytosterols and 6-O-acyl derivatives and it was hypothesized that uptake of these lipids through nutrition might lead to their accumulation in tissues of GD patients. Thus the presence of HexChol and plant-derived phytosterols (stigmasterol, β-sitosterol and campesterol) in GD and non-GD spleens was investigated using LC-MS/MS. As

illustrated in Figure 2.5, the levels of HexChol tended to be higher in GD spleens. Strikingly, also the levels of glycosyl-stigmasterol, glycosyl- β -sitosterol and glycosyl-campesterol were found to be significantly elevated in the patients' spleens. Most striking was the elevation of plant-derived glycosyl- β -sitosterol.

Next, the presence of 6-*O*-acyl-glycosyl-cholesterol and plant 6-*O*-acyl-glycosyl-phytosterols in spleens from control subjects and GD patients was examined (Figure 2.4). For these measurements an appropriate structurally related standard (6-*O*-palmitoyl- $^{13}\text{C}_6$ -GlcChol (**31**), Scheme S2.1) was synthesized and used. Importantly, 6-*O*-acyl-glycosyl-sterols were elevated in spleens from GD patients. Various 6-*O*-acyl-glycosyl- β -sitosterols were found to be increased in GD spleens as compared to control tissue (Figure 2.5 and S2.4). The levels of various 6-*O*-acyl-glycosyl-campesterols and particularly stigmasterol were lower in the examined spleens. For some 6-*O*-acyl-glycosyl-campesterols also significantly increased levels in GD spleen were observed. As illustrated in the supplemental Figure S2.3, although a similar trend was observed, analysis of 6-*O*-acyl-glycosyl-cholesterols using non-acyl $^{13}\text{C}_6$ -GlcChol as internal standard did not always reach statistical significance. A similar issue was encountered when analysing 6-*O*-acyl-glycosyl-sterols (Figure S2.4 *versus* S2.5). Therefore, the use of structural analogues as internal standards is recommended for the best quantification (Heat map illustrated in Figure S2.6). Of note, deacylation of 6-*O*-acyl-glycosyl-sterols is feasible. As shown in Figure S2.7 microwave-assisted deacylation or incubation for 10 hours at alkaline condition led to removal of the acyl-moieties.

Finally, the ability of rhGBA1 to fragment glucosylated phytosterols was assessed. As illustrated in Figure S2.8A, incubation of GlcChol, glucosyl stigmasterol and glucosyl sitosterol with rhGBA1 in the presence of the detergents Triton X-100 and taurocholate at pH 5.2 led to a comparable deglycosylation. Degradation of 6-*O*-palmitoyl-glucosyl-cholesterol or 6-*O*-palmitoyl-glucosyl-sitosterol by rhGBA1 was not prominent. Conceivably, the poor solubility of 6-*O*-acyl-glucosyl-sterols may have contributed to this.

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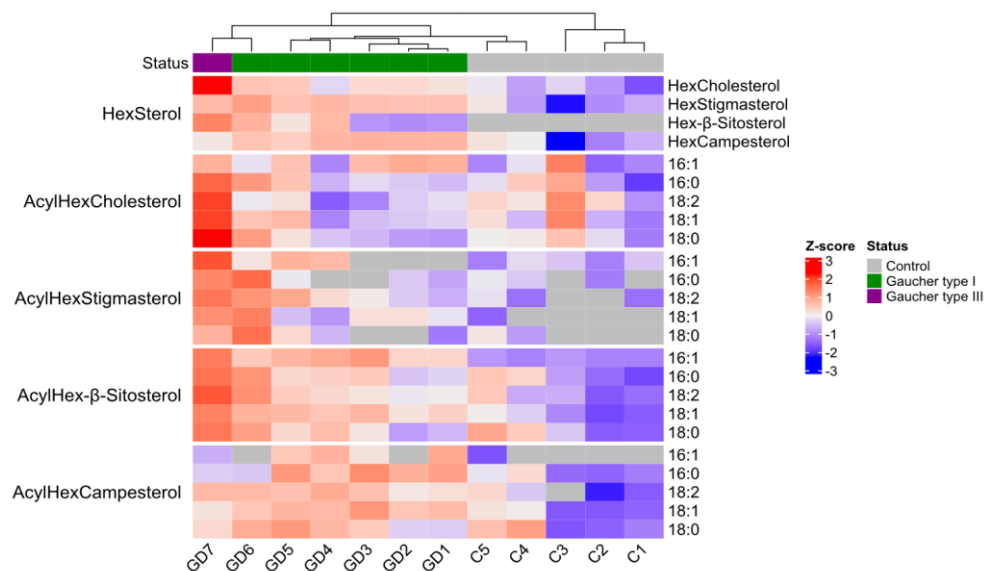


Figure 2.5. Heat map of glycosylcholesterol (HexChol) and glycosyl-stigmasterol, glycosyl- β -sitosterol, glycosyl-campesterol levels and their respective 6-O-acyl forms (16:0, 16:1, 18:0, 18:1 and 18:2) in spleens from non-GD and GD patients determined by LC-MS/MS analysis. Gaucher type 1, non-neuropathic variant of GD; Gaucher type 3, sub-acute neuronopathic GD case.

2.3 Discussion

It has been previously observed that, in contrast to the GBA2 enzyme, GBA1 tolerates a hydrophobic extension at C-6 (carbohydrate numbering) of cyclophellitol (and at C-5 according the cyclophellitol numbering). This observation led to the design of a specific suicide inhibitor for GBA1 equipped with hydrophobic moiety at the cyclophellitol C-5 (**ME656**).¹³ This inhibitor binds in a reaction mechanism-based manner to the catalytic nucleophile E340 of GBA1, but does not inactivate GBA2 or GBA3, two other cellular β -glucosidases.¹³ This specificity for GBA1 generated by instalment of a lipophilic group at cyclophellitol C-5 inspired Vocadlo and collaborators to design along this principle a sophisticated substrate for selective *in situ* cell measurement of GBA1 activity.^{17,30} Motivated by these findings, the aim here was to generate 6-O-acyl-4MU- β -glucosides with variable acyl length to investigate if GBA1 is able to process such artificial substrates and the ability of GBA1 to process related endogenous and exogenous substrates. The investigations described here confirm that 6-O-acyl-MU- β -Glc substrates, accessible through straightforward enzymatic synthesis, can indeed act as substrates for human GBA1.

To prevent the potential esterase-mediated degradation of the acyl chain of these substrates in cell lysates, the ester bond was successfully replaced by other linkages leading to five new 6-*O*-acyl-4MU- β -Glc substrate analogues (**6-10**). Subsequent investigation revealed that the modified substrate with an ether linkage (**9**) exhibited excellent kinetics, with the maximum rate of enzymatic cleavage ($V_{\max}$) being around 3-fold lower compared to 4MU- β -Glc. Moreover, **9** was found to be hydrolysed only by GBA1 and not the two other related cellular β -glucosidases GBA2 and GBA3. Thus, **9** emerged as an excellent substrate for assessing reduced GBA1 activity in cell and spleen lysates from GD patients, and therefore has great potential for diagnostic applications. Of note, it has been earlier reported that 4MU- β -xyloside is also a specific substrate for GBA1,¹⁴ however this substrate is hydrolysed at a 50-fold lower rate than 4MU- β -Glc **1**, making substrate **9** a superior choice in this aspect. This newly designed substrate is not suitable for *in situ* measurement of GBA1 activity in cells. Firstly, detergents and DMSO are required for solubility of substrate, conditions that are not compatible with cell viability. Secondly, released fluorescent 4MU is known to diffuse from lysosomes, hampering detection of lysosomal activity towards a 4MU-equipped substrate.

6-*O*-acyl modification of glucose occurs naturally and has been particularly observed in plants.²⁵ Plants contain sterols that are chemically distinct from animal cholesterol, with the most prominent phytosterols being β -sitosterol, campesterol and stigmasterol. Glucosylated forms of phytosterols, also known as steryl glycosides (SG), possess a glucose attached at the C-3 hydroxyl of the sterol similar to GlcChol. Subsequent acylation of the sugar moiety at the C-6-hydroxyl yields 6-*O*-acyl-glucosyl-sterols (ASG). High levels of SG and ASG are found in fruits, vegetables (including tomatoes and potatoes) as well as beer and wine.^{25,26} Phytosterols are nowadays widely used as food additives aiming to lower plasma LDL cholesterol and reduce cholesterol absorption by stimulation of ABCG5/ABCG8-mediated (phyto)sterol intestinal export.²⁷ Notably, β -sitosterol- β -D-glycoside (BSSG) and β -sitosterol (BSS), abundant in some foods, have been detected in human plasma and tissues.^{28,29} Upon chronic high intake, plant sterols have been found to accumulate in the brain.³¹ Exposure to BSSGs has been hypothesized to underlie the historically high prevalence of the neurodegenerative disease amyotrophic lateral sclerosis–parkinsonism dementia complex (ALS–PDC) on the island of Guam. Indeed, feeding rats with BSSGs causes several neurological signs and defects resembling those occurring in Parkinson's disease patients, such as α -synuclein aggregates, motor abnormalities and striatal dopamine loss.^{32,33} Van Kampen and co-workers successfully induced parkinsonism in Sprague Dawley rats

by feeding them with BSSG for 4 months.³⁴ Interestingly, abnormalities in the *GBA1* gene, encoding GBA1, have been linked to increased risk for Parkinson's disease and Lewy-body dementia.^{9,10}

The study described here revealed the presence of glycosylcholesterol and 6-*O*-acyl-glycosyl-cholesterol in spleens, the former being increased in the organs from GD patients. Surprisingly, plant glycosyl-phytosterols and 6-*O*-acyl derivatives were also detected in the examined spleens. These were elevated in GD patients' spleens, most strikingly 6-*O*-acyl-glycosyl- β -sitosterol. This finding is consistent with the observation that 4MU- β -Glc and 6-*O*-acyl-4MU- β -Glc serve as substrates for GBA1, the lysosomal glucocerebrosidase deficient in GD patients. The data described here suggests potential absorption of glycosylphytosterols and 6-*O*-acyl derivatives from plant-derived food. However, it cannot be excluded that plant glycosylated sterols enter the body and undergo subsequent acylation.³² Little is so far reported on the kinetics of uptake and metabolism of phytosterols. In a follow-up investigation this should be addressed by exposing healthy individuals to glycosylated phytosterol and subsequent assessment of its levels in plasma. Further topics for future investigation may include the potential excretion of glycosylated phytosterols via bile or the intestine, their binding to plasma protein, and the mechanisms by which they may cross the blood-brain barrier and enter the brain. The work described here offers the first example of increased levels of exogenous plant-derived glycolipids in GD patients. It raises the speculation that dietary factors may contribute to the increased risk for PD observed in individuals with a mutant *GBA1* allele. Further clinical investigations on this should shed light on this theory. Of note, Akiyama and colleagues earlier reported the presence of plant-type β -sitosterylglucoside in chicken brain.²⁰

To conclude, it has been shown here that human GBA1, deficient in Gaucher disease, is able to remove 6-*O*-acyl-Glc from 4-methylumbelliferone as well as from cholesterol and plant derived campesterol and β -sitosterol. The newly generated substrate **9** (6-*O*-alkyl-4MU- β -Glc), due to its specificity for GBA1, emerges as an attractive and superior tool for diagnostically assessing GBA1 activity in materials that contain the β -glucosidases GBA2 and GBA3.

2.4 Conclusion

In conclusion, GD diagnosis relies on GBA1 activity assays, typically employing 4MU- β -Glc as fluorogenic substrate. However, these assays suffer from background 4MU release by the non-lysosomal GBA2 and cytosolic GBA3 enzymes. In this chapter the development of GBA1-selective fluorogenic substrates is described by synthesizing

a series of 6-*O*-acyl-4MU- β -Glc substrates with diverse fatty acid tails. Because of chemical and enzymatic instability of the ester bonds, analogues of 6-*O*-palmitoyl-4MU- β -Glc (**3**) with different chemical linkages were synthesized. 6-*O*-alkyl-4MU- β -Glc **9**, featuring an ether linkage, emerged as the most optimal GBA1 substrate, exhibiting both a low K_m and compared to substrate **3** a high V_{max} . Importantly, substrate **9** was not hydrolysed by GBA2 and GBA3, and therefore acts as superior substrate for GD diagnosis. Realizing that plants contain glycosyl phytosterols (campesterol, β -sitosterol and stigmasterol) and 6-*O*-acyl modification of glucose naturally, uptake and build-up of these plant sterols in GD patients was investigated. Such 6-*O*-acyl-glucosyl-lipids might act as GBA1 substrates and therefore accumulate in GBA1 deficient patients. LC-MS/MS analysis revealed that 6-*O*-acylated and regular glycosylcholesterol (HexChol) tend to be increased in GD patient spleens. Moreover, significant increases in 6-*O*-acyl-glycosyl-phytosterols were detected in GD spleens. The research suggests uptake of (6-*O*-acyl)-glycosyl-phytosterols from plant diet and subsequent lysosomal processing by GBA1, and comprises the first example of accumulation of an exogenous class of glycolipids in GD. Excessive exposure of rodents to glycosylated phytosterols has been reported to induce manifestations of Parkinson's disease (PD). Further investigation is warranted to determine whether (6-*O*-acyl)-glycosyl-phytosterols could contribute to the enigmatic link between inherited defects in GBA1 and the risk for PD.

2.5 Acknowledgements:

Marta Artola is kindly acknowledged for synthesizing the 6-*O*-acyl-4MU- β -Glc substrates **2-5** and her valuable discussions and guidance throughout the project. Joosje van Weperen and Nina A.M. Ligthart are kindly acknowledged for their contribution in generating the fluorogenic activity assay data. Maria J. Ferraz and Kateryna O. Bila are kindly acknowledged in helping generate and performing the LC-MS/MS data and experiments. Daan van der Vliet is kindly acknowledged for his help with the statistics and generation of the heat maps. Johannes M.F.G. Aerts is kindly acknowledged for his valuable discussions and guidance throughout the project. Lastly, the clinics, Gaucher patients and families are kindly acknowledged for supplying the spleen samples which were used in this research.

2.6 Experimental methods

2.6.1 Biochemical and Biological Methods

Cerezyme® (rhGBA1, 1.363 mg/mL) was a kind gift from Sanofi Genzyme (Amsterdam, The Netherlands). 4-Methylumbelliferyl- β -D-glucopyranoside (4MU- β -Glc) was purchased from Glycosynth (Winwick Quay, Warrington, UK). 4MU substrates **2-10** and internal heavy isotope standard **31** were synthesized as described. Triton X-100 was purchased from Sigma-Aldrich. Taurocholic acid sodium salt and dimethyl sulfoxide (DMSO) were purchased from EMD Millipore Corporation (Billerica, Massachusetts, USA).

Spleens from GD patients and (non-GD) controls had been obtained with consent and were used in prior research.^{35, 36}

Fluorogenic assays for 4MU substrates 1-5 and 6-O-acylated and -alkylated substrates 6-10

The *in vitro* enzyme activity of rhGBA1 (Cerezyme®) using substrates **1-10** was determined by measuring the release of the fluorescent 4-methylumbelliferyl.³⁷ Substrate mixes were made in 150 mM Mcllvaine buffer pH 5.2 containing 0.2% (w/v) sodium taurocholate. Cerezyme® was diluted 1:400 in 25 mM KPI pH 5.2 and 0.1% (v/v) Triton X-100. Per well 12.5 μ L enzyme mix, 12.5 μ L 150 mM Mcllvaine buffer and 100 μ L substrate mix were incubated for 30 minutes at 37 °C. The reaction was stopped by adding 200 μ L of the stop buffer 1 M glycine-NaOH pH 10.3. As a standard and for quantification of the obtained signals 1 nmol 4MU was added. GraphPad Prism 9 was used to analyze the results. Fluorescence intensities in the fluorogenic substrate assays were measured with a fluorimeter LS55 (Perkin-Elmer, Beaconsfield, UK) at λ_{ex} 366 nm and λ_{em} 445 nm plus slit_{ex} 10 nm and slit_{em} 3.0 nm. For 4MU substrates **2-5** substrate mixtures with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM substrate were prepared as described above with additional 0.1% (v/v) Triton X-100 and a maximum of 0.8% (v/v) DMSO. For 4MU substrates **6-10** substrate mixtures with 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 2.0, 3.0 and 4.0 mM substrate were prepared with additional 0.5% (v/v) Triton X-100 and a maximum of 10% (v/v) DMSO.

Determination of pH-optima for 4MU- β -Glc 1, 3 and 9

For the pH curves, substrate mixes of **1, 3** and **9** in 150 mM Mcllvaine buffer with the appropriate pH (pH 3.0, 4.0, 4.6, 4.8, 5.0, 5.2, 5.4, 5.6, 6.0, 7.0 or 8.0) were

prepared using the fluorogenic substrate assay conditions described above except for a substrate and DMSO concentration of 1 mM and 2.5% (v/v), respectively.

Cell culture

HEK293T cells overexpressing GBA2 (GBA/GBA2 KO) and HEK293T cells overexpressing GBA3 (GBA/GBA2 KO) were cultured in DMEM medium (Sigma-Aldrich) supplemented with 10% (v/v) FCS, 0.1% (w/v) penicillin/streptomycin and 1% (v/v) Glutamax at 37 °C and 5% CO₂. Lysates of the HEK293T cell pellets were generated by diluting the samples with 800 µL 25 mM KPI lysis buffer pH 5.8 for GBA2 or 100 mM HEPES buffer pH 7.0 for GBA3. Afterwards the lysates were sonicated 5 times for 1 second at 20% amplitude with Vibra-Cell™ VCX130 (Sonics, Newton, USA) in an ice bath.

Protein concentrations were determined with a BCA protein assay (BCA kit, Pierce, Thermo Fisher), using the Emax Plus Microplate Reader (Molecular Devices, Sunnyvale, USA).

β-Glucosidase activity of substrate 9 against GBA2 and GBA3.

The enzymatic activities of GBA2 and GBA3 were measured in lysates of cells overexpressing the corresponding enzyme using the same conditions as above. The 4MU substrates were tested on HEK293T cells overexpressing GBA2 dissolved in 150 mM McIlvaine buffer pH 5.8, whereas HEK293T cells overexpressing GBA3 were dissolved in 100 mM HEPES buffer pH 7.0. The fluorogenic assay conditions described above were used except for a substrate and DMSO concentration of 1 mM and 2.5% (v/v), respectively.

Spleen lysates

Spleens from GD patients and (non-GD) controls were lysed using 1.0 mm glass beads with a MP biomedical FastPrep-24 in KPI pH 6.5 supplemented with 0.1% Triton X-100: 1 gram tissue per 2 mL buffer. Protein concentrations were determined with the BCA protein assay. Lysates were diluted to 30 mg protein/mL, aliquoted and frozen at -80 °C before use.

Analysis of Acyl-HexSterols and HexSterols in spleen homogenates by LC-MS/MS

Prior to extraction, 5 pmol of ¹³C₆-GlcChol and 6-*O*-palmitoyl-¹³C₆-GlcChol (**31**) (used as internal standards) were added to 25 µL of homogenate. Next, lipids were

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extracted according to the method of Bligh and Dyer by addition of methanol, chloroform, and water (1:1:0.9, v/v/v) and the lower phase was taken to dryness under a stream of nitrogen. Isolated lipids were further extracted by water/butanol extraction (1:1, v/v).¹⁹ Lipids were separated using a BEH C18 reversed-phase column (2.1 $\times$ 50 mm, particle size 1.7 μ m Waters Corporation) by applying an isocratic elution of mobile phases, 2-propanol:water 90:10 (v/v) containing 10 mM ammonium formate (eluent A) and methanol containing 10 mM ammonium formate (eluent B). HexSterols were eluted as previously described¹⁹ at a flow rate of 0.25 mL/min with 10% A and 90% B for 5.5 min. The column temperature was kept at 23 °C and the autosampler at 10 °C. For Acyl-HexSterols separation, the UPLC program had a duration of 6.5 min and consisted of 30% A and 70% B at a flow rate of 0.5 mL/min. In these measurements, the column temperature and the temperature of the autosampler were kept at 40 °C and 10 °C, respectively, during the run. For quantitative analysis of Acyl-HexSterols in samples of spleen, a method using the multiple reaction monitoring (MRM) mode was developed using the transitions described in the supplemental (Table S2.1). A signal to-noise ratio of three was set for the limit of detection and the limit of quantitation was processed a signal-to-noise ratio above 10. Calculation of the signal-to-noise ratio was done using the peak-to-peak method. LC-MS/MS measurements were performed using a Waters UPLC-Xevo-QS micro instrument (Waters, Corporation, Milford, USA) in positive mode using an electrospray ionization source as described before.¹⁹ Data were analysed with Masslynx 4.2 software (Waters Corporation, Milford, MA).

Statistics

p-Values were calculated by a two-sided student's *t*-test comparing Gaucher Type I and controls. Lipids for which there were less than 2 measured values per condition were excluded. Raw *p*-values were corrected for multiple testing by the Benjamini-Hochberg method, setting the false discovery rate at 0.05. Lipids for which the adjusted *p*-value was < 0.05 were considered statistically significant. Statistical calculations were performed in R programming software (v4.2.2).

Heatmap

To generate the heatmaps, the measured lipid concentrations were log2 transformed and subsequently centred and scaled to unit variance to calculate Z-scores. Heatmaps were generated used the ComplexHeatmap package (v2.14.0)³⁸ in R programming software (v4.2.2). Hierarchical clustering of samples was performed using Euclidian distances.

2.7 Chemical Synthesis

2.7.1 General Experimental Details

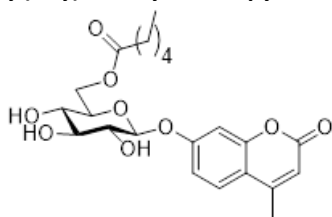
All reagents were of a commercial grade and were used as received unless stated otherwise. Dichloromethane (CH_2Cl_2), tetrahydrofuran (THF) and *N,N*-dimethylformamide (DMF) were stored over 4 Å molecular sieves, which were dried *in vacuo* before use. Triethylamine and di-isopropyl ethylamine (DIPEA) were dried over KOH and distilled before use. All reactions were performed under an argon atmosphere unless stated otherwise. Solvents used for flash column chromatography were of pro analysis quality. Reactions were monitored by analytical thin-layer chromatography (TLC) using Merck aluminium sheets pre-coated with silica gel 60 with detection by UV absorption (254 nm) and by spraying with a solution of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot\text{H}_2\text{O}$ (25 g/L) and $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4\cdot\text{H}_2\text{O}$ (10 g/L) in 10% sulfuric acid followed by charring at $\sim 150^\circ\text{C}$ or by spraying with an aqueous solution of KMnO_4 (7%) and K_2CO_3 (2%) followed by charring at $\sim 150^\circ\text{C}$. Column chromatography was performed manually using either Baker or Screening Device silica gel 60 (0.04 - 0.063 mm) or a Biotage Isolera™ flash purification system using silica gel cartridges (Screening devices SiliaSep HP, particle size 15-40 μm , 60A) in the indicated solvents. ^1H -NMR and ^{13}C -NMR spectra were recorded on Bruker AV-400 (400/100 MHz) and Bruker AV-I-500 (500/125 MHz) spectrometer in the given solvent. Chemical shifts are given in ppm relative to the residual solvent peak used or tetramethylsilane (TMS) as internal standard. Coupling constants are given in Hz. All given ^{13}C spectra are proton decoupled. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), qt (quintet), m (multiplet), br (broad), Ar (aromatic), Um (4-methylumbelliferone). 2D-NMR experiments (HSQC, COSY and HMBC) were carried out to assign protons and carbons of the new structures. High-resolution mass spectra (HRMS) of intermediates were recorded with a LTQ Orbitrap (Thermo Finnigan) and final compounds were recorded with an apex-QE instrument (Bruker). LC/MS analysis was performed on an LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI+) coupled to a Surveyor HPLC system (Thermo Finnigan) equipped with a C18 column (Gemini, 4.6 mm x 50 mm, 3 μm particle size, Phenomenex) equipped with buffers A: H_2O , B: acetonitrile (MeCN) and C: 1% aqueous TFA, or an Agilent Technologies 1260 Infinity LCMS with a 6120 Quadrupole MS system equipped with buffers A: H_2O , B: acetonitrile (MeCN) and C: 100 mM NH_4OAc . For reversed-phase HPLC-MS purifications an Agilent Technologies 1200 series prep-LCMS with a 6130 Quadrupole MS system was used equipped with buffers A: 50 mM NH_4HCO_3 in H_2O and B: MeCN.

2.7.2 Synthesis and characterization data

General procedure for the acylation of 4MU- β -Glc with various fatty acids

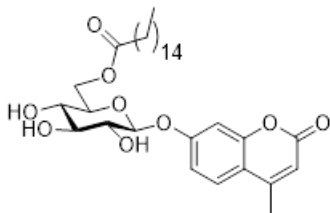
The enzymatic esterification of 4MU- β -Glc was carried out in a mixture of acetone and pyridine in a round-bottom flask (Figure 2.1A). In particular, 4MU- β -Glc (1 eq.) and the desired carboxylic acid (10 eq) were dissolved in dry acetone and pyridine (9:1, volume ratio, 0.059 M). The mixture was heated to 40 °C and stirred for 2 h. Then, Novozyme 435 (40 mg/mL, >5000 U/g) and activated 3Å molecular sieves (160 mg/mL) were added to the mixture and stirred at 50 °C for 24 h under nitrogen atmosphere. The progress of the reaction was monitored by thin layer chromatography (CH₂Cl₂/MeOH 9:1). After the reaction was completed, the mixture was filtered, the solvents were evaporated and the product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (9:1) as eluent to afford the desired acylated 4MU- β -Glc substrates **2-5** as white powders.

((2R,3S,4S,5R,6S)-3,4,5-Trihydroxy-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-2-yl)methyl hexanoate (**2**)



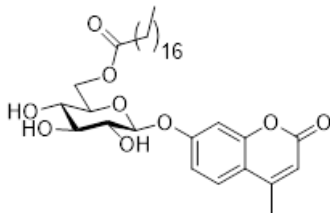
Obtained from 4MU- β -Glc (0.2 g, 0.59 mmol) and caproic acid (687 mg, 5.9 mmol) in dry acetone and pyridine (9:1, volume ratio, 10 mL) in 68% yield (175 mg, 0.40 mmol) following the general procedure described above. ¹H NMR (500 MHz, CDCl₃) δ 7.36 (d, *J* = 8.7 Hz, 1H, CH-Um), 6.91 (dd, *J* = 8.8, 2.3 Hz, 1H, CH-Um), 6.84 (d, *J* = 2.3 Hz, 1H, CH-Um), 6.07 (d, *J* = 1.3 Hz, 1H, CH-Um), 5.02 (d, *J* = 7.2 Hz, 1H, H-1), 4.40 (d, *J* = 11.4 Hz, 1H, H-6a), 4.28 (dd, *J* = 12.4, 6.7 Hz, 1H, CH, H-6b), 3.74–3.88 (m, 3H, H-2, H-3 and H-5), 3.56 (t, *J* = 8.8 Hz, 1H, H-4), 2.35 – 2.26 (m, 5H, CH₂-alkyl and CH₃-Um), 1.58 – 1.42 (m, 2H, CH₂-alkyl), 1.17 (h, *J* = 3.5 Hz, 4H, 2xCH₂-alkyl), 0.77 (td, *J* = 6.9, 5.9, 3.1 Hz, 3H, CH₃-alkyl); ¹³C NMR (126 MHz, CDCl₃) δ 174.5 (CO-acyl), 161.3, 159.8, 154.5, 152.9 (4xC_q-Um), 125.7 (CH-Um), 114.9 (C_q-Um), 113.7, 112.6, 104.1 (3xCH-Um), 100.0 (C-1), 76.3 (C-3), 74.2 (C-2), 73.2 (C-5), 70.4 (C-4), 63.4 (C-6), 34.1, 31.2, 24.6, 22.3 (4xCH₂), 18.6 (CH₃-Um), 14.0 (CH₃-alkyl); HRMS: calcd. for C₂₂H₂₈NaO₉ [M+Na]⁺ 459.1631, found: 459.1624.

((2R,3S,4S,5R,6S)-3,4,5-Trihydroxy-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-2-yl)methyl palmitate (3)



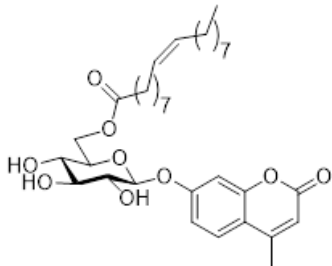
Obtained from 4MU- β -Glc (0.2 g, 0.59 mmol) and palmitic acid (1.52 g, 5.9 mmol) in dry acetone and pyridine (9:1, volume ratio, 10 mL) in 71% yield (243 mg, 0.42 mmol) following the general procedure. ^1H NMR (500 MHz, CDCl_3) δ 7.44 (d, J = 8.7 Hz, 1H, CH-Um), 6.95 (dd, J = 8.8, 2.4 Hz, 1H, CH-Um), 6.91 (d, J = 2.4 Hz, 1H, CH-Um), 6.12 (d, J = 1.3 Hz, 1H, CH-Um), 5.05 – 4.94 (m, 1H, H-1), 4.37 (d, J = 4.1 Hz, 2H, H-6a and H-6b), 4.12 (br s, 3H, 3xOH), 3.79 – 3.73 (m, 2H, H-2 and H-3), 3.70 (dt, J = 8.9, 4.3 Hz, 1H, H-5), 3.50 (t, J = 9.0 Hz, 1H, H-4), 2.39 – 2.31 (m, 5H, CH_2 -alkyl and CH_3 -Um), 1.59 – 1.50 (m, 2H, CH_2 -alkyl), 1.24 (br s, 24H, 12x CH_2 -alkyl), 0.87 (t, J = 6.9 Hz, 3H, CH_3 -alkyl). ^{13}C NMR (126 MHz, CDCl_3) δ 174.6 (Cq-alkyl), 161.1, 159.8, 154.8, 152.6 (4xCq-Um), 125.7 (CH-Um), 115.1 (Cq-Um), 113.9, 112.9, 103.7 (3xCH-Um), 99.5 (C-1), 77.3 (C-2/C-3), 74.5 (CH-5), 73.3 (C-2/C-3), 70.2 (C-4), 63.3 (C-6), 35.3, 32.1 (2x CH_2 -alkyl), 29.9 (4x CH_2 -alkyl), 29.8 (2x CH_2 -alkyl), 29.7, 29.5, 29.4, 29.3, 25.0, 23.3 (6x CH_2 -alkyl), 18.9 (CH_3 -Um), 14.3 (CH_3 -alkyl). HRMS: calcd. for $\text{C}_{32}\text{H}_{49}\text{O}_9$ $[\text{M}+\text{H}]^+$ 577.3377, found: 577.3378; HRMS: calcd. for $\text{C}_{32}\text{H}_{48}\text{NaO}_9$ $[\text{M}+\text{Na}]^+$ 599.3196, found: 577.3203.

((2R,3S,4S,5R,6S)-3,4,5-Trihydroxy-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-2-yl)methyl stearate (4)



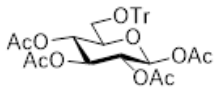
Obtained from 4MU- β -Glc (0.2 g, 0.59 mmol) and stearic acid (1.52 g, 5.9 mmol) in dry acetone and pyridine (9:1, volume ratio, 10 mL) in 60% yield (215 mg, 0.36 mmol) following the general procedure. ^1H NMR (500 MHz, CDCl_3) δ 7.35 (d, J = 8.7 Hz, 1H, CH-Um), 6.90 (d, J = 8.7 Hz, 1H, CH-Um), 6.05 (s, 1H, CH-Um), 6.82 (s, 1H, CH-Um), 5.18 (br s, 3H, 3OH), 5.00 (d, J = 7.0 Hz, 1H, H-1), 4.39 (d, J = 11.3 Hz, 1H, H-6a), 4.27 (d, J = 5.7 Hz, 1H, H-6b), 3.89 – 3.70 (m, 3H, H-2, H-3 and H-5), 3.55 (t, J = 9.0 Hz, 1H, H-4), 2.39 – 2.21 (m, 5H, CH_2 -alkyl and CH_3 -Um), 1.56 – 1.41 (m, 2H, CH_2 -alkyl), 1.34 – 1.04 (m, 28H, 14x CH_2 -alkyl), 0.86 (t, J = 7.0 Hz, 3H, CH_3 -alkyl). ^{13}C NMR (126 MHz, CDCl_3) δ 174.5 (Cq-alkyl), 161.2, 159.8, 154.5, 152.8 (4xCq-Um), 125.7 (CH-Um), 114.9 (Cq-Um), 113.8, 112.6, 104.1 (3xCH-Um), 100.1 (C-1), 76.3 (C-3), 74.3 (C-5), 73.3 (C-2), 70.5 (C-4), 63.5 (C-6), 34.2, 32.0, (2x CH_2 -alkyl), 29.9 (3x CH_2 -alkyl), 29.9 (3x CH_2 -alkyl), 29.8, 29.7, 29.5, 29.5, 29.3, 25.0, 22.8 (7x CH_2 -alkyl), 18.7 (CH_3 -Um), 14.3 (CH_3 -alkyl). HRMS: calcd. for $\text{C}_{34}\text{H}_{53}\text{O}_9$ $[\text{M}+\text{H}]^+$ 605.3690, found: 605.3696; HRMS: calcd. for $\text{C}_{34}\text{H}_{52}\text{NaO}_9$ $[\text{M}+\text{Na}]^+$ 627.3509, found: 627.3517.

((2R,3S,4S,5R,6S)-3,4,5-Trihydroxy-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-2-yl)methyl oleate (5)



Obtained from 4MU- β -Glc (0.2 g, 0.59 mmol) and stearic acid (1.52 g, 5.9 mmol) in dry acetone and pyridine (9:1, volume ratio, 10 mL) in 59% yield (210 mg, 0.35 mmol) following the general procedure. ^1H NMR (500 MHz, CDCl_3) δ 7.34 (d, J = 8.8 Hz, 1H, CH-Um), 6.89 (dd, J = 8.8, 2.3 Hz, 1H, CH-Um), 6.81 (d, J = 2.3 Hz, 1H, CH-Um), 6.03 (s, 1H, CH-Um), 5.40 – 5.20 (m, 2H, CH=CH), 4.97 (d, J = 7.1 Hz, 1H, H-1), 4.38 (d, J = 11.4 Hz, 1H, H-6a), 4.18 (dd, J = 12.0, 7.4 Hz, 1H, H-6b), 3.77 – 3.64 (m, 3H, H2, H3 and H5), 3.47 (t, J = 9.1 Hz, 1H, H-4), 2.32 – 2.18 (m, 5H, CH_3 -um and CH_2 -alkyl), 1.99 – 1.90 (m, 4H, $2\times\text{CH}_2$ -alkyl), 1.53 – 1.38 (m, 2H, CH_2 -alkyl), 1.29 – 1.12 (m, 20H, $10\times\text{CH}_2$ -alkyl), 0.84 (t, J = 7.0 Hz, 3H, CH_3 -alkyl). ^{13}C NMR (126 MHz, CDCl_3) δ 174.5 (Cq-alkyl), 161.3, 159.8, 154.5, 152.9 (4xCq-Um), 130.0, 129.7 (CH=CH), 125.6 (C-Um), 114.8 (Cq-Um), 113.9, 112.5, 104.0 (3xCH-Um), 100.0 (C-1), 76.2, 74.2, 73.2 (C-2, C-3 and C-5), 70.5 (C-4), 63.6 (C-6), 34.1, 32.0 ($2\times\text{CH}_2$ -alkyl), 29.8 ($2\times\text{CH}_2$ -alkyl), 29.6, 29.5 ($2\times\text{CH}_2$ -alkyl), 29.4 ($2\times\text{CH}_2$ -alkyl), 29.3, 29.2 ($2\times\text{CH}_2$ -alkyl), 27.3 ($2\times\text{CH}_2$ -alkyl), 24.9, 22.8 ($2\times\text{CH}_2$ -alkyl), 18.6 (CH_3 -Um), 14.2 (CH_3 -alkyl). HRMS: calcd. for $\text{C}_{34}\text{H}_{51}\text{O}_9$ $[\text{M}+\text{H}]^+$ 603.3533, found: 603.3538; HRMS: calcd. for $\text{C}_{34}\text{H}_{50}\text{NaO}_9$ $[\text{M}+\text{Na}]^+$ 625.3353, found: 625.3362.

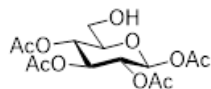
(3R,4S,5R,6R)-6-((Trityloxy)methyl)tetrahydro-2H-pyran-2,3,4,5-tetraol tetraacetate (11)



D-Glucose (30 g, 180 mmol) was dissolved in dry pyridine (900 mL) under protected atmosphere. Trityl chloride (50 g, 180 mmol, 1.1 eq) was added and the reaction was left to stir at 80 °C overnight. The reaction was cooled to 0 °C and acetic anhydride (72 mL, 760 mmol, 4.2 eq) was added. The reaction mixture was stirred for 24 h allowing to reach room temperature. The mixture was poured in 5% acetic acid aqueous solution (2.3 L) and stirred for 1 hour. The suspension was filtered and washed with water. The filtrate was resuspended in diethyl ether (200 mL) and filtered once more. The product was recrystallized from warm ethanol giving the product as white solids. (40 g, 68 mmol, 38%). ^1H NMR (400 MHz, CDCl_3) δ 7.46 – 7.19 (m, 15H, Ar-H, OTr), 5.79 – 5.67 (m, 1H, H-1), 5.30 – 5.23 (m, 1H, H-3), 5.22 – 5.15 (m, 2H, H-2 and H-4), 3.69 (ddd, J = 9.8, 4.2, 2.5 Hz, 1H, H-5), 3.34 (dd, J = 10.6, 2.5 Hz, 1H, H-6a), 3.06 (dd, J = 10.7, 4.2 Hz, 1H, H-6b), 2.16 (s, 3H, CH_3 -OAc), 2.04 (s, 3H, CH_3 -OAc), 2.00 (s, 3H, CH_3 -OAc), 1.73 (s, 3H, CH_3 -OAc). ^{13}C NMR (101 MHz, CDCl_3) δ 170.4, 169.5, 169.14, 169.09 (4xCq-OAc), 146.6 (3xCq-OTr), 128.9, 127.9, 127.2 ($15\times\text{CH}_{\text{Ar}}$), 92.1 (C-1), 74.2 (C-5), 73.3 (C-2), 71.8 (C-3), 68.9 (C-4), 61.8 (C-6), 21.0, 20.79,

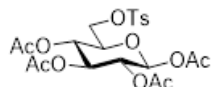
20.76, 20.6 (4xCH₃-OAc). HRMS: calcd. for C₃₃H₃₄O₁₀ [M+NH₄]⁺ 608.2490, found: 608.2487.

(2S,3R,4S,5R,6R)-6-(Hydroxymethyl)tetrahydro-2H-pyran-2,3,4,5-tetrayl tetraacetate (12)



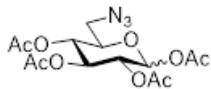
The tritylated glucose **11** (11.8 g, 19.98 mmol) was dissolved in glacial acetic acid (70 mL) and cooled to 0 °C. HBr (33% HBr in AcOH solution, 4 mL, 24.31 mmol) was added and the reaction mixture was stirred for 5 minutes and the formed triphenylbromine was immediately filtered off. The solid was washed with water and the water layers were collected extracted with CH₂Cl₂ (2 x 80 mL). The combined organic layers were washed with cold water and dried (MgSO₄), filtered and concentrated under reduced pressure. The crude was purified by silica column chromatography (1:2 EtOAc:Pentane) yielding the product (4.00 g, 11.48 mmol, 58% yield) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 5.71 (d, *J* = 8.3 Hz, 1H, H-1), 5.29 (t, *J* = 7.5 Hz, 1H, H-3), 5.17 – 5.00 (m, 2H, H-2 and H-4), 3.75 (d, *J* = 12.7 Hz, 1H, H-6a), 3.63 (ddd, *J* = 9.9, 4.1, 2.1 Hz, 1H, H-5), 3.57 (dd, *J* = 12.6, 4.1 Hz, 1H, H-6b), 2.10 (s, 3H, CH₃-OAc), 2.06 (s, 3H, CH₃-OAc), 2.02 (s, 3H, CH₃-OAc), 2.01 (s, 3H, CH₃-OAc). ¹³C NMR (101 MHz, CDCl₃) δ 170.4, 170.2, 169.4, 169.2 (4xCq-OAc), 91.8 (C-1), 75.0 (C-5), 72.7 (C-3), 70.5 (C-2), 67.8 (C-4), 60.2 (C-6), 20.9, 20.8, 20.72, 20.69 (4xCH₃-OAc). HRMS: calcd. for C₁₄H₂₀O₁₀ [M+Na]⁺ 371.09487, found: 371.09442.

(2S,3R,4S,5R,6R)-6-((Tosyloxy)methyl)tetrahydro-2H-pyran-2,3,4,5-tetrayl tetraacetate (13)



1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose **12** (3.50 g, 10.1 mmol) was dissolved in dry pyridine (60 mL) under protected atmosphere and cooled 0 °C. Tosyl chloride (2.49 g, 13.1 mmol) was added and the reaction mixture was stirred overnight allowing to reach room temperature. The mixture was diluted with EtOAc and washed with water, 1 M HCl, sat. aq. NaHCO₃ and Brine. All water layers were extracted with EtOAc and the combined organic layers were dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was crystallized from warm ethanol giving a white solid (3.54 g, 10.1 mmol, 70%). *R*_f = 0.19 (20% EtOAc in pentane); ¹H NMR (400 MHz, CDCl₃) δ 7.77 (d, 2 H, *J* = 8.0 Hz, Ar-H-Tosyl), 7.36 (d, 2 H, *J* = 8.0 Hz, Ar-H-Tosyl), 5.65 (d, 1 H, *J* = 8.0 Hz, H-1), 5.20 (t, 1 H, *J* = 9.6 Hz, H-3), 5.09-5.01 (m, 2 H, H-2 and H-4), 4.31 (m, 2 H, H-6), 3.83 (m, 1 H, H-5), 2.46 (s, 3 H, CH₃-Tosyl), 2.09 (s, 3 H, CH₃-OAc), 2.04-1.98 (m, 9 H, 3x CH₃-OAc); ¹³C NMR (101 MHz, CDCl₃) δ 170.2, 169.4, 169.2, 168.9 (4xCq-OAc), 145.2, 132.4 (Cq-Tosyl), 129.9, 128.2 (4xCH-Tosyl), 91.6 (C-1), 72.6 (C-3), 72.2 (C-5), 70.1, 67.9 (C-2 and C-4), 66.8 (C-6), 21.8 (CH₃-Tosyl), 20.8, 20.61, 20.59, 20.55 (4xCH₃-OAc). HRMS: calcd. for C₂₁H₂₆O₁₂S [M+NH₄]⁺ 520.14832, found: 520.14797.

(3R,4S,5R,6R)-6-(Azidomethyl)tetrahydro-2H-pyran-2,3,4,5-tetraol tetraacetate (14)

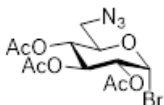


Tosylated glucose **13** (3.25 g, 6.47 mmol) was dissolved in dry DMF (60 mL). Sodium azide (0.84 g, 12.9 mmol) and 15-crown-5 (1.41 mL, 7.11 mmol) were added and the reaction was left to stir overnight at 50 °C. The reaction was diluted with EtOAc and extracted with water and brine. The water layers were extracted with EtOAc and the combined organic layers were dried (MgSO₄), filtered and concentrated *in vacuo*. The product was purified by silica column chromatography (50% EtOAc in pentane to 100% EtOAc) giving a white solid (2.25 g, 6.02 mmol, 93% yield).

α -anomer = ¹H NMR (CDCl₃, 400 MHz): δ 6.38 (d, 1 H, *J* = 3.6 Hz, H-1), 5.49 (t, 1H, H-3), 5.13 (t, 1H, *J* = 9.8 Hz, H-4), 5.12 (dd, 1H, *J* = 10.4 Hz, H-2), 4.11 (dddd, 1H, H-5), 3.43 (dd, 1H, *J* = 2.8 Hz, *J* = 3.6 Hz, H-6a), 3.33 (dd, 1H, *J* = 5.2 Hz, H-6b), 2.22 (s, 3H, CH₃-OAc), 2.09 (s, 3H, CH₃-OAc), 2.06 (s, 3H, CH₃-OAc), 2.05 (s, 3H, CH₃-OAc); ¹³C NMR (101 MHz, CDCl₃): δ 170.2, 169.7, 169.5, 168.7 (4x Cq-OAc), 88.9 (C-1), 70.9 (C-5), 69.7 (C-3), 69.2, 69.0 (C-2 and C-4), 50.7 (C-6), 20.9, 20.7, 20.6, 20.5 (4x CH₃-OAc).

β -anomer = ¹H NMR (CDCl₃, 400 MHz): δ 5.75 (1 H, d, H-1), 5.27 (1 H t, H-3), 5.16 (1 H, dd, *J* = 9.4 Hz, H-2), 5.10 (1 H, t, *J* = 9.4 Hz, H-4), 3.84 (1 H, ddd, *J* = 9.4 Hz, H-5), 3.40 (1 H, dd, *J* = 3.3 Hz, H-6b), 3.37 (1 H, dd, *J* = 5.3 Hz, H-6a), 2.23 (s, 3H, CH₃-OAc), 2.09 (s, 3H, CH₃-OAc), 2.07 (s, 3H, CH₃-OAc), 2.04 (s, 3H, CH₃-OAc); ¹³C NMR (101 MHz, CDCl₃) δ 170.1, 169.5, 169.2, 169.0 (4x Cq-OAc), 91.5 (C-1), 73.8 (C-5), 72.7 (C-3), 70.1, 69.0 (C-2 and C-4), 50.6 (C-6), 20.8, 20.60, 20.57, 20.55 (4x CH₃-OAc). HRMS: calcd. for C₁₄H₁₉O₉ [M+Na]⁺ 396.10135, found: 396.10064.

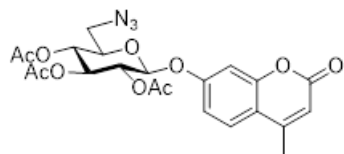
(2R,3R,4S,5R,6R)-2-(Azidomethyl)-6-bromotetrahydro-2H-pyran-3,4,5-triol triacetate (15)



6-Azido-glucose **14** (0.37 g, 1.0 mmol, 1 eq) was dissolved in dry CH₂Cl₂ (10 mL) under protected atmosphere. The solution was cooled to 0 °C followed by the addition TMSBr (0.52 mL, 4.0 mmol, 4 eq) and BiBr₃ (22 mg, 0.05 mmol, 0.05 eq). The reaction mixture was left stirring overnight allowing to reach room temperature. The reaction mixture was diluted with CH₂Cl₂ and washed with sat. aq. NaHCO₃ and Brine. The water layers were extracted with CH₂Cl₂ and the combined organic layers were dried (MgSO₄), filtered and concentrated *in vacuo*. The product was purified by silica column chromatography (30% EtOAc in pentane) giving a white solid (0.36 mg, 0.91 mmol, 91%). *R*_f = 0.7 (50% EtOAc in pentane). ¹H NMR (400 MHz, CDCl₃) δ 6.62 (d, *J* = 4.0 Hz, 1H, H-1), 5.53 (t, *J* = 9.7 Hz, 1H, H-3), 5.18 – 5.09 (m, 1H, H-4), 4.82 (dd, *J* = 10.0, 4.1 Hz, 1H, H-2), 4.29 – 4.22 (m, 1H, H-5), 3.46 (dd, *J* = 13.7, 2.7 Hz, 1H, H-6a), 3.35 (dd, *J* = 13.7, 5.1 Hz, 1H, H-6b), 2.09 (s, 3H, CH₃-OAc), 2.05 (s, 3H, CH₃-OAc), 2.03 (s, 3H, CH₃-OAc); ¹³C NMR (101 MHz, CDCl₃) δ 170.0, 169.9, 169.6

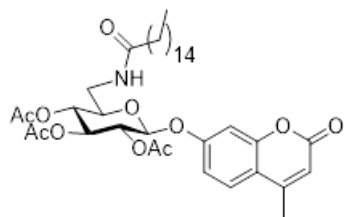
(3xCq-OAc), 86.2 (C-1), 73.1 (C-5), 70.7 (C-2), 70.1 (C-3), 68.4 (C-4), 50.4 (C-6), 30.8 (CH₃-OAc), 30.71 (CH₃-OAc), 30.69 (CH₃-OAc). HRMS: calcd. for C₁₂H₁₆BrN₃O₇ [M+NH₄]⁺ 411.05099, found 411.05089.

(2R,3R,4S,5R,6S)-2-(Azidomethyl)-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (16)



4-Methylumbelliferone (0.13 g, 0.75 mmol, 1.5 eq) was added to a solution of NaOH (28 mg, 0.7 mmol, 1.4 eq) in water (1.5 mL). To this solution was added the sugar bromide **15** (0.5 mmol, 1.0 eq) in Acetone (2 mL). The mixture was stirred in the dark overnight at room temperature. The reaction was diluted with CH₂Cl₂ and washed with 1 M NaOH and Brine. The water layers were extracted with CH₂Cl₂ and the combined organic layers were dried (MgSO₄), filtered and concentrated *in vacuo*. The product was purified by silica column chromatography (30% to 50% EtOAc in pentane) giving a white solid (152 mg, 0.31 mmol, 62%). R_f = 0.4 (50% EtOAc in Pentane). ¹H NMR (400 MHz, CDCl₃) δ 7.53 (d, J = 8.5 Hz, 1H, CH-um), 6.96 – 6.92 (m, 2H, 2x CH-um), 6.19 (d, J = 1.4 Hz, 1H, CH-um), 5.33 – 5.26 (m, 2H, H-2 and H-3), 5.20 (d, J = 7.5 Hz, 1H, H-1), 5.12 – 5.07 (t, 1H, H-4), 3.83 (ddd, J = 9.8, 6.9, 2.7 Hz, 1H, H-5), 3.43 (dd, J = 13.5, 6.9 Hz, 1H, H-6a), 3.37 (dd, J = 13.5, 2.7 Hz, 1H, H-6b), 2.40 (d, J = 1.4 Hz, 3H, CH₃-Um), 2.06 (d, J = 2.5 Hz, 6H, 2xCH₃-OAc), 2.03 (s, 3H, CH₃-OAc); ¹³C NMR (101 MHz, CDCl₃) δ 170.3, 169.6, 169.3 (3x Cq-OAc) 161.0, 159.1, 154.8, 152.3 (4x Cq-um), 126.0 (CH-um), 115.8 (Cq-um), 113.9, 113.4, 104.3 (3xCH-um), 98.4 (C-1), 73.8, 72.4, 71.0 (C-2, C-3 and C-5), 69.2 (C-4), 51.1 (C-6), 20.72, 20.70 (3xCH₃-OAc), 18.8 (CH₃-um); IR (neat) 2100, 1732, 1761, 1612, 1367, 1240 1211, 1066, 1033 cm⁻¹. HRMS: calcd. for C₂₂H₂₃N₃O₁₀ [M+Na]⁺ 512.12756, found: 512.12728.

(2S,3R,4S,5R,6R)-2-((4-Methyl-2-oxo-2H-chromen-7-yl)oxy)-6-(palmitamidomethyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (17)

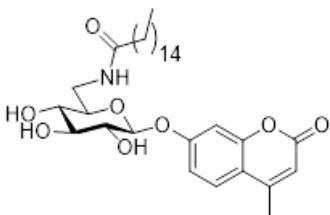


To a solution of palmitic acid (314 mg, 1.23 mmol, 2 eq) and HOBt (188 mg, 1.23 mmol, 2 eq) in acetonitrile (20 mL) and CH₂Cl₂ (20 mL) at 0 °C under nitrogen atmosphere was added EDC (235 mg, 1.23 mmol, 2 eq). After stirring for 10 minutes the solution was treated with the 6-azido sugar **16** (300 mg, 0.61 mmol) followed by a toluene solution of tributylphosphine (0.6 M, 0.23 mL, 0.92 mmol, 1.5 eq). The mixture was stirred in the dark for 5.5 h after which it was concentrated under reduced pressure. The crude was purified by silica column chromatography (12:8 EtOAc:Pentane) yielding the pure product as a white amorphous solid (341 mg, 0.49

6-O-Alkyl 4-methylumbelliferyl-β-D-glucosides as selective substrates for GBA1 in the discovery of glycosylated sterols

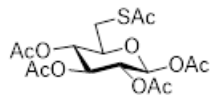
mmol, 79 % yield). ¹H NMR (400 MHz, CDCl₃) δ 7.52 (d, *J* = 9.2 Hz, 1H, CH-Um), 6.90 (d, *J* = 8.3 Hz, 2H, 2xCH-Um), 6.19 (d, *J* = 1.3 Hz, 1H, CH-Um), 5.78 (t, *J* = 6.2 Hz, 1H, NH), 5.32 – 5.23 (m, 2H, H-2 and H-3), 5.17 (d, *J* = 7.6 Hz, 1H, H-1), 5.01 (t, *J* = 9.6 Hz, 1H, H-4), 3.86 – 3.78 (m, H-5) 3.61 (ddd, *J* = 14.5, 5.7, 2.8 Hz, 1H, H-6a), 3.49 (dt, *J* = 14.6, 6.4 Hz, 1H, H-6b), 2.40 (d, *J* = 1.3 Hz, 3H, CH₃-um), 2.22 – 2.16 (m, 2H, CH₂-alkyl), 2.08 (s, 3H, CH₃-OAc), 2.06 (s, 3H, CH₃-OAc), 2.03 (s, 3H, CH₃-OAc), 1.59 (s, 2H, CH₂-alkyl), 1.24 (d, *J* = 5.9 Hz, 24H, 12xCH₂-alkyl), 0.89 – 0.84 (t, 3H, CH₃-alkyl); ¹³C NMR (101 MHz, CDCl₃) δ 173.5 (Cq-alkyl, 170.8, 169.8, 169.4 (3xCq-OAc), 161.4, 159.2, 155.5, 151.5 (4xCq-Um), 126.5 (CH-Um), 115.0 (Cq-Um), 113.6, 113.5, 104.9 (3xCH-Um), 97.4 (C-1), 73.7 (C-5), 72.6, 71.1 (C-2 and C-3), 68.7 (C-4), 39.1 (C-6), 36.8, 32.1, 29.83, 29.79, 29.7, 29.51, 29.49, 29.4, 25.7, 22.8 (14xCH₂-alkyl), 20.81, 20.75 (3xCH₃-OAc), 17.9 (CH₃-Um), 14.3 (CH₃-alkyl). HRMS: calcd. for C₃₈H₅₅N₃O₁₁ [M+H]⁺ 702.38479, found: 702.38445.

***N*-(((2*R*,3*S*,4*S*,5*R*,6*S*)-3,4,5-trihydroxy-6-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)tetrahydro-2*H*-pyran-2-yl)methyl)palmitamide (6)**

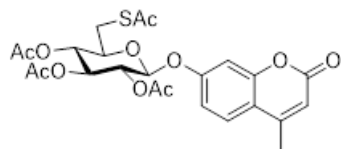


The protected sugar **17** (30 mg, 0.043 mmol) was dissolved in MeOH (3 mL) followed by the addition of sodium methoxide (30% in MeOH solution, 3 drops). The mixture was stirred for 4 h at room temperature after which TLC indicated complete conversion. The reaction was quenched with amberlite after which it was filtered and washed with MeOH. The solution was

concentrated yielding the crude product which was purified by silica column chromatography (5% MeOH in CH₂Cl₂) yielding the desired product (17 mg, 0.030 mmol, 77 % yield) as a white solid. ¹H NMR (400 MHz, MeOD) δ 7.64 – 7.57 (d, 1H, CH-Um), 7.03 (dd, *J* = 8.8, 2.4 Hz, 1H, CH-Um), 7.00 (d, *J* = 2.4 Hz, 1H, CH-Um), 6.17 (d, *J* = 1.4 Hz, 1H, CH-Um), 5.02 – 4.94 (m, 1H, H-1), 3.64 – 3.46 (m, 5H, H-2, H-3, H-4, H-6a and H-6b), 3.28 – 3.18 (m, 1H, H-5), 2.43 (d, *J* = 1.2 Hz, 3H, CH₃-Um), 2.20 (td, *J* = 7.4, 2.1 Hz, 2H, CH₂-alkyl), 1.60 – 1.50 (m, 2H, CH₂-alkyl), 1.22 (d, *J* = 8.0 Hz, 24H, 12xCH₂-alkyl), 0.88 – 0.82 (t, 3H, CH₃-alkyl). ¹³C NMR (101 MHz, MeOD) δ 176.05 (Cq-alkyl), 162.8, 161.1, 155.3, 154.4 (4xCq-Um), 126.5 (CH-Um), 115.6 (Cq-um), 114.6, 112.7, 105.5 (3xCH-Um), 101.1 (C-1), 76.4, 75.6, 74.0 (C-2, C-3 and C-4), 71.7 (C-5), 40.2 (C-6), 36.2, 32.9, 30.24, 30.21, 30.1, 29.94, 29.92, 29.86, 25.6, 23.8 (14xCH₂-alkyl), 18.9 (CH₃-Um), 14.3 (CH₃-alkyl). HRMS: calcd. for C₃₂H₄₉NO₈ [M+H]⁺ 576.3531, found: 523.3529.

(2S,3R,4S,5S,6S)-6-((Acetylthio)methyl)tetrahydro-2H-pyran-2,3,4,5-tetraol tetraacetate (18)

Tosylated glucose **13** (2.08 g, 4.12 mmol) was dissolved in dry DMF (50 mL) and potassium ethanethioate (1.41 g, 12.4 mmol, 3 eq) was added at room temperature under nitrogen atmosphere. The reaction mixture was stirred overnight after which it was diluted with EtOAc and extracted with NaHCO₃, water and brine. The water layers were extracted with EtOAc and the combined organic layers were dried (MgSO₄), filtered and concentrated under reduced pressure. The product was purified by silica column chromatography (EtOAc: Pentane 6:14) yielding the product (1.22 g, 3.00 mmol, 73% yield) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 5.66 (d, *J* = 8.3 Hz, 1H, H-1), 5.21 (t, *J* = 9.4 Hz, 1H, H-3), 5.14 – 5.06 (t, 1H, H-2), 5.06 – 4.98 (t, 1H, H-4), 3.83 – 3.76 (ddd, 1H, H-5), 3.21 (dd, *J* = 14.5, 3.1 Hz, 1H, H-6a), 3.14 (dd, *J* = 14.4, 6.0 Hz, 1H, H-6b), 2.33 (s, 3H CH₃-S-Ac), 2.11 (s, 3H CH₃-OAc), 2.08 (s, 3H CH₃-OAc), 2.02 (s, 3H CH₃-OAc), 2.00 (s, 3H, CH₃-OAc).; ¹³C NMR (101 MHz, CDCl₃) δ 195.4 (Cq-SAc), 170.2, 169.8, 169.3, 169.0 (4xCq-OAc), 91.0 (C-1), 75.0 (C-5), 72.8 (C-3), 70.3 (C-2), 69.8 (C-4), 30.5 (CH₃-S-Ac), 29.1 (C-6), 20.9, 20.8, 20.7, 20.6 (4xCH₃-OAc). HRMS: calcd. for C₁₆H₂₂O₁₀S [M+Na]⁺ 429.08259, found: 429.08205.

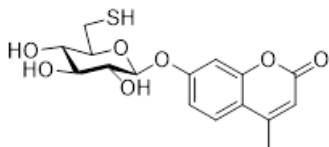
(2S,3S,4S,5R,6S)-2-((Acetylthio)methyl)-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (19)

The acetyl protected sugar **18** (208 mg, 0.51 mmol) was dissolved in CH₂Cl₂ (2.5 mL) and cooled to 0 °C. HBr (33% in AcOH, 1.21 mL, 22.3 mmol, 10.8 eq) was added dropwise and the reaction mixture was stirred in the dark for 5 h. The mixture was diluted with CH₂Cl₂ and washed with ice water (3x 50 mL), NaHCO₃ (50 mL) and water again (50 mL). The water layers were extracted with CH₂Cl₂ and the combined organic layers were dried (MgSO₄), filtered and concentrated under reduced pressure. The crude (160 mg, 0.37 mmol, 73% yield) was used in the next step without further purification. 4MU (114 mg, 0.65 mmol) was added to a solution of NaOH (24.9 mg, 0.62 mmol) in H₂O (2.15 mL). The crude bromo sugar (111 mg, 0.26 mmol) was added to this solution dissolved in acetone (2.8 mL). The mixture was stirred in the dark for 18 h at room temperature. After reaching completion the reaction was diluted with CH₂Cl₂ and washed with 1 M NaOH and brine. The water layers were extracted with CH₂Cl₂ and the combined organic layers were dried (MgSO₄), filtered and concentrated under reduced pressure. The product was purified by silica column chromatography (30-50% EtOAc in Pentane) yielding the product (60 mg, 0.114 mmol, 32% yield over 2 steps) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.51 (d, *J* = 8.7 Hz, 1H, CH-Um), 6.95 (d, 1H, CH-Um), 6.91 (dd,

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$J = 8.7, 2.5$ Hz, 1H, CH-Um), 6.18 (d, $J = 1.3$ Hz, 1H, CH-Um), 5.32 – 5.21 (m, 2H, H-1, H-3), 5.14 – 5.01 (m, 2H, H-2 and H-4), 3.79 (ddd, $J = 10.1, 7.7, 2.8$ Hz, 1H, H-5), 3.29 (dd, $J = 14.4, 2.8$ Hz, 1H, H-6a), 3.03 (dd, $J = 14.4, 7.7$ Hz, 1H, H-6b), 2.40 (d, $J = 1.2$ Hz, 3H, CH₃-Um), 2.38 (s, 3H, CH₃-SAC), 2.10 (s, 3H, CH₃-OAc), 2.04 (s, 3H, CH₃-OAc), 2.02 (s, 3H, CH₃-OAc). ¹³C NMR (101 MHz, CDCl₃) δ 195.3 (Cq-SAC), 170.2, 169.9, 169.4 (3xCq-OAc), 160.9, 159.3, 154.9, 152.3 (4xCq-Um), 124.4 (CH-Um), 115.6 (Cq-Um), 114.0, 112.4, 104.2 (3xCH-Um), 99.6 (C-1), 73.8 (C-5), 72.6, 71.1, 70.5 (C-2, C-3 and C-4), 30.6 (CH₃-SAC), 30.1 (C-6), 20.8, 20.71, 20.70 (3xCH₃-OAc), 18.8 (CH₃-Um). HRMS: calcd. for C₂₄H₂₆O₁₁S [M+H]⁺ 523.1269, found: 523.1269.

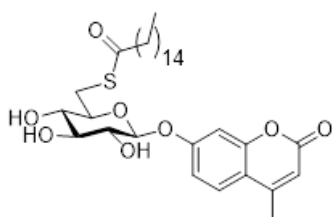
4-Methyl-7-(((2S,3R,4S,5S,6S)-3,4,5-trihydroxy-6-(mercaptomethyl)tetrahydro-2H-pyran-2-yl)oxy)-2H-chromen-2-one (20)



The acetylated thiosugar **19** (60 mg, 0.114 mmol) was dissolved in MeOH (3 mL) followed by the addition of sodium methoxide (30% in MeOH solution, 3 drops). The mixture was stirred for 4 h at room temperature after which TLC indicated complete conversion. The

reaction was quenched with amberlite after which it was filtered and washed with MeOH. The solution was concentrated yielding the crude product which was purified by silica column chromatography (5% MeOH in CH₂Cl₂) yielding the desired product (50 mg, 0.14 mmol, 78% yield). ¹H NMR (400 MHz, MeOD) δ 7.70 (d, $J = 8.8$ Hz, 1H, CH-Um), 7.13 (dd, $J = 8.8, 2.5$ Hz, 1H, CH-Um), 7.09 (d, $J = 2.4$ Hz, 1H, CH-Um), 6.20 (d, $J = 1.3$ Hz, 1H, CH-Um), 5.10 – 5.01 (m, 1H, H-1), 3.58 – 3.44 (m, 3H, H-2, H-4 and H-5), 3.35 (dd, $J = 9.4, 4.5$ Hz, 1H, H-3), 3.01 (dd, $J = 14.1, 2.4$ Hz, 1H, H-6a), 2.66 (dd, $J = 14.1, 8.1$ Hz, 1H, H-6b), 2.45 (d, $J = 1.2$ Hz, 3H); ¹³C NMR (101 MHz, MeOD) δ 163.3, 161.9, 156.0, 155.4 (4xCq-Um), 127.3 (CH-um), 116.1 (Cq-Um), 114.9, 112.9, 105.0 (3xCH-Um), 101.2 (C-1), 78.7, 77.6, 74.8 (C-2, C-4 and C-5), 73.8 (C-3), 26.9 (C-6), 18.7 (CH₃-Um). HRMS: calcd. for C₁₆H₁₈O₇S [M+H]⁺ 355.0846, found: 355.0846.

S-(((2S,3S,4S,5R,6S)-3,4,5-Trihydroxy-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-2-yl)methyl) hexadecanethioate (7)

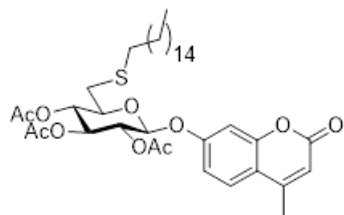


The deacetylated thio-sugar **20** (89 mg, 0.25 mmol) was dissolved in dry CH₂Cl₂ (1 mL) and DMF (2 mL) and was cooled to 0 °C. To this triethylamine (42 μ L, 0.30 mmol, 1.2 eq) and palmitoyl chloride (91 μ L, 0.30 mmol, 1.2 eq) were added. The reaction was allowed to warm up to room temperature and was stirred for 6 h after which the solvent was evaporated. The crude

was purified on silica column chromatography (5% MeOH in CH₂Cl₂) and the residual

starting material was recovered yielding the product (64 mg, 0.11 mmol, 43% yield, 95% brsm) as a white solid. ^1H NMR (400 MHz, MeOD/ CDCl_3) δ 7.57 (d, J = 9.3 Hz, 1H, CH-Um), 7.02 (m, J = 7.8 Hz, 2H, 2xCH-Um), 6.16 (d, J = 1.3 Hz, 1H, CH-Um), 4.89 (d, J = 7.6 Hz, 1H, H-1), 3.60 – 3.42 (m, 4H, H-2, H-4, H-5, H-6a), 3.35 – 3.26 (m, 1H, H-3), 2.99 (dd, J = 14.0, 8.7 Hz, 1H, H-6b), 2.64 – 2.49 (m, 2H, CH_2 -alkyl), 2.42 (d, J = 1.2 Hz, 3H, CH_3 -Um), 1.64 – 1.51 (m, 2H, CH_2 -alkyl), 1.30 – 1.16 (m, 24H, 12x CH_2 -alkyl), 0.88 – 0.79 (m, 3H, CH_3 -alkyl). ^{13}C NMR (101 MHz, MeOD/ CDCl_3) δ 198.9 (Cq-SAc), 162.8, 160.7, 155.4, 153.6 (Cq-Um), 125.7 (CH-Um), 114.9 (Cq-Um), 113.9, 112.2, 104.2 (CH-Um), 100.4 (C-1), 76.1, 75.5, 73.2 (C-2, C-4 and C-5), 72.9 (C-3), 43.2, 31.8, 30.3, 29.6, 29.5 (5x CH_2 -alkyl), 29.5 (C-6), 29.31, 29.25, 29.2, 28.8, 25.5, 22.6 (9x CH_2 -alkyl), 19.6 (CH_3 -Um), 13.8 (CH_3 -alkyl). HRMS: calcd. for $\text{C}_{32}\text{H}_{48}\text{O}_8\text{S}$ $[\text{M}+\text{H}]^+$ 593.3143, found: 593.3142.

(2S,3S,4S,5R,6S)-2-((Hexadecylthio)methyl)-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (21)



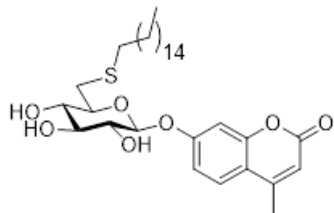
To a solution of the 6-thio sugar **19** (50 mg, 0.10 mmol) in DMF (2 mL) were added AcOH (6.57 μL , 0.12 mmol) and hydrazine hydrate (3.60 μL , 0.12 mmol), after stirring the reaction mixture for 1 h, the solution was concentrated to halve the original volume. The resulting crude was diluted with EtOAc and washed

with water brine. The organic layer was dried with MgSO_4 , filtered and concentrated *in vacuo*. The crude product was used in the next step without further purification. The crude intermediate (46 mg, 0.10 mmol) was dissolved in dry CH_2Cl_2 (1 mL) and cooled to 0 °C. To this solution, triethylamine (27 μL , 0.19 mmol) and 1-bromohexadecane (146 μL , 0.48 mmol) were added. The reaction was allowed to warm up to room temperature and was stirred for 18 h. The reaction was quenched with methanol and concentrated under reduced pressure. The crude was purified on silica column chromatography (6:14 EtOAc:pentane) and the residual starting material was recovered yielding the product (14 mg, 0.02 mmol, 21% yield, 87% brsm) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 7.52 (d, J = 9.1 Hz, 1H, CH-Um), 7.04 – 6.94 (m, 2H, 2xCH-Um), 6.19 (d, J = 1.3 Hz, 1H, CH-Um), 5.32 – 5.25 (m, 2H, H-2 and H-3), 5.18 – 5.11 (m, 1H, H-1), 5.11 – 5.04 (m, 1H, H-4), 3.80 (ddd, J = 9.8, 8.4, 2.9 Hz, 1H, H-5), 2.72 (dd, J = 14.4, 2.9 Hz, 1H, H-6a), 2.62 (dd, J = 14.4, 8.4 Hz, 1H, H-6b), 2.51 (t, J = 7.5 Hz, 2H, CH_2 -alkyl), 2.40 (d, J = 1.2 Hz, 3H, CH_3 -Um), 2.07 (s, 3H, CH_3 -OAc), 2.06 (s, 3H, CH_3 -OAc), 2.03 (s, 3H, CH_3 -OAc), 1.55 – 1.43 (m, 2H, CH_2 -alkyl), 1.23 (d, J = 9.2 Hz, 26H, 13x CH_3 -OAc), 0.91 – 0.81 (m, 3H, CH_3 -alkyl). ^{13}C NMR (101 MHz, CDCl_3) δ 170.4, 169.7, 169.5 (3xCq-OAc), 161.0, 159.5, 155.0, 152.2 (4xCq-Um), 125.9 (CH-Um), 115.7 (Cq-Um), 113.7, 113.4, 104.5 (3xCH-Um), 98.5 (C-1), 75.9 (C-5), 72.7 (C-3), 71.4 (C-4), 71.2 (C-2), 33.9 (CH_2 -alkyl), 33.2 (C-6), 32.1, 29.9, 29.80, 29.75,

6-O-Alkyl 4-methylumbelliferyl-β-D-glucosides as selective substrates for GBA1 in the discovery of glycosylated sterols

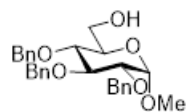
29.68, 29.66, 29.4, 29.0, 22.8 (14xCH₂-alkyl), 20.9, 20.79, 20.77 (3xCH₃-OAc), 18.9 (CH₃-Um), 14.28 (CH₃-alkyl). HRMS: calcd. for C₃₈H₅₆O₁₀S [M+H]⁺ 705.3667, found: 705.3670.

7-(((2S,3R,4S,5S,6S)-6-((Hexadecylthio)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)oxy)-4-methyl-2H-chromen-2-one (9)



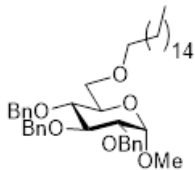
The acetylated intermediate **21** (14 mg, 0.02 mmol) was dissolved in MeOH (3 mL) followed by the addition of sodium methoxide (30% in MeOH solution, 3 drops). The mixture was stirred for 4 h at room temperature after which TLC indicated complete conversion. The reaction was quenched with amberlite after which it was filtered and washed with MeOH. The solution was concentrated yielding the crude product which was purified by silica column chromatography (5% MeOH in CH₂Cl₂) yielding the desired product (19 mg, 0.03 mmol, 77% yield) as a white solid. ¹H NMR (400 MHz, MeOD/CDCl₃) δ 7.60 (d, *J* = 8.7 Hz, 1H, CH-Um), 7.11 (dd, *J* = 8.7, 2.4 Hz, 1H, CH-Um), 7.08 (d, *J* = 2.4 Hz, 1H, CH-Um), 6.17 (d, *J* = 1.3 Hz, 1H, CH-Um), 4.96 (d, *J* = 7.6 Hz, 1H, H-1), 3.56 (dd, *J* = 9.2, 7.8 Hz, 2H, H-2 and H-5), 3.47 (t, *J* = 9.0 Hz, 1H, H-4), 3.36 – 3.32 (m, 1H, H-3), 3.04 (dd, *J* = 14.4, 2.2 Hz, 1H, H-6a), 2.60 (dd, *J* = 14.3, 8.8 Hz, 1H, H-6b), 2.56 – 2.46 (m, 2H, CH₂-alkyl), 2.43 (d, *J* = 1.2 Hz, 3H, CH₃-Um), 1.48 (m, *J* = 7.5, 7.0 Hz, 2H, CH₂-alkyl), 1.18 (m, *J* = 40.3 Hz, 26H, 13xCH₂-alkyl), 0.89 – 0.81 (t, 3H, CH₃-alkyl).; ¹³C NMR (101 MHz, MeOD/CDCl₃) δ 162.9, 161.01, 155.3, 154.5 (4xCq-Um), 126.5 (CH-Um), 115.6 (Cq-Um), 114.5, 112.7, 104.8 (3xCH-Um), 101.1 (C-1), 78.6 (C-2), 77.0 (C-4), 74.0 (C-5), 73.5 (C-3), 34.0 (CH₂-alkyl), 33.9 (C-6), 32.5, 30.23, 30.20, 30.15, 30.1, 29.9, 29.8, 29.5, 23.2 (14xCH₂-alkyl), 18.9 (CH₃-Um), 14.3 (CH₃-alkyl). HRMS: calcd. for C₃₂H₅₀O₇S [M+H]⁺ 579.3350, found: 579.3350.

((2R,3R,4S,5R,6S)-3,4,5-Tris(benzyloxy)-6-methoxytetrahydro-2H-pyran-2-yl)methanol (22)



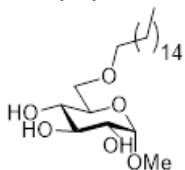
2,3,4-tri-*O*-benzyl-6-hydroxyl-1-methoxyl-α-D-glucopyranoside was synthesized starting from α-methyl-D-glucose as described in the literature and its spectroscopic data are in accordance with published data (21).

(2R,3R,4S,5R,6S)-3,4,5-Tris(benzyloxy)-2-((hexadecyloxy)methyl)-6-methoxytetrahydro-2H-pyran (23)



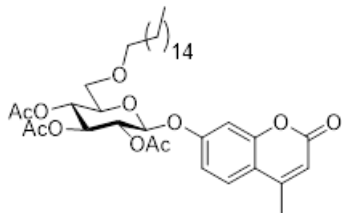
A solution of methyl 2,3,4-tri-*O*-benzyl- α -D-glucopyranoside **11** (1.40 g, 3.01 mmol) in DMF (20 mL) and 1-bromohexadecane (1.2 mL, 3.92 mmol) was added slowly with vigorous stirring to a suspension of NaH (289 mg, 7.23 mmol, 60% in paraffin oil) in DMF (10 mL) at 0 °C. The reaction mixture was stirred for 16 h allowing to reach room temperature. After completion of the reaction, the reaction mixture was quenched with methanol, diluted with diethyl ether and water and extracted. Afterwards the organic layer was washed with brine (3 x 50 mL), dried over MgSO₄, filtered and concentrated. The crude residue was purified with column chromatography to yield the product (1.85 g, 2.69 mmol, 89 % yield). ¹H NMR (400 MHz, CDCl₃) δ 7.38 – 7.26 (m, 15H, CH_{Ar}), 4.98 (d, *J* = 10.8 Hz, 1H, CH₂-OBn), 4.89 (d, *J* = 10.9 Hz, 1H, CH₂-OBn), 4.85 – 4.78 (m, 2H, CH₂-OBn), 4.67 (d, *J* = 12.2 Hz, 1H, CH₂-OBn), 4.63 – 4.58 (m, 2H, CH₂-OBn and H-1), 3.98 (t, 1H, H-3), 3.75 – 3.45 (m, 6H, H-2, H-4, H-5, H-6a and CH₂-alkyl), 3.37 (s, 3H, CH₃-OMe), 3.37 – 3.31 (m, 1H, H6b), 1.58 (m, *J* = 6.3 Hz, 2H, CH₂-alkyl), 1.25 (d, *J* = 9.7 Hz, 26H, 13xCH₂-alkyl), 0.89 (d, *J* = 13.7 Hz, 3H, CH₃-alkyl). ; ¹³C NMR (101 MHz, CDCl₃) δ 138.9, 138.6, 138.3 (3xCq-OBn), 128.6, 128.54, 128.52, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7 (15xCH-OBn), 98.3 (C-1), 82.3 (C-3), 80.0, 77.8 (C-2 and C-5), 75.9, 75.2, 73.5 (3xCH₂-OBn), 71.9 (C-6), 70.1 (C-4), 69.3 (CH₂-alkyl), 55.3 (CH₃-OMe), 32.1, 29.83, 29.80, 29.77, 29.75, 29.72, 29.6, 29.5, 26.3, 22.8 (15xCH₂-alkyl), 14.3 (CH₃-alkyl). HRMS: calcd. for C₄₄H₆₄O₆ [M+NH₄]⁺ 706.50412, found: 706.50325.

(2R,3S,4S,5R,6S)-2-((Hexadecyloxy)methyl)-6-methoxytetrahydro-2H-pyran-3,4,5-triol (24)



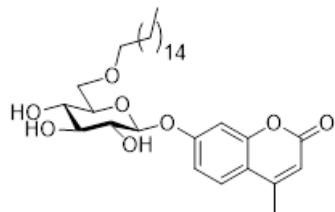
Pd/C (85 mg, 0.08 mmol, 10% on charcoal) was added to a stirred solution of the benzyl protected sugar **12** (110 mg, 0.16 mmol) with concentrated HCl (86 μ L, 1.05 mmol) in ethanol/ethyl acetate (1/1, v/v, 5 mL). The reaction mixture was then purged with H₂ and stirred for 4 h at rt. Upon completion, the solid was filtered off and the filtrate was concentrated *in vacuo* and dried to yield the deprotected sugar (64 mg, 0.12 mmol, 96% yield) as a colorless film. ¹H NMR (400 MHz, CDCl₃) δ 4.74 (d, *J* = 3.6 Hz, 1H, H-1), 3.74 (t, *J* = 9.3 Hz, 1H, H-3), 3.69 – 3.61 (m, 3H, H-4 and CH₂-alkyl), 3.56 – 3.43 (m, 4H, H-2, H-5, H6a and H6b), 3.40 (s, 3H, CH₃-OMe), 1.57 (q, *J* = 7.0 Hz, 2H, CH-alkyl), 1.24 (s, 26H, 13xCH₂-alkyl), 0.91 – 0.83 (t, 3H, CH₃-alkyl).; ¹³C NMR (101 MHz, CDCl₃) δ 99.6 (C-1), 74.4 (C-3), 72.3 (C-6), 72.1, 71.1 (C-2 and C-5), 70.5 (C-4), 70.4 (CH₂-alkyl), 55.3 (CH₃-OMe), 32.1, 29.85, 29.82, 29.80, 29.74, 29.69, 29.5, 26.2, 22.8 (14xCH₂-alkyl), 14.3 (CH₃-alkyl). HRMS: calcd. for C₂₃H₄₆O₆ [M+Na]⁺ 441.3187, found: 441.3188.

(2R,3R,4S,5R,6S)-2-((Hexadecyloxy)methyl)-6-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (25)



The 2,3,4 deprotected sugar **13** (386 mg, 0.92 mmol) was dissolved in 1:1 AcOH/Ac₂O (18 mL), and cooled down to 0 °C in an ice bath. Concentrated H₂SO₄ (199 μ L, 3.55 mmol) was added dropwise into the reaction. The reaction was then removed from the ice bath and stirred at room temperature overnight. After 18 h, the reaction was cooled to 0 °C, and saturated NaHCO₃ was added dropwise until the reaction was neutralized. The aqueous phase of the reaction was extracted with CH₂Cl₂, and the organic layer was recovered, dried over MgSO₄, filtered, and evaporated to dryness to afford the crude acetylated sugar as a white solid which was used in the next step without further purification. The crude sugar (352 mg, 0.62 mmol) was subsequently dissolved in dry CH₂Cl₂ (12 mL) under protected atmosphere. The solution was cooled to 0 °C followed by addition of TMS-Br (0.38 mL, 2.89 mmol) and tribromobismuthane (13.8 mg, 0.03 mmol). The reaction mixture was left stirring overnight allowing to reach room temperature. The reaction mixture was diluted with CH₂Cl₂ and washed with sat. aq. NaHCO₃ and brine. The water layers were extracted with CH₂Cl₂ and the combined organic layers were dried (MgSO₄), filtered and concentrated under reduced pressure. The crude product was used in the next step without further purification. 4MU (90 mg, 0.51 mmol) was added to a solution of NaOH (19.6 mg, 0.49 mmol) in H₂O (2 mL). To this solution was added the crude sugar bromide (121 mg, 0.20 mmol) dissolved in acetone (2 mL). The mixture was stirred in the dark for 18 h at room temperature. After reaching completion the reaction was diluted with CH₂Cl₂ and washed with 1 M NaOH and brine. The water layers were extracted with CH₂Cl₂ and the combined organic layers were dried (MgSO₄), filtered and concentrated under reduced pressure. The product was purified by silica column chromatography (30-50% EtOAc in Pentane) yielding the product (84 mg, 0.12 mmol, 37% yield over 3 steps) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.53 – 7.47 (m, 1H, CH-Um), 6.97 – 6.91 (m, 2H, 2xCH-Um), 6.18 (d, *J* = 1.3 Hz, 1H, CH-Um), 5.36 – 5.22 (m, 2H, H-2 and H-3), 5.20 – 5.09 (m, 2H, H1 and H-4), 3.85 – 3.77 (m, 1H, H-5), 3.59 (dd, *J* = 10.9, 3.0 Hz, 1H, H-6a), 3.51 (dd, *J* = 10.9, 5.7 Hz, 1H, H-6b), 3.48 – 3.31 (m, 2H, CH₂-alkyl), 2.39 (s, 3H, CH₃-Um), 2.05 (d, *J* = 0.8 Hz, 6H, 2xCH₃-OAc), 2.03 (s, 3H, CH₃-OAc), 1.58 – 1.47 (m, 2H, CH₂-alkyl), 1.22 (d, *J* = 13.2 Hz, 26H, 13xCH₂-alkyl), 0.91 – 0.81 (m, 3H, CH₃-alkyl).; ¹³C NMR (101 MHz, CDCl₃) δ 170.4, 169.6, 169.4 (3xCq-OAc), 161.5, 159.5, 154.9, 152.3 (4xCq-Um), 125.8 (CH-Um), 115.5 (Cq-Um), 113.9, 113.2, 104.3 (3xCH-Um), 98.5 (C-1), 74.0 (C-5), 72.9 (C-2), 72.3 (CH₂-alkyl), 71.2 (C-3), 69.5 (C-6), 69.1 (C-4), 32.0, 29.81, 29.77, 29.73, 29.67, 29.6, 29.5, 26.1, 22.8 (14xCH₂-alkyl), 20.80, 20.76 (3xCH₃-OAc), 18.8 (CH₃-Um), 14.2 (CH₃-alkyl). HRMS: calcd. for C₃₈H₅₆O₁₁ [M+H]⁺ 689.3895, found: 689.3898.

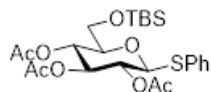
7-(((2S,3R,4S,5S,6R)-6-((hexadecyloxy)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)oxy)-4-methyl-2H-chromen-2-one (9)



The general procedure for acetyl deprotection with sodium methoxide was followed yielding the final alkylated sugar (51 mg, 0.09 mmol, 74% yield) as a white solid. ^1H NMR (400 MHz, MeOD/ CDCl_3) δ 7.56 (d, J = 8.8 Hz, 1H, CH-Um), 7.05 (dd, J = 8.8, 2.4 Hz, 1H, CH-Um), 7.01 (d, J = 2.4 Hz, 1H, CH-Um), 6.15 (d, J = 1.4 Hz,

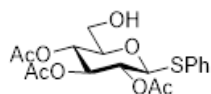
1H, CH-Um), 4.95 (d, J = 7.5 Hz, 1H, H-1), 3.85 – 3.75 (m, 1H, H-3), 3.64 – 3.35 (m, 7H, H-2, H-4, H-5, H-6a, H-6b and CH_2 -alkyl), 2.41 (d, J = 1.2 Hz, 3H, CH_3 -Um), 1.60 – 1.47 (m, 2H, CH_2 -alkyl), 1.20 (d, J = 20.1 Hz, 26H, 13xCH_2 -alkyl), 0.88 – 0.80 (m, 3H, CH_3 -alkyl). ^{13}C NMR (101 MHz, MeOD/ CDCl_3) δ 162.6, 160.9, 154.7, 154.1 (4x Cq-Um), 126.2 (CH-Um), 115.4 (Cq-Um), 114.3, 112.6, 104.7 (3x CH-Um), 100.4 (C-1), 77.0, 76.2, 73.6 (C-2, C-3 and C-5), 72.4 (CH_2 -alkyl), 70.8 (C-4), 70.4 (C-6), 32.3, 30.1, 30.0, 30.0, 29.9, 29.7, 26.4, 23.0 (14xCH_2 -alkyl), 18.9 (CH_3 -Um), 14.3 (CH_3 -alkyl). HRMS: calcd. for $\text{C}_{32}\text{H}_{50}\text{O}_8$ $[\text{M}+\text{H}]^+$ 563.3578, found: 563.3577.

(2R,3R,4S,5R,6S)-2-(((Tert-butyldimethylsilyl)oxy)methyl)-6-(phenylthio)tetrahydro-2H-pyran-3,4,5-triyl triacetate (26)



Intermediate **26** was synthesized over 5 steps starting from D-glucose following described procedures and its spectroscopic data was in agreement with the published literature (22, 23).

(2R,3R,4S,5R,6S)-2-(Hydroxymethyl)-6-(phenylthio)tetrahydro-2H-pyran-3,4,5-triyl triacetate (27)



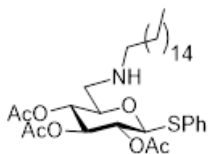
Compound **26** (4.01 g, 7.82 mmol) was dissolved in a dry methanol and CH_2Cl_2 mixture (1:1 v/v 10 mL). To it a catalytic amount of *p*-TsOH (149 mg, 0.78 mmol) was added and the reaction was stirred

for 2 h at room temperature. The reaction was quenched with triethylamine and washed with sat. NaHCO_3 and brine. The combined water layers were washed an additional time with CH_2Cl_2 and the combined organic layers were dried over MgSO_4 , filtered and concentrated. The crude was purified by silica-gel column chromatography which yielded the product (2.79 g, 6.97 mmol, 89% yield) as a colorless syrup. ^1H NMR (400 MHz, CDCl_3) δ 7.46 (m, J = 6.5, 3.2 Hz, 2H, 2xCH_{Ar}), 7.33 – 7.30 (m, 3H, 3xCH_{Ar}), 5.25 (t, J = 9.4 Hz, 1H, H-3), 5.02 – 4.92 (m, 2H, H-2 and H-4), 4.73 (d, 1H, H-1), 3.73 (dd, J = 11.4, 6.8 Hz, 1H, H-6a), 3.62 – 3.52 (m, 2H, H-5 and H-6a), 2.07 (s, 3H, CH_3 -OAc), 2.03 (s, 3H, CH_3 -OAc), 1.99 (s, 3H, CH_3 -OAc). ^{13}C NMR (101 MHz, CDCl_3) δ 170.3, 170.1, 169.4 (3x Cq-OAc), 133.0 (CH_{Ar}), 131.7 (Cq-SPh), 129.2, 129.0, 128.5, 128.3 (4xCH_{Ar}), 85.7 (C-1), 78.7

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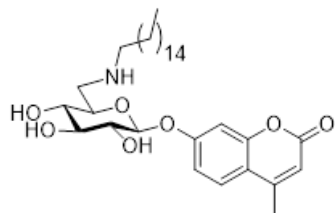
(C-5), 73.9 (C-3), 70.2, 68.5 (C-2 and C-4), 61.6 (C-6). HRMS: calcd. for $C_{18}H_{22}O_8S$ $[M+NH_4]^+$ 416.1374, found: 416.1375.

(2R,3R,4S,5R,6S)-2-((Hexadecylamino)methyl)-6-(phenylthio)tetrahydro-2H-pyran-3,4,5-triyl triacetate (28)



Dess-martin periodinane (4.44 g, 10.5 mmol) was added to a solution of **27** (2.78 g, 6.97 mmol) in CH_2Cl_2 (25 mL). The mixture was stirred for 16 h at room temperature. The reaction mixture was quenched with sat. aq. $Na_2S_2O_3$ and diluted with EtOAc. The organic layer was washed with sat. aq. $NaHCO_3$ and H_2O , dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude aldehyde was used in the next step without further purification. hexadecan-1-amine (2.53 g, 10.5 mmol) was dissolved in 2 mL anhydrous THF. The crude aldehyde (2.76 g, 6.97 mmol) was added to the solution and stirred for 2 h at room temperature. After 2 h the reaction mixture was cooled to 0 °C and a mixture of sodium cyanoborohydride (876 mg, 14.0 mmol) and AcOH (0.80 mL, 14.0 mmol) was added. After stirring overnight allowing to reach rt, the reaction was quenched with the addition of saturated aqueous ammonium chloride solution, and the mixture was extracted with ethyl acetate. The combined organic layers were washed with water, dried over $MgSO_4$ and evaporated. The residue was purified by silica column chromatography yielding the product (735 mg, 1.18 mmol, 17% yield over 2 steps) as a yellow oil. 1H NMR (400 MHz, $CDCl_3$) δ 7.46 (dd, J = 7.3, 2.3 Hz, 2H, CH-Ar), 7.35 – 7.28 (m, 3H, CH-Ar), 5.14 (t, J = 9.4 Hz, 1H, H-3), 5.06 (s, 1H, NH), 4.95 – 4.87 (m, 1H, H-2), 4.65 (d, J = 10.0 Hz, 1H, H-1), 4.26 – 4.16 (m, 1H, H-6a), 3.45 (dt, J = 9.8, 2.5 Hz, 1H, H-5), 3.41 – 3.24 (m, 3H, H-4 and CH_2 -alkyl), 3.18 (dd, J = 15.0, 2.5 Hz, 1H, H-6b), 2.15 (s, 3H, CH_3 -OAc), 2.08 (s, 3H, CH_3 -OAc), 2.05 (s, 3H, CH_3 -OAc), 1.59 (s, 2H, CH_2 -alkyl), 1.25 (s, 26H, CH_2 -alkyl), 0.92 – 0.84 (m, 3H, CH_3 -alkyl). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.3, 170.6, 169.9 (3x Cq-OAc), 133.5 (CH-Ar), 131.9 (Cq-Ar), 129.1, 128.5 (2x CH-Ar), 86.2 (C-1), 80.7 (C-5), 74.9 (C-3), 70.6 (C-2), 68.3 (C-4), 51.1 (CH_2 -alkyl), 45.8 (C-6), 32.1, 29.8, 29.81, 29.77, 29.73, 29.70, 29.51, 29.45, 28.4, 27.0, 22.8 (14x CH_2 -alkyl), 21.2, 21.01, 20.99 (3x CH_3 -OAc), 14.3 (CH_3 -alkyl).; HRMS: calcd. for $C_{34}H_{55}NO_7S$ $[M+H]^+$ 622.3772, found: 622.3774.

7-(((2S,3R,4S,5S,6R)-6-((Hexadecylamino)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)oxy)-4-methyl-2H-chromen-2-one (10)



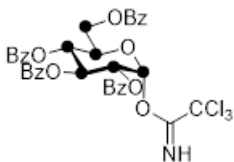
To a solution of the amino sugar **28** (735 mg, 1.18 mmol) and DIPEA (330 μ L, 1.89 mmol) in THF (6.5 mL) benzyl chloroformate (253 μ L, 1.77 mmol) was added dropwise at 0 $^{\circ}$ C. The temperature was raised to ambient after 10 minutes and stirring was continued until complete conversion of the starting material was

observed after stirring the reaction overnight. All the volatiles were removed per rotatory evaporation. Excess reagent was flushed off over a small silica plug and the crude product was used in the next step without further purification. The crude Cbz protected compound (168 mg, 0.22 mmol) was dissolved in CH_2Cl_2 (2.8 mL), and cooled to 0 $^{\circ}$ C, in the dark, then bromine (17 μ L, 0.33 mmol) was added. After 1.5 h the reaction mixture was diluted with CH_2Cl_2 and washed with 10% aqueous $\text{Na}_2\text{S}_2\text{O}_3$, sat. aq. NaHCO_3 , and brine. The organic layer was dried over MgSO_4 , filtered, and concentrated. The crude bromide was used in the next step without further purification. 4MU (78 mg, 0.44 mmol) was added to a solution of NaOH (16.8 mg, 0.42 mmol) in water (2.2 mL). To this solution was added TBABr (72 mg, 0.22 mmol) and the crude intermediate sugar (161 mg, 0.22 mmol) dissolved in chloroform (2.2 mL). The mixture was stirred in the dark for 18 h at room temperature. After reaching completion the reaction was diluted with CH_2Cl_2 and washed with 1 M NaOH and brine. The water layers were extracted with CH_2Cl_2 and the combined organic layers were dried (MgSO_4), filtered and concentrated under reduced pressure. The CBz protected amine crude was filtered over a short silica-gel plug leading to a mixture of product and some impurities which were deprotected in the next steps without further purification. This crude (37 mg) was deacetylated using the general procedure for acetyl deprotection with sodium methoxide. In a second step, Pd/C (100 mg, 0.09 mmol) was added to a stirred solution of the crude Cbz protected amino sugar (32 mg, 0.05 mmol) and AcOH (10 μ L, 0.14 mmol) in ethanol/ethyl acetate (1/1, v/v, 4 mL). The reaction mixture was then purged with H_2 and stirred for 1.5 h at rt. Upon completion, the solid was filtered off and the filtrate was concentrated *in vacuo*. The crude was purified using silica column chromatography (10% MeOH in CH_2Cl_2) yielding the product (11 mg, 0.02 mmol, 2% yield over 5 steps) as a white solid. ^1H NMR (500 MHz, MeOD/ CDCl_3) δ 7.61 (d, J = 8.8 Hz, 1H, CH-Um), 7.08 (dd, J = 8.8, 2.5 Hz, 1H, CH-Um), 7.00 (d, J = 2.4 Hz, 1H, CH-Um), 6.17 (d, J = 1.2 Hz, 1H, CH-Um), 5.08 (d, J = 7.4 Hz, 1H, H-1), 3.80 – 3.72 (m, 1H, H-5), 3.56 – 3.48 (m, 2H, H-2 and H-3), 3.30 – 3.25 (m, 2H, H-4 and H-6a), 2.95 (dd, J = 13.1, 8.4 Hz, 1H, H-6b), 2.83 – 2.73 (m, 2H, CH_2 -alkyl), 2.43 (s, 3H, CH_3 -Um), 1.60 – 1.49 (m, 2H, CH_2 -alkyl), 1.20 (d, J = 22.0 Hz, 26H, $13\times\text{CH}_2$ -alkyl), 0.88 – 0.82 (t, 3H, CH_3 -alkyl). ^{13}C NMR (126 MHz, MeOD/ CDCl_3) δ 162.6, 160.6,

6-O-Alkyl 4-methylumbelliferyl-β-D-glucosides as selective substrates for GBA1 in the discovery of glycosylated sterols

155.6, 154.2 (4xCq-Um), 126.6 (CH-Um), 115.5 (Cq-Um), 114.1, 112.7, 104.3 (3xCH-Um), 100.4 (C-1), 76.5 (C-2), 73.6 (C-3), 73.5 (C-5), 72.4 (C-4), 49.8 (C-6), 49.6, 32.4, 30.10, 30.07, 30.05, 30.0, 29.9, 29.8, 29.7, 27.2, 23.1 (15xCH₂-alkyl), 18.9 (CH₃-Um), 14.3 (CH₃-alkyl). HRMS: calcd. for C₃₂H₅₁NO₇ [M+H]⁺ 562.3738, found: 562.3739.

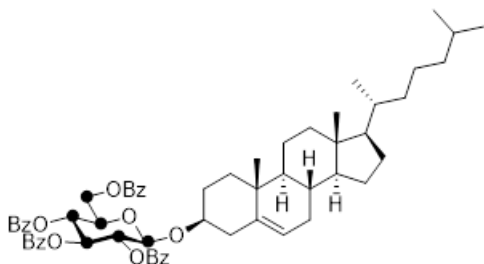
(2R,3R,4S,5R,6R)-2-((Benzoyloxy)methyl-¹³C)-6-(2,2,2-trichloro-1-iminoethoxy)tetrahydro-2H-pyran-3,4,5-triyl-2,3,4,5,6-¹³C5 tribenzoate (29)



To a solution of ¹³C₆-D-glucose (1.0 g, 5.55 mmol) in pyridine (100 mL) at 0 °C, BzCl (3.87 mL, 33.3 mmol) and a catalytic amount of DMAP (68 mg, 0.56 mmol) were added. The reaction was stirred for 20 h at room temperature after which the mixture of the reaction was diluted with EtOAc (100 mL) and washed with water (1 x 100 mL) and a 1M HCl (3 x 100 mL). The organic layer was dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was recrystallized from ethanol and immediately used in the next step. The benzoylated intermediate (2.5 g, 3.57 mmol) was dissolved in dry THF (7.14 mL) and was cooled to 0 °C under argon atmosphere. DMAPA (7.25 mL, 10.7 mmol) is added to the reaction flask and is stirred for 2.5 hours at 0 °C. Upon completion of the reaction the mixture was diluted with 75 mL EtOAc and the organic layer was washed twice with 75 mL 1 M HCL and brine. The combined water layers were extracted with EtOAc and the combined organic layers were dried with MgSO₄, filtered and then concentrated *in vacuo*. The crude intermediate (2.13 g, 3.57 mmol) is dissolved in dry CH₂Cl₂ (12 mL) under argon atmosphere and CCl₃CN (1.07 mL, 10.7 mmol) is added to the reaction flask. Cs₂CO₃ powder (465 mg, 1.43 mmol) is then added to the solution and the reaction mixture is left to stir for 3.5 hours at rt. The solution was then diluted with 75 mL of CH₂Cl₂ and washed twice with 100 mL NaHCO₃ and once with brine. The organic layers are dried with MgSO₄, filtered and then concentrated *in vacuo*. The crude mixture is purified on a silica column (15:5 pentane: EtOAc + 2% triethylamine). Compound **29** (2.03 g, 2.73 mmol, 77% yield over 3 steps) is collected as a yellowish oil. ¹H NMR (400 MHz, CDCl₃) δ 8.66 (s, 1H, NH), 8.08 – 8.03 (m, 2H, CH-OBz), 8.00 – 7.94 (m, 4H, CH-OBz), 7.91 – 7.86 (m, 2H, CH-OBz), 7.56 (t, *J* = 7.4 Hz, 1H, CH-OBz), 7.51 (t, *J* = 7.5 Hz, 2H, CH-OBz), 7.43 (t, *J* = 7.9 Hz, 3H, CH-OBz), 7.36 (d, *J* = 7.7 Hz, 4H, CH-OBz), 7.30 (t, *J* = 7.8 Hz, 2H, CH-OBz), 6.86 (d, *J* = 3.5 Hz, 1H, H-1), 6.30 (t, *J* = 10.2 Hz, 1H, H-3), 5.85 (t, *J* = 10.2 Hz, 1H, H-4), 5.65 (dd, *J* = 10.1, 3.5 Hz, 1H, H-2), 4.66 (d, *J* = 11.0 Hz, 2H, H-5 and H-6a), 4.51 (dd, *J* = 12.5, 4.8 Hz, 1H, H-6b). ¹³C NMR (101 MHz, CDCl₃) δ 166.1, 165.7, 165.5, 165.3 (4xCq-OBz), 160.6 (Cq-Imidate), 1337, 133.4, 133.3, 130.0, 129.9, 129.8 (6xCH-OBz), 129.6, 128.9, 128.7, 128.6 (4xCq-OBz), 128.54, 128.48, 128.45 (CH-OBz), 93.2 (d, *J* = 44.7 Hz, ¹³C-1), 71.4 – 69.7 (m, ¹³C-2, ¹³C-3 and ¹³C-

5), 69.2 – 68.1 (m, ^{13}C -4), 62.5 (d, $J = 44.2$ Hz, ^{13}C -6). HRMS: calcd. for $\text{C}_{30}^{13}\text{C}_6\text{H}_{28}\text{Cl}_3\text{NO}_{10}$ $[\text{M}+\text{H}]^+$ 768,08723, found: 768,08723.

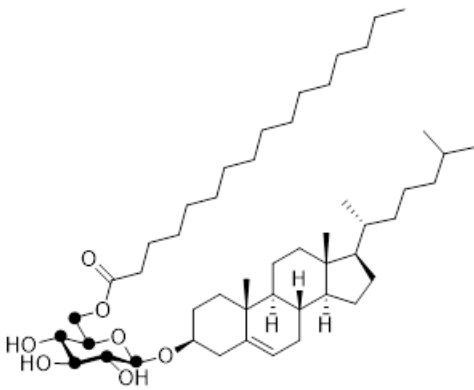
(2*R*,3*R*,4*S*,5*R*,6*R*)-2-((Benzoyloxy)methyl- ^{13}C)-6-(((3*R*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl)oxy)tetrahydro-2*H*-pyran-3,4,5-triyl-2,3,4,5,6- $^{13}\text{C}_5$ tribenzoate (30)



The imidate sugar donor **29** (150 mg, 202.4 μmol) was dissolved in dry CH_2Cl_2 (2 mL) and cholesterol (94 mg, 242.9 μmol) was added at 0 °C. The reaction mixture was stirred at 0 °C on activated 3 Å molecular sieves under argon and boron trifluoride etherate (8 μL , 60 μmol) was added and stirring was

maintained for 3 h. After neutralization with triethylamine (10 μL) and filtration over celite, the mixture was evaporated under reduced pressure and purified by silica gel chromatography giving compound **30** (51 mg, 71 μmol , 35%) as an off-white solid. ^1H NMR (400 MHz, CDCl_3) δ 8.04 – 7.99 (m, 2H, CH-OBz), 7.98 – 7.93 (m, 2H, CH-OBz), 7.93 – 7.88 (m, 2H, CH-OBz), 7.86 – 7.80 (m, 2H, CH-OBz), 7.57 – 7.47 (m, 3H, CH-OBz), 7.45 – 7.25 (m, 9H, CH-OBz), 5.90 (t, $J = 9.5$ Hz, 1H, CH-Chol), 5.63 (t, $J = 9.5$ Hz, 1H, H-3), 5.55 – 5.45 (t, 1H, H-4), 5.23 (d, $J = 5.6$ Hz, 1H, H-2), 4.94 (d, $J = 7.7$ Hz, 1H, H-1), 4.60 (dd, $J = 11.8$ Hz, 1H, H-6a), 4.52 (dd, $J = 10.7$, 4.5 Hz, 1H, H-6b), 4.20 – 4.10 (m, 1H, H-5), 3.53 (td, $J = 10.6$, 5.8 Hz, 1H, CH-Chol), 2.17 (m, $J = 5.4$ Hz, 2H, CH_2 -Chol), 2.06 – 1.97 (m, 1H, CH-Chol), 1.96 – 1.88 (m, 2H, CH_2 -Chol), 1.86 – 1.77 (m, 1H, CH-Chol), 1.71 (m, 1H), 1.58 – 1.47 (m, 3H, CH_2 -Chol and CH-Chol), 1.45 – 1.30 (m, 8H, CH_2 -Chol and CH-Chol), 1.26 (s, 3H, CH_3 -Chol), 1.19 – 0.95 (m, 9H, CH_2 -Chol and CH-Chol), 0.92 (m, 2H, CH-Chol), 0.90 (s, 3H, CH_3 -Chol), 0.87 (d, $J = 1.8$ Hz, 3H, CH_3 -Chol), 0.86 (d, $J = 1.8$ Hz, 3H, CH_3 -Chol), 0.65 (s, 3H, CH_3 -Chol). ^{13}C NMR (101 MHz, CDCl_3) δ 166.2, 166.0, 165.4, 165.2 (4xCq-OBz), 140.5 (Cq-Chol), 133.6, 133.34, 133.29, 133.2 (4xCH-OBz), 130.0, 129.91, 129.88, 129.85 (4xCH-OBz), 129.8, 129.6, 129.0, 128.9 (4xCq-OBz), 128.54, 128.50, 128.47, 128.4 (4xCH-OBz), 122.1 (CH-Chol), 100.3 (d, $J = 49.3$ Hz, C-1), 95.2, 94.7 (2xCq-Chol), 80.6 (CH-Chol), 74.0 – 71.5 (m, ^{13}C -2, ^{13}C -3 and ^{13}C -5), 70.8 – 69.5 (m, ^{13}C -4), 63.5 (d, $J = 44.6$ Hz, ^{13}C -6), 56.9, 56.3, 50.2 (3xCH-Chol), 42.4, 39.9, 39.6, 38.9, 37.2, 36.7, 36.3 (7xCq and CH_2 -Chol), 35.9 (CH-Chol), 32.0 (CH_2 -Chol), 31.9 (CH-Chol), 29.8, 29.7, 28.4 (3xCq or CH_2 -Chol), 28.2 (3xCH or CH_3 -Chol), 24.4, 23.9 (2xCq or CH_2 -Chol), 23.0, 22.7 (2x CH_3 -Chol), 21.1 (CH_2 -Chol), 19.4, 18.9, 12.0 (3x CH_3 -Chol). HRMS: not detected. LCMS: calcd. for $\text{C}_{55}^{13}\text{C}_6\text{H}_{72}\text{O}_{10}$ $[\text{M}+\text{NH}_4]^+$ 988.57, found: 988.5

((2R,3S,4S,5R,6R)-6-(((3R,8S,9S,10R,13R,14S,17R)-10,13-Dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl-2,3,4,5,6- $^{13}\text{C}_5$)methyl- ^{13}C -palmitate (31)



Intermediate **30** (205 mg, 212 μmol) was dissolved in a mixture of MeOH (2.1 mL) and CH_2Cl_2 (2.1 mL) followed by the addition of a catalytic amount of sodium methoxide (30% in MeOH, 1 drop). The mixture was stirred for 4 h at room temperature. After reaching completion the reaction was quenched with amberlite, which was subsequently filtered off and washed with MeOH and CH_2Cl_2 . The solution was concentrated *in vacuo*

yielding the crude intermediate which was used in the next step without further purification. Palmitic acid (47 mg, 181 μmol), HATU (69 mg, 181 μmol), and diisopropyl ethylamine (53 μL , 302 μmol) were dissolved in dry pyridine (2 mL) and the resulting mixture was stirred at rt for 30 minutes under a nitrogen atmosphere. The crude deprotected $^{13}\text{C}_6$ -Glucosylated cholesterol intermediate (83 mg, 151 μmol) was dissolved in pyridine (1 mL) was then injected into the reaction mixture via syringe and stirring was continued at rt for 72 h. The pyridine was removed *in vacuo* and the resulting solid was purified and residual starting material recovered using silica column chromatography to give a white solid (32 mg, 88% yield brsm). ^1H NMR (400 MHz, $\text{MeOD}/\text{CDCl}_3$) δ 5.33 (d, J = 5.4 Hz, 1H, CH-Chol), 4.39 – 4.31 (m, 2H, H-1 and H-6a), 4.19 (dd, J = 11.7, 7.0 Hz, 1H, H-6b), 3.51 (m, 1H, CH-Chol), 3.44 (t, J = 8.3 Hz, 1H, H-5), 3.36 (t, J = 8.9 Hz, 1H, H-3), 3.23 (dt, J = 16.6, 8.9 Hz, 2H, H-2 and H-4), 2.36 (m, 1H, CH-Chol), 2.29 (t, J = 7.7 Hz, 2H), 2.27 – 2.21 (m, 1H, CH-Chol), 2.03 – 1.87 (m, 3H, CH-Chol and CH_2 -Chol), 1.81 (m, 2H, CH_2 -Chol), 1.65 – 1.53 (m, 4H, CH_2 -pamityl and CH_2 -Chol), 1.52 – 1.40 (m, 4H, CH_2 -Chol), 1.23 (m, 30H, 13xCH_2 -palmityl and 2xCH_2 -Chol), 1.15 – 1.02 (m, 6H, CH-Chol and CH_2 -Chol), 0.98 (m, 5H, CH_3 -Chol and CH_2 -Chol), 0.91 – 0.86 (m, 4H, CH-Chol and CH_3 -Chol), 0.85 – 0.83 (m, 4H, CH-Chol and CH_3 -Chol), 0.82 (d, J = 1.7 Hz, 3H, CH_3 -Chol), 0.65 (s, 3H, CH_3 -Chol). ^{13}C NMR (101 MHz, $\text{MeOD}/\text{CDCl}_3$) δ 174.9 (Cq-Palmityl), 141.0 (Cq-Chol), 122.4 (CH-Chol), 101.8 (dt, J = 47.1, 4.2 Hz, ^{13}C -1), 97.6, 97.1 (2xCq-Chol), 80.1 (CH-Chol), 77.0 (t, J = 39.2 Hz, ^{13}C -3), 74.8 – 73.1 (m, ^{13}C -5 and ^{13}C -2 or ^{13}C -4), 70.2 (t, J = 40.0 Hz, ^{13}C -2 or ^{13}C -4), 64.3 (dt, J = 45.3, 4.5 Hz, ^{13}C -6), 57.2, 56.6, 50.6 (3xCH-Chol), 42.7, 40.2, 39.9, 39.1, 37.8, 37.1, 36.6 (7x CH_2 -Chol), 36.2 (CH-Chol), 34.7

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(CH₂-Chol), 32.3 (CH₂-Chol), 32.3 (CH-Chol), 30.13, 30.10, 30.06, 30.0, 29.78, 29.75, 29.7, 28.6 (8xCH₂-Chol), 28.4 (CH-Chol), 25.4, 24.6, 24.2, 23.0 (4xCH₂-Chol), 23.0 (CH₃-Chol), 22.7 (CH-Chol), 21.5 (CH₂-Chol), 20.0, 19.0, 14.3, 12.1 (4xCH₃-Chol). HRMS: not detected. LCMS: calcd. for C₅₅¹³C₆H₇₂O₁₀ [M+Na]⁺ 815.65, found: 815.6

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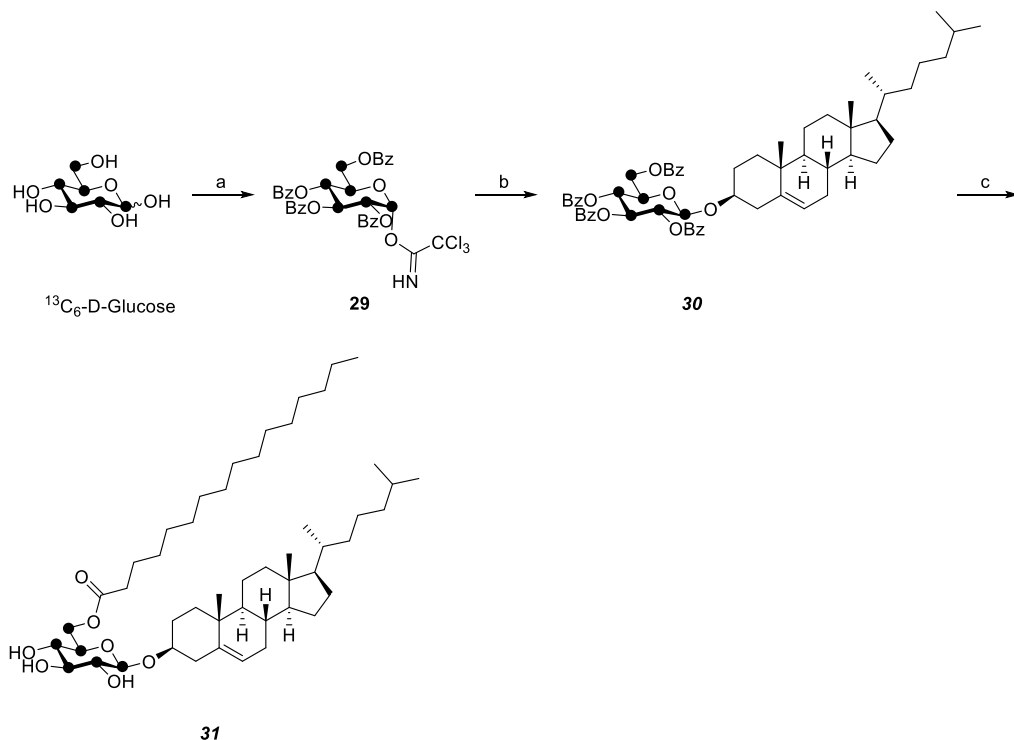
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2.9 Appendix

Synthesis of 6-O-Palmitoyl- $^{13}\text{C}_6$ -Glc-Chol **31** as an internal standard for the measurement of 6-O-acyl-glucosyl-sterols in GD spleens



Scheme S2.1. Synthesis of 6-O-palmitoyl- $^{13}\text{C}_6$ Glc-Chol. Reagent and conditions: a) i. BzCl, DMAP, pyridine, r.t., 18 h; ii. DMAPA, THF, 0 °C, 3.5 h; iii. TCA, Cs₂CO₃, CH₂Cl₂, r.t., 18 h, 77% (3 steps). b) Cholesterol, BF₃·Et₂O, CH₂Cl₂, 0 °C, 3 h, 35% c) i. NaOMe, MeOH/CH₂Cl₂, r.t., 4 h ii. Palmitic acid, DIPEA, HATU, pyridine, r.t., 72 h, 27% (2 steps, 88% brsm).

For the measurement of the natural occurrence of 6-O-acyl-glucosyl-sterols in non-GD and GD spleens a close structural internal standard of 6-O-acyl-glucosyl-sterols was generated for accurate determinations. For this purpose, C-6-O-palmitoyl- $^{13}\text{C}_6$ Glc-Chol **31** was synthesized as illustrated in Scheme S2.1. Synthesis started from commercially available $^{13}\text{C}_6$ -D-Glucose which was converted to benzoyl protected imidate donor **29** over three steps. Per-benzoylation with benzoylchloride and catalytic amounts of DMAP in pyridine, followed by deprotection of the anomeric benzoyl group with DMAPA in THF at 0 °C, and final installation of the imidate moiety using standard conditions with trichloroacetonitrile and cesium carbonate as a base yielded imidate **29**. Cholesterol was subsequently glycosylated

using **29** and boron trifluoride as an activator to yield benzoyl protected $^{13}\text{C}_6$ -Glc-Chol **30**. Finally, the benzoyl protecting groups were removed using sodium methoxide and C-6 was selectively palmitoylated using palmitic acid, DIPEA and HATU in pyridine to yield **31** in 27% over two steps while recovering a considerable amount of starting material (88% yield brsm over 2 steps).

Table S2.1. Molecular species, transitions and retention times used to analyze the presence of the (Acyl)HexSterols measured in spleens of non-GD and GD patients.

Molecular Species $[\text{M}+\text{NH}_4]^+$	Transition m/z	Retention (min)
$^{13}\text{C}_6$ -GlcChol	572.6 > 369.4	1.57
HexChol	566.6 > 369.4	1.57
HexCampesterol	580.4 > 383.4	1.70
HexStigmasterol	592.4 > 395.4	1.67
HexSitosterol	594.5 > 397.4	1.79
C-6-O-palmitoyl $^{13}\text{C}_6$ -GlcChol	810.7 > 369.4	2.19
AcylHexChol 16:1	802.7 > 369.4	1.77
AcylHexChol 16:0	804.7 > 369.4	2.18
AcylHexChol 18:2	828.7 > 369.4	1.83
AcylHexChol 18:1	830.7 > 369.4	2.18
AcylHexChol 18:0	832.7 > 369.4	2.71
AcylHexCampesterol 16:1	816.7 > 383.4	1.49
AcylHexCampesterol 16:0	818.7 > 383.4	1.74
AcylHexCampesterol 18:2	842.7 > 383.4	1.53
AcylHexCampesterol 18:1	844.7 > 383.4	1.79
AcylHexCampesterol 18:0	846.7 > 383.4	2.16
AcylHexStigmasterol 16:1	828.7 > 395.4	1.82
AcylHexStigmasterol 16:0	830.7 > 395.4	1.74
AcylHexStigmasterol 18:2	854.7 > 395.4	1.51
AcylHexStigmasterol 18:1	856.7 > 395.4	1.77
AcylHexStigmasterol 18:0	858.7 > 395.4	2.13
AcylHexSitosterol 16:1	830.7 > 397.4	1.60
AcylHexSitosterol 16:0	832.7 > 397.4	1.88

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AcylHexSitosterol 18:2	856.7 > 397.4	1.63
AcylHexSitosterol 18:1	858.7 > 397.4	1.90
AcylHexSitosterol 18:0	860.7 > 397.4	2.29

Footnote: AcylHexCholesterol data was measured on a Waters UPLC-Xevo-QS micro instrument where the C-6-O-palmitoyl 13C6-GlcChol 16:0 (**31**) internal standard showed a retention time of 2.19 min. AcylHexSterols were measured on a second identical machine and we observed a shift in retention times (13C6-palmitoylGlcChol 16:0 ; rt = 1.65 min).

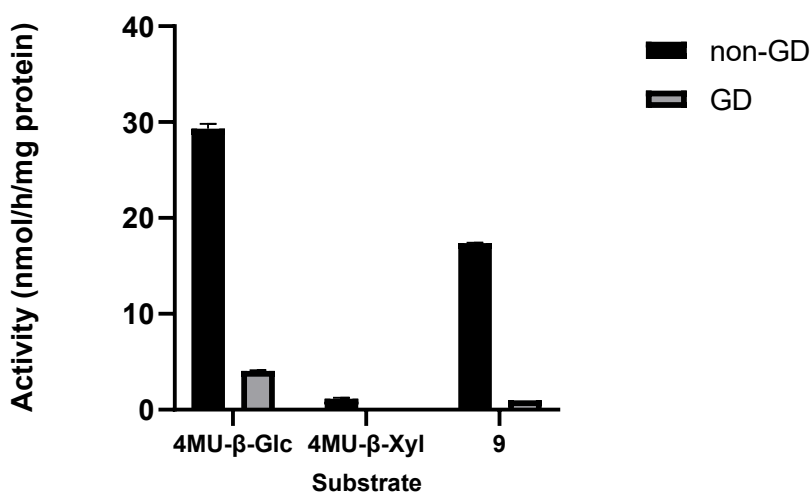


Figure S2.2. GBA activity measured in lysates (28.1 mg protein/mL) of a non-GD and GD spleen using 4MU- β -Glc **1**, 4MU- β -Xyl and 4MU-C-6-ether **9** as fluorogenic substrates. 4MU- β -Xyl did not show significant 4MU signal (~1 nmol/h/mg protein).

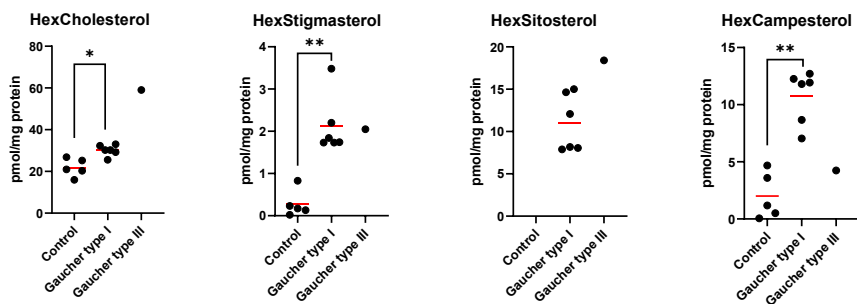


Figure S2.3. Levels (pmol/mg protein) of glycosylcholesterol (HexChol) and sterolins (glycosyl-stigmasterol, glycosyl- β -sitosterol, glycosyl-campesterol) in lysates of spleens (30 mg protein/mL) from non-GD or GD patients determined by LC-MS/MS analysis. $^{13}\text{C}_6$ -GlcChol was used as an internal standard. The red line represents the mean value of the measured samples and the p -values are denoted as ns (>0.05), * (≤ 0.05), ** (≤ 0.01) and *** (≤ 0.001).

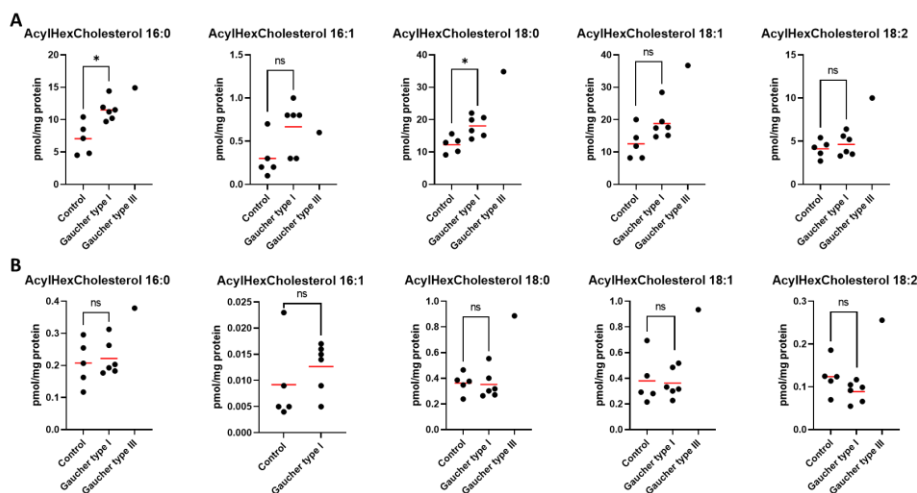


Figure S2.4. Levels (pmol/mg protein) of 6-*O*-acyl-glycosyl-cholesterol (6-*O*-palmitoyl and stearoyl versions) in lysates of spleens (30 mg protein/mL) from healthy or GD patients using (A) $^{13}\text{C}_6$ -GlcChol as an internal standard or (B) 6-*O*-palmitoyl- $^{13}\text{C}_6$ -GlcChol (31) as an internal standard. The red line represents the mean value of the measured samples and the p -values are denoted as ns (>0.05), * (≤ 0.05), ** (≤ 0.01) and *** (≤ 0.001).

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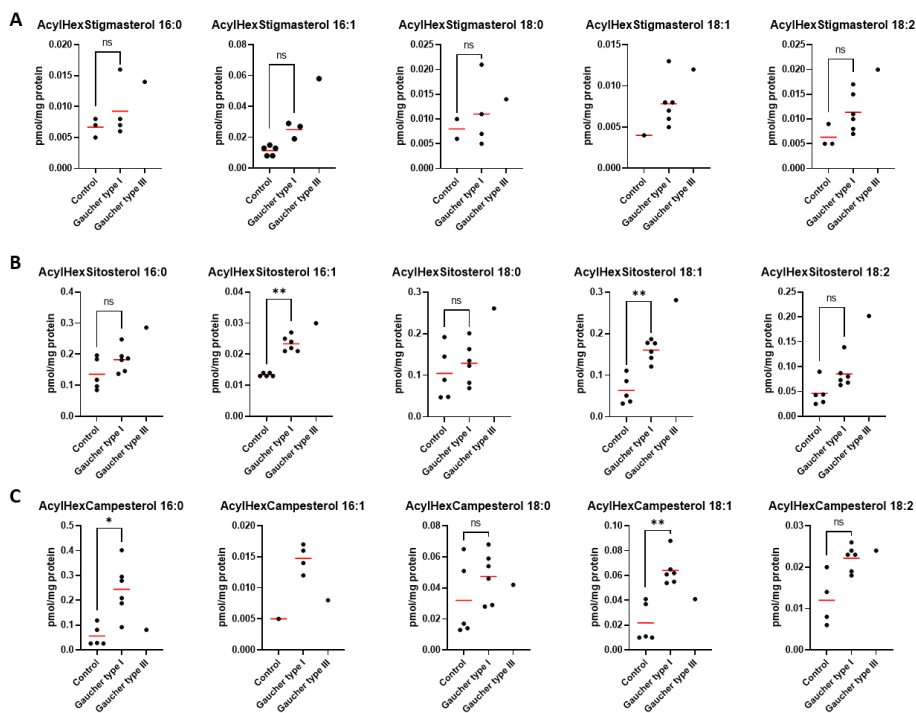


Figure S2.5. Levels (pmol/mg protein) of (A) 6-*O*-acyl-glycosyl-stigmasterol (AcylHexStigmasterol), (B) 6-*O*-acyl-glycosyl- β -sitosterol (AcylHexSitosterol) or (C) 6-*O*-acyl-glycosyl-campesterol (AcylHexCampesterol) in lysates of spleens (30 mg protein/mL) from non-GD or GD patients determined by LC-MS/MS analysis. Data was analyzed using 6-*O*-palmitoyl- $^{13}\text{C}_6$ -GlcChol (**31**) as an internal standard. The red line represents the mean value of the measured samples and the *p*-values are denoted as ns (>0.05), * (≤ 0.05), ** (≤ 0.01) and *** (≤ 0.001).

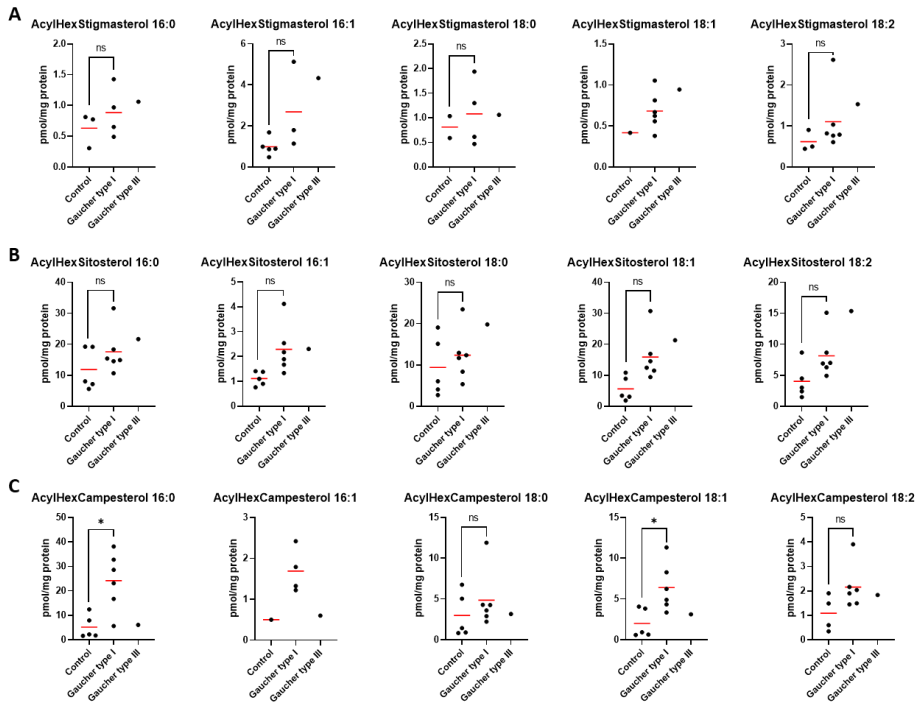


Figure S2.6. Levels (pmol/mg protein) of (A) 6-*O*-acyl-glycosyl-stigmasterol (AcylHexStigmasterol), (B) 6-*O*-acyl-glycosyl- β -sitosterol (AcylHexSitosterol) or (C) 6-*O*-acyl-glycosyl-campesterol (AcylHexCampesterol) in lysates of spleens (30 mg protein/mL) from non-GD or GD patients determined by LC-MS/MS analysis. Data was analyzed using $^{13}\text{C}_6$ -GlcChol as an internal standard. The red line represents the mean value of the measured samples and the *p*-values are denoted as ns (>0.05), * (≤ 0.05), ** (≤ 0.01) and *** (≤ 0.001).

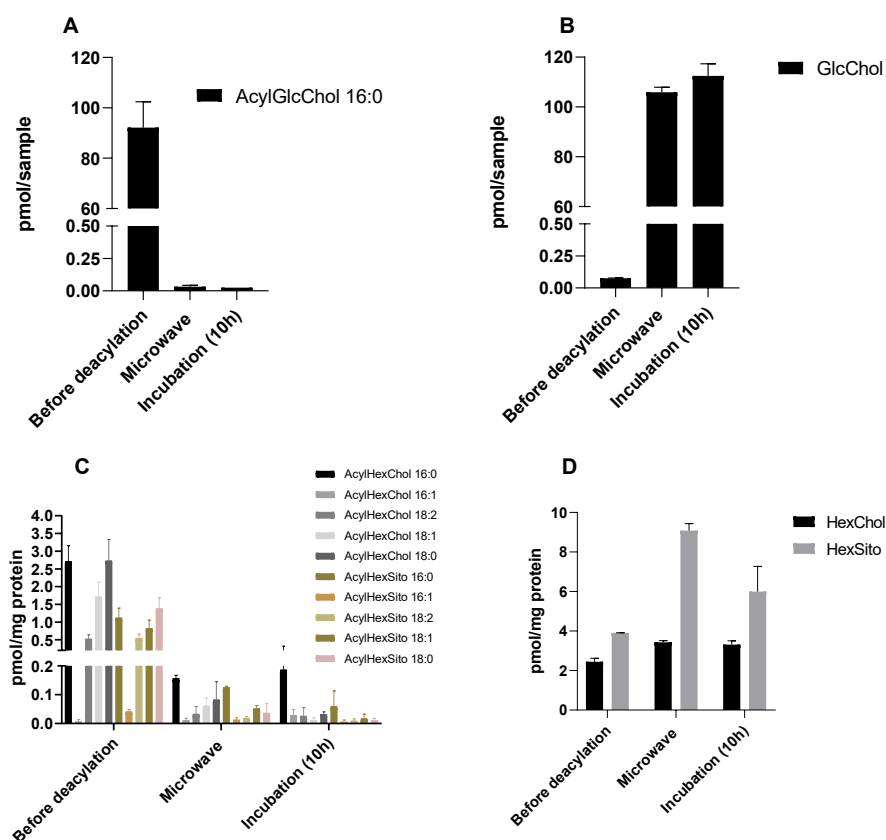


Figure S2.7. (A) 100 pmol of C-6-O-acyl-glucosyl-cholesterol before and after deacylation (microwave or 10 h incubation). (B) Formed GlcChol by deacylation of 100 pmol of C-6-O-acyl-glucosyl-cholesterol. (C) C-6-O-acyl-glycosyl-cholesterol (AcylHexChol) and 6-O-acyl-glycosyl- β -sitosterol (AcylHexSito) in spleen sample with and without diacylation. (D) Glycosylated sterols (cholesterol, sitosterol) in GD patient spleen, with and without deacylation. $^{13}\text{C}_6$ -GlcChol was used as an internal standard. Levels are expressed in pmol/mg protein. Samples were extracted as described earlier, followed by deacylation using a microwave-assisted saponification method³⁹ or incubated in a 37 °C water basin for 10 hours.

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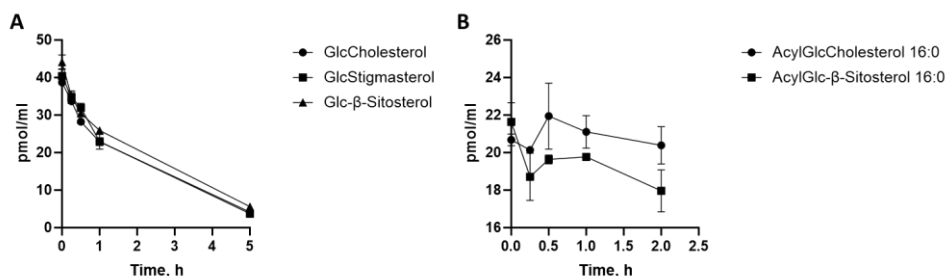


Figure S2.8. (A) Degradation of 40 pmol/ml glucosyl cholesterol and glucosyl stigmasterol, β -sitosterol by 272.6 ng/ml rhGBA1 (Cerezyme 1:800x (stock)). Samples of different sterols were incubated for 15 min, 30 min, 1 h and 5 h at 37 °C in water solution containing 150 mM Mcllvaine buffer (pH 5.2), 0.1% BSA, 0.1% Triton X-100, 0.2% sodium taurocholate, 5% EtOH and (B) 20 pmol/ml 6-O-palmitoyl-glucosyl-cholesterol or 6-O-palmitoyl-glucosyl- β -sitosterol by 272.6 ng/ml rhGBA1 (Cerezyme 1:5000x (final)). Samples of the two acylated sterols were incubated for 15 min, 30 min, 1 h and 2 h at 37 °C. Data was measured by LC-MS/MS analysis using (A) $^{13}\text{C}_6$ -GlcChol or (B) 6-O-palmitoyl- $^{13}\text{C}_6$ -GlcChol (**31**) as internal standards. Number of technical duplicates n=2.